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Mapping the double-strand breaks at the mating-type locus in fission yeast by genomic sequencing

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In fission yeast mating-type switching is initiated by the formation of a double-strand DNA break at the mating-type locus. A prerequisite for generation of the break is some 'imprinting' of the DNA in the previous cell cycle. We have used the technique of genomic sequencing to map the position of the break directly on chromosomal DNA cleaved *in vivo*. On one strand the break is situated very close to the right-hand border of the expressed *mat1* cassette. Cells of opposite mating type, *P* and *M*, have their breaks at slightly different positions on this strand. On the other DNA strand of both alleles the ends are probably masked by tightly bound proteins and therefore the precise nature of the break could not be determined. Since the break is stable throughout the cell cycle, these proteins may function *in vivo* to confer structural stability on the chromosomes having the break. The implications of these findings for models of mating-type switching are discussed.

Key words: double-strand breaks/genomic sequencing/mating-type switching/recombination/*Schizosaccharomyces pombe*

Introduction

Cells of the fission yeast *Schizosaccharomyces pombe* exist in two different mating types called plus (*P*) and minus (*M*) (Leupold, 1950). Upon deprivation of a nitrogen source, cells of opposite mating types are induced to form a diploid zygote by conjugation (Egel, 1971). In the homothallic wild-type strain (h^{90}), vegetatively growing cells switch their mating type once every few cell divisions (Egel, 1977). This is accomplished by a unidirectional gene conversion process referred to as a 'cassette' mechanism (Hicks *et al.*, 1977; Egel and Gutz, 1981). The switching process is initiated by the formation of a DNA double-strand break (DSB) at the *mat1* locus to which genetic information is transposed (Beach, 1983). Only certain cells in a pedigree are allowed to switch their mating type (Miyata and Miyata, 1981). It is argued that the ability to develop the DSB is programmed into the chromosome already one cell cycle ahead such that only one of two sister cells generates a break (Klar, 1987).

There are three copies of mating-type information present in fission yeast, all closely linked on chromosome II. The mating type of a cell is determined by the *mat1* locus. A haploid cell exhibits the *P* or *M* mating type depending on whether the *P* or *M* allele is present at *mat1* (Leupold, 1958; Beach, 1983). In addition to the expressed copy at *mat1*, unexpressed *P* information is located at *mat2-P* and unexpressed *M* information is located at *mat3-M*. These two loci are situated centromere-distal to *mat1*: *mat2-P* is 15 kb away from *mat1* and *mat3-M* is another 15 kb away from *mat2-P* (Beach and Klar, 1984). The three loci, *mat1*, *mat2-P* and *mat3-M*, share regions of homology called *H1* (59 bp) and *H2* (135 bp), which flank the allele-specific regions *P* (1104 bp) and *M* (1128 bp) (Kelly *et al.*, 1988).

The process of mating-type switching entails replacement of the allele present at *mat1* with a copy of the alternate allele resident at one of the two silent loci (Egel and Gutz, 1981; Egel, 1984a). The interconversion process is linked to cell division. When a switching-competent cell divides, only one daughter cell obtains a switched mating type, while the other retains the parental mating type. Cell lineage studies have shown that switching occurs in a characteristic pattern in a pedigree. Whenever a single cell is followed to its four granddaughters, only one of these can have switched mating type in the last division (Miyata and Miyata, 1981). This suggests that it takes two generations to produce a switched progeny. After the first division only one of the two sister cells becomes switching-competent. Studies on switching in diploid cells have indicated that this asymmetrical distribution of switching potential follows the segregation of individual chromosomes (Egel, 1984b).

In a culture of switching cells a DNA DSB is present at the *mat1 H1* homology box (Beach, 1983). Approximately 20% of the cells have this break as detected by Southern blots. The DSB is presumed to initiate the switching event. Mutants that switch mating type at reduced frequency have been isolated (Gutz and Fecke, 1979; Gutz and Schmidt, 1985; Schmidt *et al.*, 1987), and these map either at the *mat1* locus (called *smt*) or in genes outside the mating-type region. The latter class has defined 10 different *swi* genes. Mutants in *swi1*, *swi3* and *swi7* as well as *mat1-smt* have a very low level of DSB and are defective in the process of initiating mating-type switching (Egel *et al.*, 1984).

The work of Klar (1987) suggests that 'imprinting' of the *mat1* DNA is necessary for the break to be generated. This imprinting is specific for a particular DNA strand, which after DNA replication ends up in one of the two daughter cells. Only the cell inheriting the imprinted strand develops a DSB. In molecular terms this provides an explanation for the observed difference in switching potential between two sister cells (Miyata and Miyata, 1981).

In the distantly related budding yeast, *Saccharomyces cerevisiae*, the mating-type interconversion is also initiated by the generation of a DSB, and it has been possible to identify an endonuclease activity present in crude cell extracts that will cut the *MAT* DNA target *in vitro* (Kostriken *et al.*, 1983). However, a search for an *S.pombe* enzyme activity that will cut *mat1* DNA *in vitro* has not been successful (A.Klar, personal communication). The failure to identify such an activity is compatible with the notion that the target DNA has to be modified *in vivo* in some manner before an exonuclease can catalyze a cleavage.

In order to study the mechanism by which the DSB is generated *in vivo*, we decided to map its precise position directly on *S.pombe* chromosomal DNA by genomic sequencing. Here we report the position of the break in both mating types in one of the DNA strands. Our data indicate that the ends of the other strand are protected by covalently bound proteins.

Results

The DSB at *mat1* apparently cannot be generated *in vitro* by crude cell extracts, presumably because the cloned target DNA used in such assays lacks a modification which is a prerequisite for the cutting to occur. The nature of this modification is unknown, but it is probably conferred upon the *mat1* DNA a complete cell cycle before the cut is generated (Klar, 1987).

The genomic sequencing procedure of Church and Gilbert (1984) has been applied in the past to study aspects of DNA structure that are lost upon cloning, e.g. *in vivo* methylation (Church and Gilbert, 1984; Saluz and Jost, 1986) and binding of regulatory proteins (Nick and Gilbert, 1985; Church *et al.*, 1985). It is a high resolution hybridization technique that can provide information about genomic DNA at the level of individual nucleotides. Hence, the key features of this technique should be applicable to mapping the DSB at *mat1*. To do this, *S.pombe* DNA digested with an appropriate restriction enzyme was run in a sequencing gel. The position of the break is defined by the length of a fragment extending from the nearest restriction enzyme cleavage site to the break point. In order to visualize this fragment, the DNA in the sequencing gel was electroblotted onto a nylon membrane and hybridized to a single-stranded probe covering the region of the break. In principle, this is a Southern experiment (Southern, 1975) with the resolution power of a denaturing sequencing gel. Reference DNA, cut with the same restriction enzyme and subjected to limited base-specific chemical cleavage, was run in parallel, allowing the precise position of the break to be determined. By choosing different restriction enzymes cutting on either side of the DSB and by using hybridization probes of both polarities, it should be possible to map all four DNA strand ends in both the *P* and the *M* mating type.

In Figure 1 are shown the *P*- and *M*-specific hybridization probes used for mapping the DSB in the two mating types. Single-stranded RNA probes of both polarities were synthesized *in vitro*. Template for the *P* probe is a 336-bp *Rsa*I–*Ssp*I fragment from *mat1-P* and template for the *M* probe is a 440-bp *Ssp*I–*Ssp*I fragment from *mat1-M*. Probes of opposite polarities were used in separate experiments to light up the bands corresponding to the breaks on either the top strand or the bottom strand. The position of the DSB in the genomic DNA was determined relative to the restriction sites indicated by arrows in Figure 1. The left-hand ends of the DSBs were determined relative to the *Mlu*I site in the *P* cassette and the *Rsa*I site in the *M* cassette. The right-hand ends were determined relative to the *Ssp*I site situated outside the cassette in the region common to both mating types. In principle both the *P* and the *M* probe are applicable for mapping the right-hand ends because the probes are identical between the *Ssp*I site and the cassette border. However, to avoid hybridization with fragments beginning at the other *Ssp*I site inside the *M* cassette, the *P* probe was used. Reference fragments were obtained by chemical cleavage of plasmid DNA fragments. We found that the mobility of such fragments was applicable to determine the break points in genomic DNA (see Materials and methods).

Since the h^{90} wild-type strain can switch its mating type, a culture of this strain is a mixture of *P* and *M* cells and both cell types can have a DSB at *mat1*. To be able to discern these two types of DSB, DNA for genomic

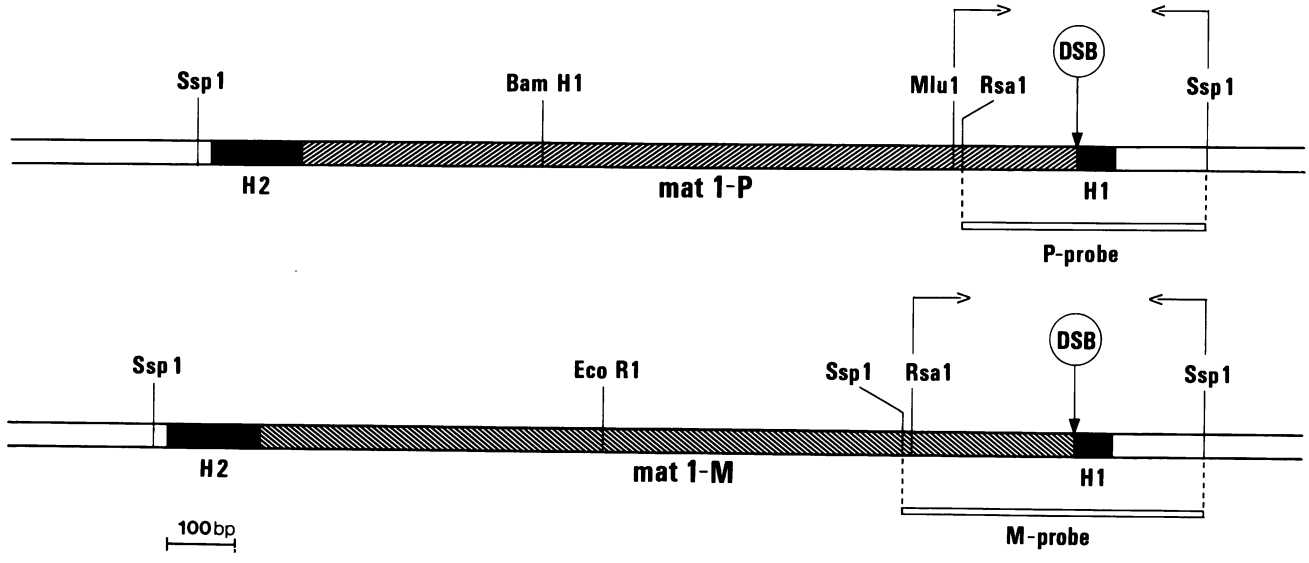

Fig. 1. Schematic representation of the *P* and *M* alleles of the *mat1* locus. The *H1* and *H2* homology boxes are indicated in black. These are also flanking the silent *mat2-P* and *mat3-M* cassettes which are located, respectively, 15 kb and 30 kb to the right of *mat1*. Below each allele are shown the hybridization probes used in genomic sequencing experiments. The DSB is at the border between the *H1* box and the cassette in both mating types. Its precise position was mapped relative to the restriction sites set off by horizontal arrows. The left-hand ends were mapped relative to the *Mlu*I site in *P* cells and the *Rsa*I site in *M* cells. The right-hand ends were mapped relative to the common *Ssp*I site in both mating types. Not all *Rsa*I and *Ssp*I sites are indicated.

sequencing experiments was prepared from heterothallic strains that carry an *in vitro*-generated deletion of the silent donor cassettes and hence cannot switch their mating types (Klar and Miglio, 1986). Depending on the actual mating type at the time of the construction of the deletion, cells can be stuck with a *P* or an *M* cassette at *mat1* giving rise to genetically stable heterothallic strains. Although these strains are incapable of switching their mating type, they still produce a DSB at *mat1* and this has no apparent consequence for cell viability.

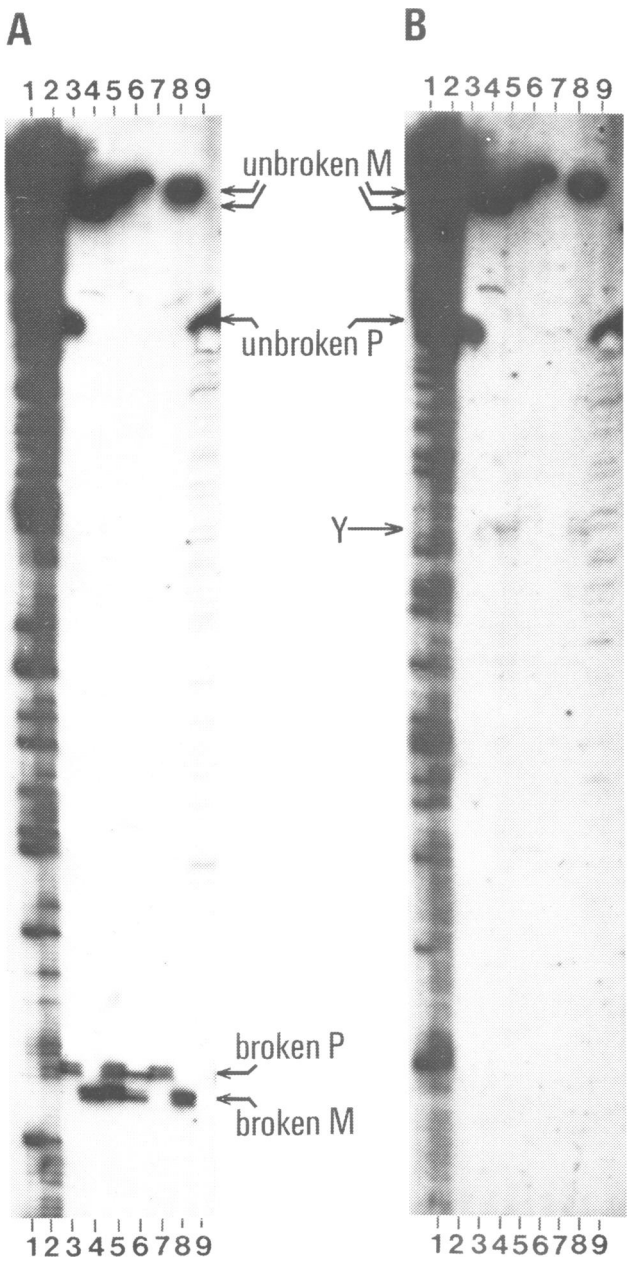

Fig. 2. Genomic sequencing experiments mapping the right-hand ends of the DSB in both mating types. DNA extracted from various yeast strains was treated with proteinase K (except for lane 6), cleaved by restriction enzymes and resolved by electrophoresis in a denaturing sequencing gel. The relevant fraction of the gel was electroblotted onto a nylon membrane and allowed to hybridize to the *P* probe of bottom-strand polarity (**panel A**). After autoradiography the probe was washed off, and the DNA blot was re-hybridized to the *P* probe of opposite polarity (**panel B**). The following DNA was loaded on the gel. **Lanes 1** and **2**: *mat1-M* control DNA digested with *Ssp*I and subjected to G- and G+A-specific chemical cleavage, respectively. **Lane 3**: Eg281 (h^+) DNA+*Ssp*I+*Mlu*I. **Lane 4**: Eg280 (h^-) DNA+*Ssp*I+*Rsa*I. **Lane 5**: Eg282 (homothallic h^{90}) DNA+*Ssp*I. **Lane 6**: same as lane 5, but with omission of proteinase K treatment. **Lane 7**: Eg281 (h^+) DNA+*Ssp*I. **Lane 8**: Eg280 (h^-) DNA+*Ssp*I. **Lane 9**: Eg310 (h^+ *swi7*) DNA+*Ssp*I.

Mapping the break of the upper strand

In Figure 2A is shown the result of a genomic sequencing experiment mapping the break points of the upper strand. The DNA blot was hybridized to the *P* probe of bottom strand polarity (i.e. transcribed in the direction from *Ssp*I to *Mlu*I in Figure 1). This experiment focuses on the right-hand ends of the break. Their position is defined by fragments extending from the rightmost *Ssp*I site in Figure 1 to the break. The DNA in all lanes has been digested with *Ssp*I. In certain lanes the DNA was digested with a second restriction enzyme (*Mlu*I or *Rsa*I) in order to see the unbroken molecules within the resolution limits of the gel. Lanes 1 and 2 contain cloned *mat1-M* reference DNA which after *Ssp*I cleavage was subjected to partial chemical cleavage specific for G and G+A, respectively. Since the G+A reaction is producing faint bands at the positions of pyrimidine residues as well, this was sufficient to assign the position of the break points unambiguously to the known sequence (Kelly *et al.*, 1988).

Lane 3 contains DNA from the heterothallic h^+ strain (Eg281) digested with *Ssp*I and *Mlu*I. Besides the unbroken molecules running at the top of the gel, a shorter band that appears to be a doublet can be seen. This doublet band is also present when the DNA was only digested with *Ssp*I (lane 7), demonstrating that it is extending from the rightmost *Ssp*I site towards the cassette. Furthermore, DNA extracted from an h^+ *swi7* strain (Eg310), which has an undetectable level of DSB at *mat1* (Egel *et al.*, 1984), does not give rise to this doublet band (lane 9). In this lane numerous faint bands are scattered over a range below the unbroken molecules. The origin of these is not known (see Discussion). We conclude that the appearance of the doublet in lanes 3 and 7 is due to DNA molecules having a break at *mat1* in *P* cells. A similar doublet is seen in the heterothallic h^- strain Eg280 (lane 4: *Ssp*I + *Rsa*I digestion; lane 8: *Ssp*I digestion), and this band is not present in the h^- *swi7* strain Eg309 (data not shown). The position of this *M* cell break appears to be three bases closer to the rightmost *Ssp*I site than the *P* cell break (see below). DNA extracted from a homothallic h^{90} strain exhibits both the *P*- and *M*-specific doublets (lane 5: Eg282 + *Ssp*I), which is in agreement with this strain being a mixture of *P* and *M* cells. This observation demonstrates that the position of the break is the same whether the strain is switching mating type or not.

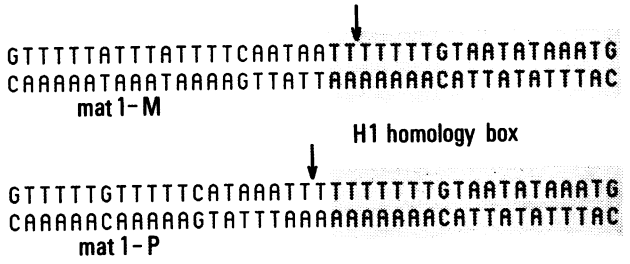

Fig. 3. Position of the breaks found on the upper strand at *mat1-M* and *mat1-P*. The *H1* homology box on the right-hand side of the cassettes is indicated.

Lane 6, too, contains *Ssp*I-digested DNA from the homothallic strain Eg282. As opposed to lane 5, however, the DNA in this lane was not treated with proteinase K prior to *Ssp*I digestion (see Materials and methods). The only observable difference between lane 5 and lane 6 is a relative amplification of the upper band in each of the two doublets when the DNA has been treated with proteinase K. However, this change seems not to be caused specifically by proteinase K, since the same effect can be seen after prolonged incubation of the genomic DNA preparation at 37°C prior to *Ssp*I digestion (data not shown). The size difference between the two bands in both the *P* and the *M* doublet appears to be slightly less than one base, suggesting that this splitting of a band is due to partial removal of a phosphate group from the terminal nucleotide. We assume that the breaks on this strand are generated with 5′ phosphate groups at a unique position giving rise to the lower band seen in each doublet in Figure 2A. A phosphatase activity present in the DNA preparation would lead to the gradual appearance of a new band, running at the position of the upper band seen in each doublet.

Assuming that the breaks of the upper strand have 5′ phosphates, and bearing in mind that the *mat1-M* reference fragments terminate with 5′ phosphates on the base next to the one that has been chemically cleaved (Maxam and Gilbert, 1980), the position of the breaks in the two mating types are as indicated in Figure 3. The *M* break is given by the reference sequence and the *P* break is displaced by three bases towards the inside of the cassette. Thus, the breaks are introduced at slightly different positions in the two mating types with respect to the *H1* homology box, but they are equally situated in the AATTTTTTT motif present in both mating types.

The left-hand ends of the upper strand are not seen in Figure 2A: the fragments from *M* cells are not hybridizing to the *P* probe used, and the fragments from *P* cells ran off the fraction of the sequencing gel that was electroblotted. The experiment shown in Figure 4A identifies the left-hand end point on the upper strand in *M* cells. The membrane was hybridized to the *M* probe of bottom-strand polarity. Lane 2 contains h^- DNA digested with *Rsa*I and *Ssp*I. Besides the right-hand doublet described above the left-hand end point band can now be seen. Neither of these signals is seen in the h^- *swi7* strain (lane 1). In this experiment the sequencing reaction is not very clear (lanes 3 and 4) but evidently the position of the left-hand end point is very close to its right-hand counterpart (compare with the sequence of *mat1-M* given in Figure 3). This suggests that the breaks are simple cuts without any bases deleted. While the right-hand band is a doublet, the left-hand end appears as a single band. This is in agreement with the interpretation that the break is generated with 3′ hydroxyls and 5′ phosphates, and that the appearance of the doublet is due to partial removal of the phosphate groups from the right-hand end.

In Figure 4B the left-hand end point on the upper strand in *P* cells is mapped. This membrane was hybridized to the *P* probe of bottom-strand polarity. Lane 2 contains *Mlu*I-digested h^+ DNA and a band corresponding to the left-hand end point is seen. This band is not present in the h^+ *swi7* strand (lane 1). Assuming that the left-hand ends terminate with 3′ hydroxyls, this band should have a mobility that is approximately one base slower than its corresponding Maxam–Gilbert fragment terminating with 3′ phosphates. This gives a position of the left-hand end corresponding exactly to the right-hand end defined in Figure 2A (see Figure 3). The right-hand end is not seen in this experiment because the DNA was not digested with *Ssp*I.

The breaks on the lower strand are not seen as discrete bands

In order to determine the position of the breaks on the lower strand, the blot from Figure 2A was stripped for the first probe and re-hybridized with the *P* probe of opposite polarity (Figure 2B). The unbroken molecules are at the same positions as in Figure 2A, while lanes 1 and 2 now refer to the sequence on the lower strand. However, no discrete bands originating from broken molecules can be identified. One interpretation of this result is that the breaks on the lower strand are at a different position. To investigate this an

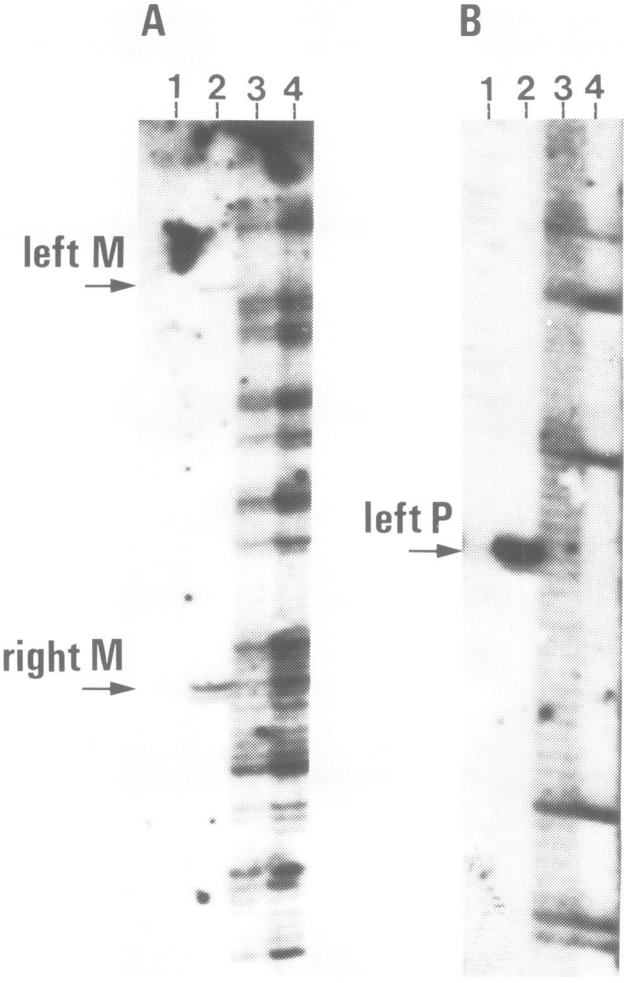

Fig. 4. Genomic sequencing experiments mapping the left-hand ends of the DSB on the upper strand in *M* cells (**panel A**) and *P* cells (**panel B**). The experimental procedure was as described in Figure 2. **Panel A**: the following DNA was loaded on this gel. **Lane 1**: Eg309 (h^- *swi7*) DNA+*Rsa*I+*Ssp*I. **Lane 2**: Eg280 (h^-) DNA+*Rsa*I+*Ssp*I. **Lanes 3** and **4**: *mat1-M* control DNA digested with *Rsa*I and subjected to, respectively, G+A- and G-specific chemical cleavage. After DNA transfer the membrane was hybridized to the *M* probe of bottom-strand polarity. **Panel B**: the lanes contained the following DNA. **Lane 1**: Eg310 (h^+ *swi7*) DNA+*Mlu*I. **Lane 2**: Eg281 (h^+) DNA+*Mlu*I. **Lanes 3** and **4**: *Mlu*I-digested *mat1-P* DNA subjected to, respectively, G+A- and G-specific cleavage. This membrane was hybridized to the *P* probe of bottom-strand polarity.

experiment was carried out in which the DNA in the sequencing gel was run for a short distance only, allowing DNA transfer from full-length lanes (Figure 5). The DNA was from the h^- strain Eg280 (lanes 1 and 4) and its *swi7* derivative Eg309 (lanes 2 and 5)—all digested with *Ssp*I and *Rsa*I. Lane 3 is a G-specific cleavage of *Rsa*I-digested *mat1-M* plasmid DNA. After transfer the membrane was divided and separate lanes were hybridized to the *M* probe of bottom-strand polarity (lanes 1 and 2) or top-strand polarity (lanes 3, 4 and 5). The broken molecules of the upper strand are giving rise to discrete bands as explained above (lane 1). In this lane the left-hand band corresponding to the break is much weaker than its right-hand counterpart. This appears to be an artefact caused by premature transcription termination during probe synthesis, leading to an under-representation of sequences in the 3′ end of the probe. This seems to become an increasing problem when the concentration of the labelled NTP is lowered; in other experiments both DSB bands were of equal intensity (see Figure 4A, lane 2). When the same DNA digest is hybridized to the upper strand, however, no discrete bands corresponding to broken molecules can be identified at all (lane 4). Instead, some material in the slot as well as a broad band 'X' is hybridizing to the probe. These signals are much weaker in the *swi7* strain which has a reduced level of DSB (lane 5). We attribute this to gel retardation of the broken molecules by tightly bound proteins. In *P* cells, too, the breaks on the lower strand are masked (Figure 2B and data not shown).

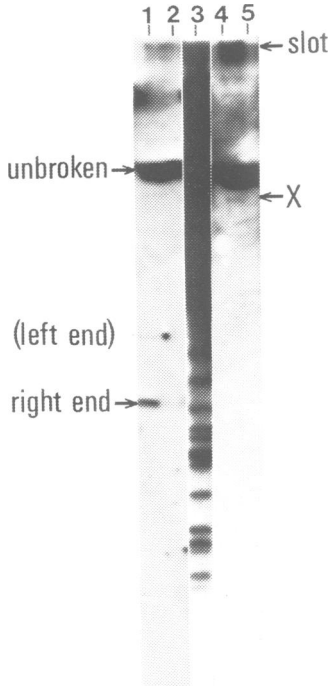

Fig. 5. Genomic sequencing experiment where the DNA in the sequencing gel was run for a short distance only. The entire gel was blotted onto the membrane, and separate lanes were hybridized to the *M* probe of bottom-strand polarity (**lanes 1** and **2**) or top-strand polarity (**lanes 3, 4** and **5**). DNA from the h^- strain (Eg280, **lanes 1** and **4**) and the h^- *swi7* strain (Eg309, **lanes 2** and **5**) were digested with *Rsa*I and *Ssp*I. **Lane 3** is a G-reaction of *Ssp*I-digested *mat1-M* DNA.

Discussion

In several fungi double-strand DNA breaks have been reported to play a specific role as initiators of recombination (for a review, see Orr-Weaver and Szostak, 1985). In fission yeast, switching of mating type is initiated by a DSB at the *mat1* locus. This break also represents a hotspot for mitotic and meiotic recombination (Egel, 1981; Klar and Miglio, 1986). We have identified the precise break point in both *P* and *M* cells on the upper DNA strand (Figure 3). There are no indications of attached proteins on this strand. The breaks are at the same position whether the strand is switching its mating type or not (Figure 2A, lanes 3, 4 and 5), suggesting that they represent the initial cutting process rather than a switching intermediate. On the lower DNA strand we have not been able to detect any defined break points by genomic sequencing. We believe this is due to masking of the ends by attached proteins. The experiment described in Figure 5 indicates that some DNA extracted from cells having the break cannot migrate into the sequencing gel (lane 4). Treatment with proteinase K was not sufficient to unmask the ends on this strand, and therefore we presume that the proteins are attached by covalent bonds. A blurred band 'Y' appears only when the DNA has been treated with proteinase K (Figure 2B, compare lanes 5 and 6) indicating that the protein has been partially removed from one end.

Structure of the break

The sequence around the break has symmetric properties in both *M* and *P* cells. In Figure 6 the palindromic bases have been underlined. The break point on the upper strand is located very close to the palindrome centre in both mating types.

In the budding yeast, *S.cerevisiae*, a restriction endonuclease *Sce*I has been described (Watabe *et al.*, 1983). This enzyme generates specific double-strand cuts with four bases 3′ extensions. Based on statistical considerations, Shibata *et al.* (1984) have suggested a 26-bp consensus sequence for *Sce*I cleavage sites (Figure 7). This sequence is asymmetrical, and a few mismatches are allowed for all sites indicating that the recognition mechanism is somewhat 'sloppy', resembling, for example, prokaryotic promoter recognition (Hoopes and McClure, 1987). The *HO* gene product, which generates the double-strand break at the *YZ*

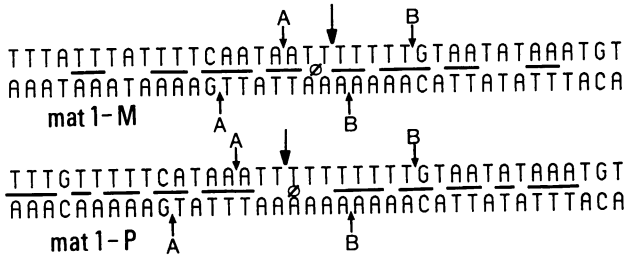

Fig. 6. Symmetric properties of the sequence around the break points at *mat1-M* and *mat1-P*. Bases underlined are palindromic with respect to the symmetry centres (ϕ). The breaks found on the upper strand in *S.pombe* are indicated by large arrows. Break points compatible with the cleavage pattern for the *S.cerevisiae* enzymes *HO* and *Sce*I are shown by small arrows marked A and B, respectively.

border initiating mating-type switching in *S.cerevisiae*, is also producing a cut with four bases 3′ extension (Kostriken *et al.*, 1983; Kostriken and Heffron, 1984) and the sequence around this cut shows homology to the proposed *Sce*I recognition sequence (Shibata *et al.*, 1984).

We found that the sequence around the breaks at *mat1-M* and *mat1-P* in *S.pombe* has some homology to the *Sce*I consensus sequence as well as to the *HO* recognition sequence (Figure 7). Because the *S.pombe* sequences are palindromic, homology is found on both strands, and two sets of break points would be compatible with the *Sce*I/*HO* pattern of cleavage as indicated by small arrows, A and B, in Figure 6. Obviously, the breaks we have mapped on the upper strand (Figure 6, large arrows) are at another position.

The recognition sequence for the cutting mechanism evidently extends into the mating-type-specific sequences. Mutants in the genes *swi1*, *swi3* and *swi7* have a reduced level of DSB at *mat1* (Egel *et al.*, 1984) and these genes may encode components of the endonuclease and/or the strand-specific imprinting system required for generating the cut (Klar, 1987). The faint bands seen in the *swi7* strain (Figure 2A and 2B, lane 9) may be caused by nicks in the DNA: the fragments are smaller than the unbroken molecules and their position is not affected by proteinase K treatment (data not shown). One possibility is that these bands result from cutting with reduced fidelity, i.e. that the *swi7* mutant is defective in guiding an endonuclease to the appropriate position.

The process of mating-type interconversion in *S.pombe* is formally similar to that of *S.cerevisiae*, also proceeding by a cassette mechanism (for a review, see Herskowitz and Oshima, 1981). However, the role played by DSBs in switching appears to be quite different in the two yeasts, and this may point to differences in the biochemical structure of the breaks. The DSB generated by the *HO* endonuclease at the *MAT* locus in *S.cerevisiae* has no proteins bound to its ends (Kostriken *et al.*, 1983). It is only present for a short period in the G1 phase of the budding yeast cell cycle (Nasmyth, 1983). In consequence, only 2% of the *MAT* DNA molecules have a DSB as detected by Southern blots, in spite of the fact that 40% of the cells in a population actually switch their mating type (Strathern *et al.*, 1982). This is in contrast to the situation in *S.pombe*, where the DSB at *mat1* is stable throughout the cell cycle and 20% of the *mat1* DNA molecules have a DSB at any time (Beach, 1983; O.Nielsen and P.Nurse, unpublished results). We assume that the cells can survive having a stable DSB at *mat1* only because this break is protected by appropriate structural features. Specifically, the distal part of the right arm of chromosome II might be lost if the ends at *mat1* were not attached to each other. We propose that the ends of the DSB at *mat1* are held together by a protein complex. Our data suggest that this is mediated by covalent linkages to the lower DNA strand.

Sce1	CAnPYnnAnnCYYGTTnnnPnnnnYA
upperM	CAATAATTTTTTTGTAATATAAATGT
lowerM	CAAAAAAATTATTGAAAATAAATAAA
upperP	CATAAATTTTTTTTTGTAATATAAA
lowerP	CAAAAAAAAAAATTTATGAAAAACAAA
HO	CAACAGTATAATTTTATAAACCCTGG

Fig. 7. Comparison of 23 bases from both DNA strands around the break point in *P* and *M* cells with the consensus sequence for *Sce*I cleavage and with the *HO* recognition site. Bases fitting the *Sce*I consensus sequence are overlined. Bases identical to the *HO* recognition sequence are underlined. P = purine, Y = pyrimidine, n = G, A, T or C.

Function of the break in mating type switching

In *S.cerevisiae*, deletion of the silent donor cassettes results in the segregation of dead cells in strains harbouring an active *HO* gene, presumably because the cells fail to repair the breaks in the absence of the donor loci (Klar *et al.*, 1984). In fission yeast, on the other hand, deletion of the silent donor cassettes, *mat2-P* and *mat3-M*, does not lead to lethal sectoring (Klar and Miglio, 1986). These observations are consistent with the idea that the DSBs initiate mating-type switching by different mechanisms in the two yeasts. In

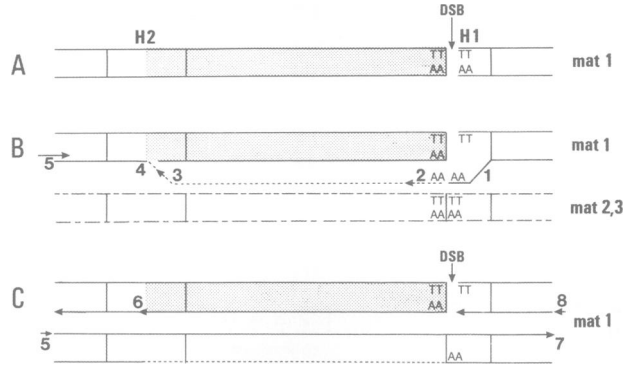

Fig. 8. A model for the unilateral conservation of the DSB during mating-type switching. (**A**) Starting situation with the DSB close to the border of the *H1* box. The exact position of the break in the lower strand is still unknown. Blunt ends are drawn for simplicity. For constraints on the position of this break, see text. (**B**) Copy-choice replication using a silent cassette as template. (**1**) Still in G1 phase the lower strand of the *mat1 H1* box (hereafter called the 'A' strand, referring to the 7−10 run of A residues) interacts with the 'T' strand of the silent cassette *H1* box. (**2**) The 3′ end of the *H1* 'A' strand is used as a primer for DNA synthesis into the silent cassette of opposite mating type. (**3**) Copy synthesis continues into *H2*, yielding a single-strand product not hybridized to the template (analogous to transcription). (**4**) Inside *H2* the 3′ end leaves the template, invades the *H2* box of *mat1* and is ligated to the lower strand preserving the *H2* base sequence. At this point the donor and recipient cassettes are topologically separate again, and *mat1* consists of a three-stranded structure. (**C**) Resolution by continued DNA synthesis conserving the orginal mating type together with the break. (**5**) General replication takes over at the left of *H2*. The new strand approaching from outside continues into *mat1*, using the single-stranded bypass of opposite mating type as a template. (**6**) The 3′ end of the original *mat1* cassette can serve as a primer for outward synthesis to the left. (**7**) The growing strand that has entered *mat1* from the left continues its synthesis beyond *mat1* into the right-hand spacer. This strand does not see an interruption at *H1* since its template, the newly switched cassette, is already attached to the *H1* 'A' strand. (**8**) The externally primed strand approaching from the right copies the *H1* 'T' strand until it is stopped by the break in the template. This model leads to a newly switched *mat1* cassette without interruption, starting from the 'virgin state' in the switching cycle, and a fully conserved original cassette (shaded area) together with the DSB. Therefore, the non-switched daughter cell inheriting the cut is predisposed to repeat the switching cycle immediately. It is inherent in this scheme that no length of DNA sequence is ever discarded during mating-type switching.

S.cerevisiae the break itself can trigger mating-type switching immediately. Both mother and daughter cells obtain a switched mating type, indicating that transposition of the cassette occurs prior to DNA replication.

In *S.pombe*, although required for initiation of switching, the DSB is a more permanent feature of the chromosome structure at *mat1*. The work of Klar and Miglio (1986) implies that the cells can replicate the DNA in spite of the break at *mat1*. This can be accomplished by healing the break before replication, or perhaps the replication machinery is able to preserve the structure with the break.

The strand-specific imprinting at *mat1* allows only one of two sister cells to carry out a switch (Miyata and Miyata, 1981; Klar, 1987). When dividing, this switching-competent cell produces a switched and an unswitched daughter cell (in fission yeast both cells resulting from a division are referred to as daughters). Egel and Eie (1987) found that the unswitched daughter cell has an elevated probability of switching again in the next division. This pattern of 'recurrent switching', where a stem cell of the original mating type can give rise to a switched daughter in each of many successive divisions, was observed through at least eight generations. This can be explained by a switching mechanism where the DSB at *mat1* is preserved in the unswitched daughter cell rendering this cell switching competent.

The break points of the upper strand reported here suggest that the cells utilize the entire *H1* homology for cassette interaction between *mat1* and *mat2/mat3* during switching. We propose a specific model for mating-type interconversion, where this break is preserved in the unswitched daughter cell (Figure 8). Since this mechanism is linking switching to DNA replication, no genetic material may ever be discarded at the *mat1* locus. According to this scheme, copying of the silent cassette is primed by the 3′ end of the lower break at the *mat1* DSB. We do not know the precise position of the break point on the lower strand, but we presume it is not too far away from the upper-strand break. Certain restrictions are imposed if the appropriate *mat1* sequence is to be generated in the switched cell. Flush ends may suffice for the DSB at *mat1-M*, since the 3′ terminus created by such a cut would be completely homologous to the *mat2-P* sequence (which is identical to the *mat1-P* sequence shown in Figure 3). In the opposite direction, however, a blunt-end cut at *mat1-P* would generate a 3′ terminus with an extra A residue relative to the *mat3-M* sequence (which is as the *mat1-M* sequence in Figure 3). Therefore, a 5′ overhang of at least one base should be expected for the DSB at *mat1-P*.

The model presented here does not explain how the first DSB starting a new chain of switching events is generated from the 'virgin state' of unbroken molecules. Conceivably DNA replication at *mat1* is organized in a way that generates a DSB in one of the two copies, not only during mating-type switching (i.e. conservation of a pre-existing break) but also during replication of an unbroken *mat1* molecule incapable of switching. If this break is always generated in the copy derived from replication of the same strand, this can explain the imprinting mechanism observed by Klar (1987).

Materials and methods

Strains and DNA

The *S.pombe* strains used had the following genotypes. Eg280 *mat1-M mat2,3Δ::LEU2 leu1-32* (Klar and Miglio, 1986); Eg281 *mat1-P mat2,3Δ::LEU2 leu1-32* (Klar and Miglio, 1986); Eg282 968 h^{90} wild-type (Leupold, 1950); Eg309 *mat1-M mat2,3Δ::Leu2 leu1-32 swi7* (this work); Eg310 *mat1-P mat2,3Δ::LEU2 leu1-32 swi7* (this work).

S.pombe chromosomal DNA was isolated from 50 ml YEL cultures (late log phase) in the following way. Cells were harvested and resuspended in 5 ml of 20 mM citrate/phosphate buffer (pH 5.6) containing 1.2 M sorbitol and 3 mg/ml novozyme (Novo Industries). After incubation for 60 min at 37°C, protoplasts were transferred to 15 ml TE and lysed by addition of 1.5 ml 10% SDS. Debris was precipitated by addition of 5 ml 5 M potassium acetate followed by incubation at 0°C for 30 min and centrifugation at 17 000 *g* for 15 min. Nucleic acids were recovered from the supernatant by isopropanol precipitation. Pellets were washed in 70% ethanol, dried and resuspended in 2 ml of 5 × TE. The DNA was treated with RNase A (10 μg/ml) for 1 h and then with proteinase K (100 μg/ml) for 2 h at 37°C in the presence of 0.5% SDS. In certain experiments this step was omitted. RNase A and proteinase K were then removed by phenol purification. Finally the DNA was recovered by ethanol precipitation, and the dried pellets were resuspended in 50 μl of TE.

Media and standard genetic techniques for *S.pombe* were as described by Gutz *et al.* (1974).

The plasmids *EM5* and *EP6* (Beach, 1983) were used as substrates for Maxam–Gilbert sequencing reactions. *EM5* contains a 2.4-kb *Eco*RI fragment of *mat1-M* comprising the *H1* homology box cloned into pBR322. *EP6* contains the corresponding 2.5-kb *Bam*HI–*Eco*RI fragment of *mat1-P*.

Substrates for *in vitro* transcription of strand-specific hybridization probes were constructed by cloning the relevant fragments into the *Sma*I site of *pGEM3* (Promega Biotec). The *M*-derived template is a 440-bp *Ssp*I fragment and the *P*-derived template is a 336-bp *Rsa*I–*Ssp*I fragment (Figure 1). These fragments were cloned in both orientations, so hybridization probes of either polarity could be obtained by using the T7 RNA polymerase. Standard techniques for DNA manipulations were according to Maniatis *et al.* (1982).

Genomic sequencing

Plasmid DNA digested with appropriate restriction enzymes was subjected to limited base-specific chemical cleavage as described by Church and Gilbert (1984). Approximately 100 ng of plasmid DNA was used together with 5 μg of carrier DNA. About 5 μg of *S.pombe* chromosomal DNA was digested with the same restriction enzymes and run in parallel on a 5% sequencing gel that was 0.4 mm thick (Maniatis *et al.*, 1982). Experiments where the reference DNA and the *S.pombe* chromosomal DNA were mixed in the same slot did not change the relative position of the observed DSB bands (data not shown). The relevant fraction of the gel was electroblotted onto a Gene Screen membrane (New England Nuclear) and UV-cross-linked as described by Saluz and Jost (1986). Gel fragments up to 28 cm in length could be electroblotted with the equipment used.

Probe synthesis

Strand-specific RNA probes were synthesized using T7 RNA polymerase (Pharmacia) according to Melton *et al.* (1984) with modifications. ^{32}P-labelled UTP (800 Ci/mmol; NEN) was partially dried down to obtain a final concentration of 16 μM without addition of cold UTP. The reaction volume was 10 μl containing 0.5 μg of template. After incubation 5 μg of carrier RNA was added and the template was degraded by incubation at 37°C for 15 min with 1 unit of *RQ1* DNase (Promega Biotec). The probe was then purified on a Nensorb column (NEN), from which it was recovered in 200 μl of 50% methanol. This was added directly to the hybridization buffer.

Hybridization conditions

The hybridization conditions were essentially those described by Church and Gilbert (1984) with the modifications introduced by Amasino (1986): 50% formamide, 0.25 M Na/HPO_4, pH 7.2, 0.25 M NaCl, 7% SDS (Sigma, recrystallized), 10% PEG 6000 (Merck), 1 mM EDTA and 100 μg/ml carrier RNA. Due to the increase in hybridization rate caused by the PEG (Amasino, 1986), the hybridization volume could be increased from 10 to 200 ml without any detectable loss of signal (data not shown). For convenience the hybridization reactions were carried out in flat-bottom plastic boxes containing 150–200 ml of hybridization solution. The probe was added directly without pre-hybridization and the boxes were incubated on a rocker platform at 42°C overnight. After hybridization, blots were washed according to Amasino (1986) followed by treatment with RNase A as described by Church and Gilbert (1984). The dried membranes were exposed without intensifying screens to Agfa Curix X-ray films for 1–10 days. Removal of one probe prior to rehybridization with another was according to Thomas (1980).

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