

A site- and strand-specific DNA break confers asymmetric switching potential in fission yeast

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Mating-type switching in the fission yeast *Schizosaccharomyces pombe* results in the transfer of genetic information from one of the two silent cassettes (*mat2P* or *mat3M*) to the transcriptionally active locus (*mat1*). The switching pattern is programmed by an imprinting event which restricts *mat1* gene conversion to only one of the two sister cells, leading to asymmetric cell division. Biochemical analysis indicated that the *mat1* locus contains a fragile chromosomal site. Southern hybridization and primer extension experiments showed that the fragility consists of a single-strand break (SSB). The nicked DNA is stable throughout the cell cycle. The features of the nick fulfil all the requirements for the ‘epigenetic’, site and strand-specific chromosome modification at the *mat1* locus, providing strong evidence that an SSB can initiate mitotic and meiotic gene conversion during replication.

Keywords: imprinting/nick/recombination/replication/*Schizosaccharomyces pombe*

Introduction

The budding and fission yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, mitotically switch their mating-types, producing a cell population containing an equal proportion of both mating-types (for reviews, see Herskowitz *et al.*, 1992; Klar, 1992). The switching pattern in both yeast is highly regulated and asymmetrically distributed in a cell lineage (Figure 1). Mating-type switching results in the transfer of genetic information from a silent cassette to a transcriptionally active locus. The mechanism of programmed genetic rearrangement is initiated by DNA cleavage of the transcriptionally active locus. The major differences in both yeast lies in the asymmetric distribution of the mechanism controlling DNA cleavage and in the timing of the gene conversion event relative to DNA replication. In *S.cerevisiae* the endonuclease HO is asymmetrically expressed in only one of the two sister cells (the mother) at the end of the G₁ phase (Nasmyth, 1983), triggering mating-type switching before S phase. Following mitosis, both sister cells inherit a switched chromatid and then a switched phenotype.

By standard Southern blot analysis and by analogy with the *S.cerevisiae* mating-type switching system, it was proposed that gene conversion at the *mat1* locus (Egel and Gutz, 1981; Egel, 1984) of *S.pombe* is also initiated by a double-strand break (DSB) (Beach, 1983). However,

pedigree analysis has shown that two asymmetric divisions (Figure 1) are required to produce one switched cell among four granddaughters (Miyata and Miyata, 1981). Furthermore, the sister of a newly switched cell is competent for switching during its next division (Egel and Eie, 1987; Klar, 1990). Genetic and molecular approaches have shown that the asymmetric cell division in fission yeast does not require differential localization of a diffusible factor, but it is a consequence of a semi-inheritable chromosomal modification (Egel, 1984; Klar, 1987). This chromosomal imprinting event restricts DNA cleavage, at the *mat1* locus, to only one of the two sister chromatids, one generation before switching. This triggers mating-type interconversion to only one of the two sister chromatids, at the time of DNA replication or just after (Klar, 1987). Following mitosis, only one of the two sister cells inherits a switched chromatid and consequently a switched phenotype. In summary, in budding yeast the pattern of switching is dictated by the pattern of the endonuclease expression and in fission yeast by the pattern of the endonuclease-target sequence accessibility.

The properties of DNA cleavage observed at the *mat1* locus, in fission yeast, is intriguing in several respects: (i) as observed by Southern hybridization, ~20% of chromosome II contained a DSB at the *mat1* locus, which is found at a constant rate throughout the entire length of the cell cycle (Egel, 1984). (ii) An imprinting event restricts the cutting machinery to only one of the two sister chromatids (Klar, 1987). (iii) The position of the break was mapped, at the nucleotide level, on only one strand. As the ends of the other strand could not be determined it was proposed that they are protected by a

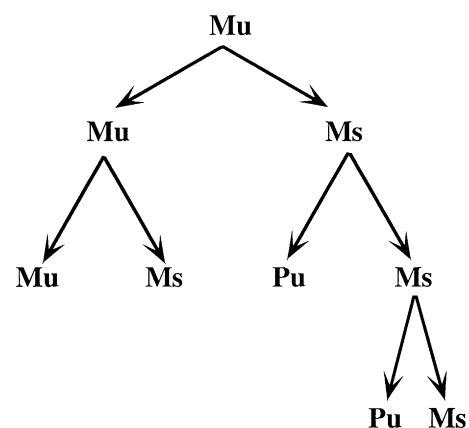


Fig. 1. Mating-type switching pattern of fission yeast. Pattern of switching showing the asymmetric cell divisions. P, M are the mating-types and the suffix ‘u’ and ‘s’ represent ‘unswitchable’ and ‘switchable’, respectively. *Schizosaccharomyces pombe* cells divide by fission generating nearly equal daughter cells, thus the suffix u or s can be attributed to the dividing cell only *a posteriori*, when the daughter cell has expressed its mating-type.

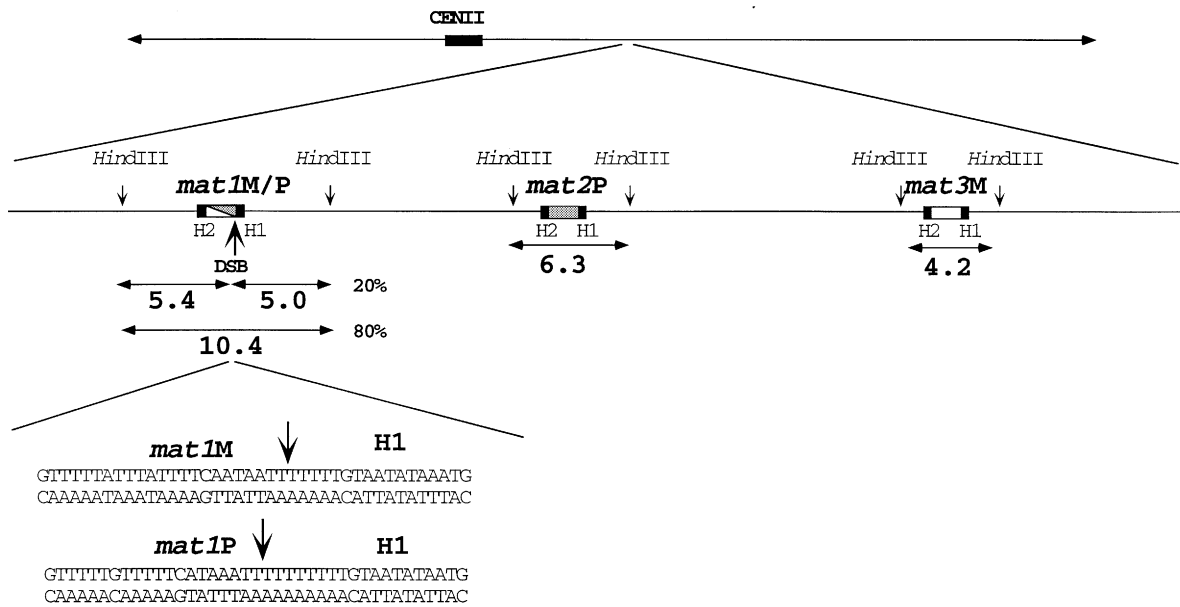


Fig. 2. Schematic representation of the mating-type region on chromosome II. The size of the *HindIII* fragments containing the mating-type loci observed after standard genomic DNA preparation are indicated. The proportion of broken *HindIII*-*mat1* fragments is shown. The black boxes labelled H1 and H2 are sequences common to each cassette, whereas H3 sequences are common only to the silent loci. An arrow indicates the position of the break site, mapped at the nucleotide level only on the upper strand for *mat1P* and *mat1M*.

covalently-bound protein (Nielsen and Egel, 1989). (iv) The 160 bp distal to *mat1* contains two *cis*-acting elements, SAS1 and SAS2 (switch-activating sequence) which are essential for either imprinting or the catalysis of break formation and consequently for mating-type switching (Egel and Gutz, 1981; Arcangioli and Klar, 1991; Klar *et al.*, 1991; Stykarsdottir *et al.*, 1993). (v) Genetic evidence suggested that the broken chromosome is sealed, replicated and cleaved again (Klar and Miglio, 1986; Arcangioli and Klar, 1991; Klar *et al.*, 1991). This suggestion was recently reinforced by the characterization of the *swi7* gene, required for a wild-type steady-state level of the DSB and encoding for the DNA polymerase α (Singh and Klar, 1993). Taken together, the properties of the DSB at *mat1* are unusual and without precedent.

These intriguing properties have encouraged us to re-examine the DNA break at the mating-type locus. Our work shows that the mating-type locus constitutes an unprecedented fragile chromosomal site, due to a site and strand-specific nick. The nick is found at a constant rate, throughout the length of the cell cycle. These results implicate a single-strand break (SSB) in the initiation of mitotic and meiotic gene conversion. The implications for the asymmetric switching between sister cells and the strand-segregation model are discussed.

Results

The mating-type locus contains a fragile site

To further investigate the DNA break at the *mat1* locus, *S.pombe* genomic DNA was prepared in solid agarose plugs, as for pulse field gel electrophoresis (Schwartz and Cantor, 1984) rather than by the standard established yeast extraction protocol (Moreno *et al.*, 1991). This method protects the chromosomes from mechanical shearing and nuclease degradation during the purification process. Following the genomic DNA preparation, the DNA was

digested with *HindIII* restriction enzyme, separated by agarose gel electrophoresis and analyzed by Southern hybridization using the 10.4 kb *mat1-P*-*HindIII* fragment as a probe. The *h⁹⁰* wild-type strain yields the typical bands of 10.4 (*mat1*), 6.3 (*mat2-P*) and 4.2 kb (*mat3-M*) plus the 5.4 and 5.0 kb fragments originating from the cleaved *mat1* locus (Figure 2). Surprisingly, the 5.4 and the 5.0 kb fragments were barely visible under these conditions (Figure 3A, lane 1). Approximately 1% of the *mat1* locus was cleaved, in contrast with the 20% usually observed with the standard yeast genomic DNA preparation (Beach, 1983; Beach and Klar, 1984).

We investigated the nature of the discrepancy between the two genomic DNA preparation methods. Figure 3A (lanes 2–5) shows that mechanical shearing upon vortexing melted agarose plugs containing *S.pombe* chromosomes DNA prior to *HindIII*-digestion strongly increased the yield of the 5.4 and 5.0 kb DNA fragments. Little effect was observed when the DNA was first digested by *HindIII* and then vortexed (data not shown). This experiment showed that the standard genomic DNA preparation introduced shear forces (phenol extraction, DNA precipitation, etc.) that specifically break a fragile chromosomal site at *mat1*, which can be avoided by handling the chromosomes in solid agarose plugs.

Figure 3B shows the effects of incubation with several proteases and nucleases on the *HindIII* digestion pattern. A striking effect was observed only with the single-strand specific mung bean nuclease (Figure 3B, lane 8), producing the two *mat1* DNA fragments *mat1*-proximal and *mat1*-distal of 5.4 and 5 kb, respectively. Quantitation of the labeled DNA fragments indicated that 27% of the 10.4 kbp *mat1* fragment had been cleaved generating the *mat1*-distal and the *mat1*-proximal signals. The DSB level obtained after mung bean treatment is similar to the level obtained after vortexing and to the level

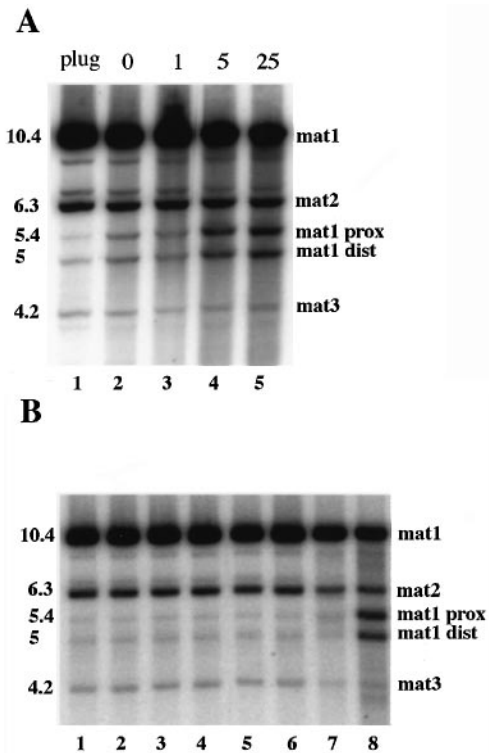


Fig. 3. Southern blot analysis of *Hind*III-digested wild-type DNA prepared in a solid agar plug and probed with *mat1P* DNA fragment. (A) Mechanical sensitivity. Lane 1: *S.pombe* genomic DNA maintained in a solid agar plug. Lanes 2–5: after DNA preparation the agar plug was melted, gently pipetted and vortexed for 1, 5 or 25 s, solidified again before *Hind*III-digestion, electrophoresis and Southern hybridization analysis. (B) Enzymatic sensitivity. *Schizosaccharomyces pombe* genomic DNA was prepared in solid agar plugs. Lane 1: control *Hind*III-digested genomic DNA. Lanes 2–8: following *Hind*III-digestion the plugs were treated with proteinase K (lane 2), pronase (lane 3), V8 protease (lane 4), ribonuclease H (lane 5), ribonuclease A (lane 6), ribonuclease T1 (lane 7) or mung bean nuclease (lane 8), respectively. Names of the mating-type cassettes and their mol. wts are indicated.

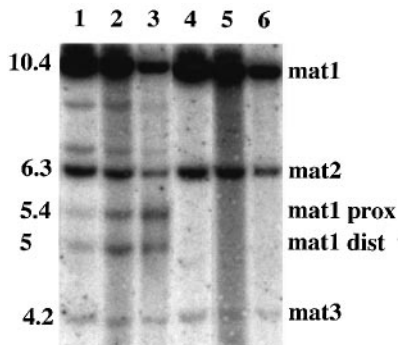


Fig. 4. *Cis*-acting elements are essential for *mat1* fragility. Southern transfer of wild-type (lanes 1–3) and *mat1M smt0* mutant (lanes 4–6) strain DNA digested with *Hind*III and probed with *mat1P* DNA fragment. Lanes 1 and 4, the DNA was maintained in solid agarose. Lanes 2 and 5, the DNA was digested with mung bean nuclease. Lanes 3 and 6, the agarose block containing DNA was melted and vortexed prior to restriction enzyme digestion.

observed using the standard genomic DNA preparation method (Beach, 1983; Beach and Klar, 1984).

We also analyzed the sensitivity of *mat1* DNA from the *mat1M smt0* mutant strain (Engelke *et al.*, 1987) containing a deletion of the *cis*-acting elements SAS1

and SAS2, which abolishes mating-type switching, while preserving the integrity of the cleavage site sequence (Arcangioli and Klar, 1991). In contrast to the wild-type strain, the *mat1* mutant DNA is refractory to vortexing and mung bean nuclease treatment (Figure 4), indicating that the *cis*-acting regulatory elements are required for the mechanism responsible for the *mat1* DNA fragility.

mat1 contains a single-strand break

The *mat1* mung bean nuclease sensitivity was weaker at 37°C (data not shown) and increased with temperature. Previous observations of Nielsen and Egel (1989) mapped the 5' and 3' ends of *mat1* upper DNA-strand, with no missing bases. Based on these findings, we favor the hypothesis that the *mat1* locus contains a nick (SSB) and not a gap, leaving an intact lower strand. Consequently, we analyzed *Ssp*I-digested *S.pombe* genomic DNA after electrophoresis through polyacrylamide gels under native and/or denaturing conditions (Figure 5). To improve the clarity of the analysis, DNA from the wild-type h⁹⁰ and the *mat1M*, Δ *mat2,3* mutant strains (in which the two donor loci have been removed and contained a stable M allele at *mat1*) were compared (Figure 5A and B). Under denaturing conditions (Figure 5B) two additional DNA fragments of 199 and 196 bp and one additional DNA fragment of 196 bp (Figure 5B, lanes 7 and 8) hybridized with the lower strand *mat1* DNA probe with the h⁹⁰ strain and with the *mat1M*, Δ *mat2,3* strains, respectively. These fragments were not observed with the upper strand *mat1* DNA probe (lanes 5 and 6), or under native conditions, irrespective of the sense of the probes used (Figure 5A, lanes 1–4). Interestingly, the *mat1M* DNA fragment of 440 bp ran as a doublet under native (lanes 1–4), but not under denaturing (lanes 5–8) conditions. For the two consecutive polyacrylamide gels (2D gel) the first migration was performed under native conditions, then the piece of polyacrylamide containing the DNA fragments was cut out and incubated into denaturing buffer at 95°C and separated through the second gel under denaturing conditions (Figure 5C). A sample of *Ssp*I-digested DNA was loaded into the first lane as an internal standard (Figure 5C). Following electrophoresis, the gel was electrotransferred and hybridized with a single-strand labeled probe homologous to the upper *mat1* strand (Figure 5D). The 2D gel electrophoresis analysis (Figure 5C) shows that the two spots of 199 and 196 bp (*mat1M/Pdist*), result from the 1450 bp *mat1P* and the 440 bp *mat1M* upon *Ssp*I-digestion. A detailed analysis of the 2D gel reveals that the *mat1M*-dist DNA fragment originates from the faster migrating band observed in the native gel shown in Figure 5A (doublet *mat1M*, see above). It is probable that the nick on the upper *mat1*-DNA strand perturbs its migrating properties. Also, we cannot exclude the possibility that some DNA degradation occurred during the DNA manipulation steps. Another interpretation relies on a hypothetical DNA modification which has been proposed to be a precondition for *mat1* cleavage (Klar, 1987). The *mat1M*- and *mat1P*-proximal upper strands are barely visible, probably due to the single-strand probe used.

The integrity of the lower strand and the nicked upper strand of *mat1*-DNA fragment was confirmed by PCR-mediated linear amplification using a single labeled oligonucleotide as a primer. Figure 6A shows that the *Taq*

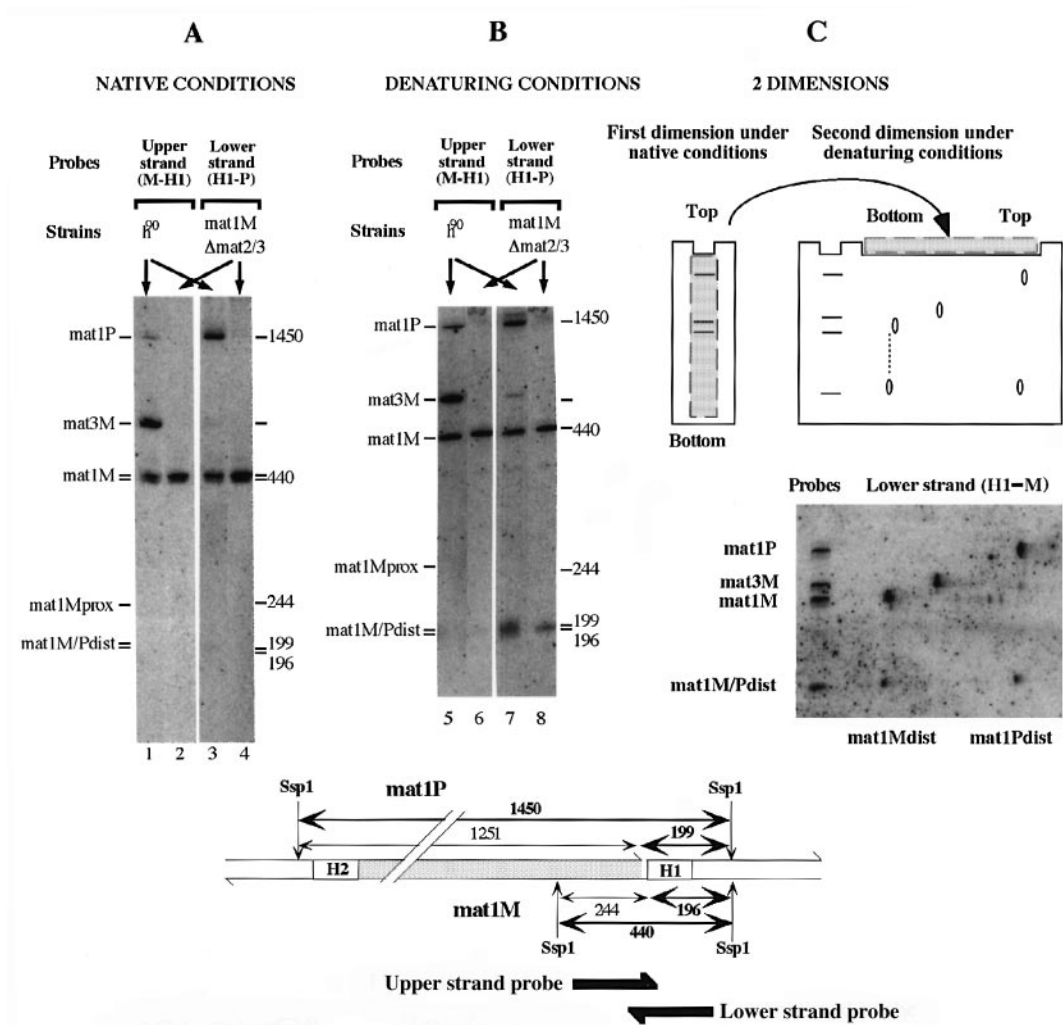


Fig. 5. Native and/or denaturing gel electrophoresis analysis of the mating-type region. (A) Autoradiogram of *SspI*-digested DNA from h^{90} and *mat1M* $\Delta mat2/3$ strains resolved in a native polyacrylamide gel, electrotransferred and probed with either a labeled lower or upper H1 strand from the *mat1P* or *mat1M* loci. (B) Same as (A), but the DNA was resolved in a denaturing polyacrylamide gel. (C) Diagram of the migration pattern of the *SspI* fragment hybridized with a *mat1* lower strand probe analyzed by 2D gel electrophoresis, where the first dimension was performed under native conditions and the second dimension under denaturing conditions (upper panel). Autoradiogram of the second gel, under denaturing conditions, after electrophoretic transfer and hybridization with a labeled lower H1 strand from the *mat1M* locus (lower panel). (D) Schematic representation of the *mat1* locus. The polarity of the probes, names of the mating-type cassettes and their mol. wts are indicated.

DNA polymerase primed with the S2 oligonucleotide (lanes 5 and 6) terminated at the DNA break present on the upper strand. This resulted in DNA fragments of 158 and 161 nt in length, from *mat1M* or *mat1P* DNA templates, respectively (Kelly *et al.*, 1988; Nielsen and Egel, 1989). On the unbroken sister chromatid the PCR products terminated at the *NsiI* or *SspI* restriction sites. Conversely, when the *Taq* DNA polymerase was primed with the labeled M1 or P1 primers, copying the lower strand, no chain termination was observed and the DNA polymerization terminated only at the *NsiI* or *SspI* restriction sites, as indicated. The additional bands may originate from low sequence specificity somewhere else in the genome or star activity on non-canonical *SspI* sequences (AATATa) or less abundant nick sites, especially into the H1 sequence. This result was confirmed by genomic Southern blot analysis at single nucleotide resolution (data not shown) and correlates with position found by Nielsen and Egel (1989). This experiment excludes the possibility that a protein complex, resistant to proteinase K treatment,

protects and joins the ends of the two lower strands of DNA. From these data we concluded that the fragility of chromosome II at *mat1* is due to a site-specific SSB on the upper strand.

The nick at *mat1* is stable relative to the cell cycle

The asymmetric mating-type switching observed in single cell pedigree analysis (Miyata and Miyata, 1981) implies that the gene conversion replacing the allele at *mat1* does not occur at random during the cell cycle, but during or after *mat1* DNA replication (Beach, 1983), allowing only one of the two sister chromatids to switch efficiently to the opposite mating-type. Therefore, the nature of *mat1* DNA along the cell cycle was investigated. The mutant strain carrying the temperature-sensitive mutation *cdc25* [arrested at the G₂/M transition at the restrictive temperature (Russell and Nurse, 1986)] was analyzed. The *cdc25* mutant strain was first grown, in minimum medium, at the permissive temperature (24°C), then shifted to the restrictive temperature (36°C) for 4 h and reincubated at

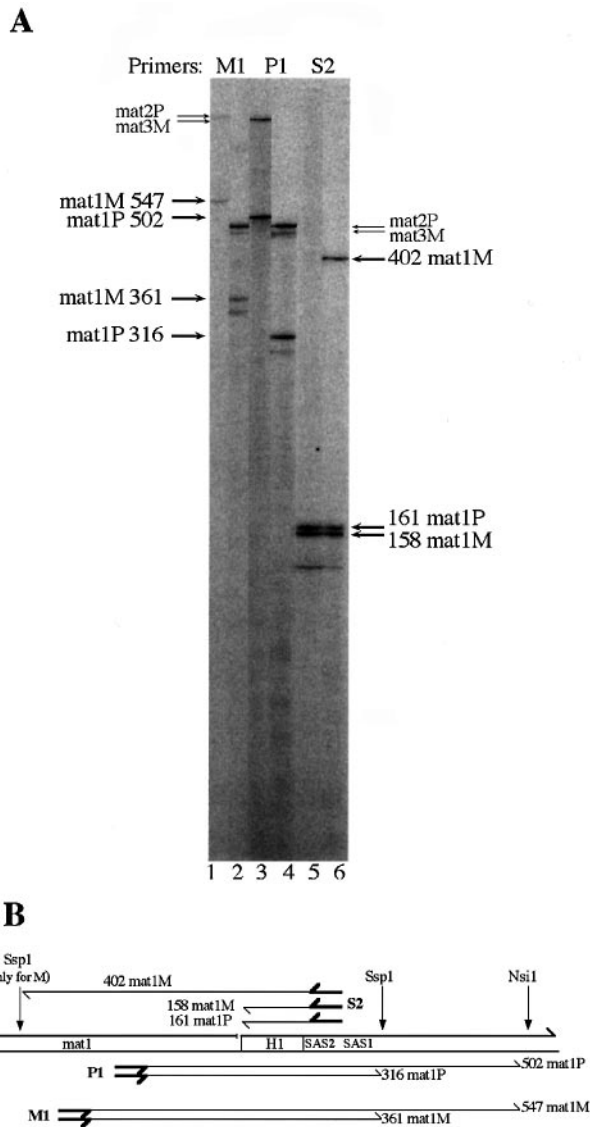


Fig. 6. Primer extension analysis of genomic DNA. (A) Primer extension products from genomic DNA prepared from wild-type strain, digested with *NsiI* (odd lanes) or *SspI* (even lanes). Three labeled primers were used; M1 hybridized to the lower strand of *mat1M* and *mat3M*; P1 to the lower strand of *mat1P* and *mat2P*; and S2 to the upper strand of *mat1M* and *mat1P* distal sequences. The arrows indicate the position of the PCR extended products reaching the position of the restriction site or the nick. The precise size of the products obtained from the silent loci are not known, because of lack of sequence information. (B) The DNA fragments and PCR extended products sizes are indicated and schematically represented.

24°C allowing a synchronous entry into the cell cycle. Samples of cell cultures were harvested at intervals, genomic DNA was prepared in agarose plugs and further analyzed (Figure 7). Cell-cycle position and degree of synchrony were monitored by flow cytometry (FACS) analysis and by determining cell number and septation index (calcofluor staining) as a function of time (Figure 7A and B). The PCR-mediated linear amplification, shown in Figure 7C indicates that the nick is present on the upper DNA strand at a constant level and that the lower strand is intact throughout the cell cycle. Figure 7D shows Southern hybridization of *HindIII*-digested DNA probed

with either *mat1*-proximal (*HindIII*–*NsiI*) or -distal (*NsiI*–*HindIII*) labeled DNA. These two probes do not overlap with the *mat1* locus sequences. Several transient high mol. wt DNA species appeared periodically with both the *mat1*-proximal and -distal probes, 80 and 280 min after G_2/M block release, concomitant with the beginning of S phase, the peak of septation and before the cell number doubled (Figure 7). The ARS1 and PCNA (Waseem *et al.*, 1992) probes were also used to follow DNA replication timing (Figure 7D). These data indicate that the modifications of *mat1*-DNA appear with the S phase and may represent replication–recombination intermediates. This issue was not investigated further. Interestingly, the *mat1*-distal broken DNA fragment (5.0 kb) increased at the beginning of the two S phases (80 and 280 min after the G_2/M block released). However, the proximal-*mat1* DNA fragment (5.4 kb) gave a weaker signal for the same periods (Figure 7D and E) and its level increased later, concomitantly with the *mat1* 10.4 kb DNA fragment upon *HindIII*-digestion. Quantification of the *mat1*-distal and *mat1*-proximal DNA fragments (Figure 7E) confirmed that *mat1*-distal DNA increased prior to the proximal region. Taken together, these data suggest that the fork of replication initiates from the distal region of *mat1*, at least for the nicked chromatid. Similar data were observed using synchronized *cdc10* mutant strains (arrested at the G_1/S transition; Aves *et al.*, 1985).

Discussion

The implications of these findings have a profound impact on the molecular mechanism responsible for the chromosomal imprinting hypothesized by Klar (1987). The features of the nick described in this work fulfil all the requirements needed for the ‘epigenetic’ site and strand-specific chromosomal modification at the *mat1* locus.

The demonstration of nicked DNA at the *mat1* locus provides an alternative explanation for the discrepancy observed between the yeasts *S.cerevisiae* and *S.pombe* when the two silent donor loci are removed. Whereas *S.cerevisiae* cells die, presumably because they fail to repair the DSB generated by HO endonuclease (Klar *et al.*, 1984), *S.pombe* cells remain viable (Klar and Miglio, 1986). Viability can be explained by a homologous recombination-dependent DNA replication (Skalka, 1974; Zou and Rothstein, 1997). In addition, when such donor-deleted strains were crossed and sporulation induced, they produced at high frequency 3:1 and 1:3 gene conversions of *mat1* in meiotic tetrads (Klar and Miglio, 1986). These factors indicate that the recombination-dependent DNA replication model may also use the homologous chromosome as a template for gene conversion.

In wild-type cells it is possible that the nick at *mat1* constitutes the initial recombination event allowing unidirectional gene conversion. Since the nick is located 3' of the convertible *mat1* DNA, gene conversion cannot be initiated by conventional 3'-end invasion into one of the homologous donors (*mat2P* or *mat3M*). A simple hypothesis is that the nick becomes recombinogenic by being transiently transformed to a DSB (Strathern *et al.*, 1982; Szostak *et al.*, 1983). However, the data shown in Figure 7C do not support this model and seem more consistent with a switching process related to a replication-stimulated

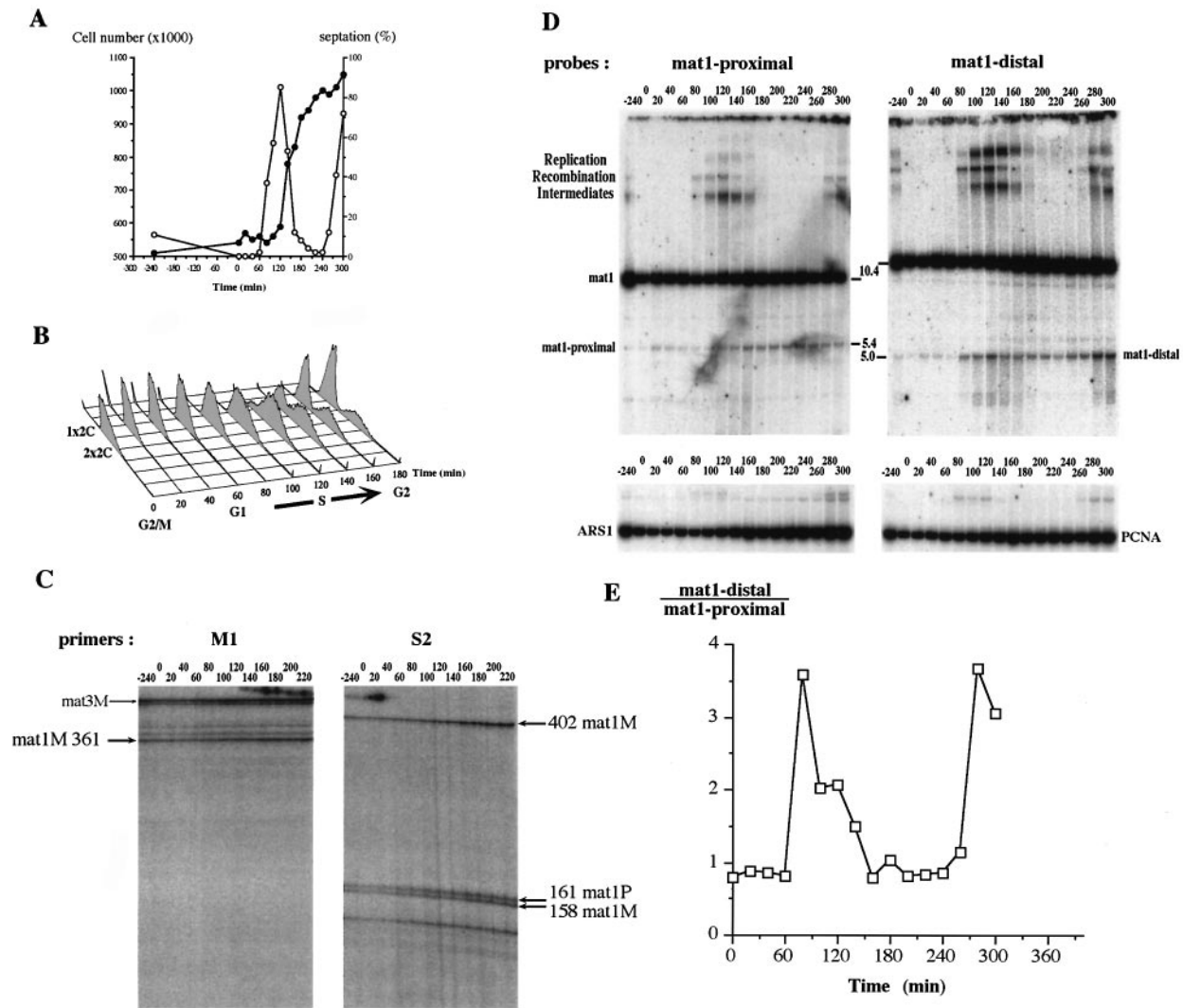


Fig. 7. Analysis of the mating-type region throughout the cell cycle. The five panels show the data obtained from the same experiment. **(A)** Degree of synchrony of the *cdc25* strain after shifting cells from restrictive (4 h at 36°C) to permissive (24°C) temperature in minimal medium. Under these conditions, the peak of septation is concomitant with S phase. **(B)** DNA content of *cdc25* synchronized mutant strain measured by flow cytometry. The phases of the cell cycle are indicated, the drifting of 2C peak at 0 and 180 min is the result of the division of the elongated cells. **(C)** Primer extension products using template DNA from the synchronized *cdc25* mutant strain digested with *Ssp*I. The name of the primers are indicated and are the same as in Figure 6. **(D)** Genomic DNA prepared in a solid agar plug from the synchronized *cdc25* mutant strain, *Hind*III-digested, resolved in agarose gel, blotted and hybridized with either the *mat1*-proximal, -distal, ARS1 or *pcn1* probes. Names of the mating-type cassettes and their mol. wts are indicated. **(E)** Relative intensity of the *mat1*-distal and *mat1*-proximal DNA fragments during the cell cycle.

recombination pathway (Figure 8). When the fork of replication, running in early S phase (80 and 280 min), through the distal-part of *mat1*, encounters the nick, the replication is interrupted. Consequently, a 5.0 kb distal-*mat1* DNA without its complementary 5.4 kb DNA fragment is observed upon *Hind*III-digestion (Figure 8B). Interestingly, this DNA structure is related to a kind of DSB. The stalled polymerase could be rescued by a recombination–replication-coupled machinery channelling the neosynthesized strand into the H1 homologous sequences of the opposite silent mating-type locus (Figure 8C). The donor-choice mechanism promoting intra-chromosomal folding of *mat2* and *mat3* onto *mat1* in a cell type-specific fashion (Thon and Klar, 1993) might prevent strand displacement into the H1 sequence of the sister chromatid (see above) by forcing invasion into one of the silent loci of the opposite mating-type. Following

branch invasion into the opposite silent cassette, DNA synthesis can take place. Interestingly, a slow migrating species is observed with the *mat1*-distal and not with the *mat1*-proximal probes (Figure 7D) and may reveal the transient gene conversion structure represented in Figure 8C. When reaching the H2 homologous box, the replicating strand returned to the *mat1* locus, allowing the progression of the replication fork (Figure 8D). The opposite intact strand can be replicated and nicked, producing an unswitched chromatid which is marked for switching at the following S phase, starting the cycle all over again (Figure 8E). The increase in the level of the *mat1*-distal DNA fragment (5.0 kb) independently of its complementary *mat1*-proximal DNA fragment (5.4 kb) is more spectacular when a mutant strain carrying a deletion of the *mat2P* cassette (*h^{-s}*; Beach and Klar, 1984) is used, consistent with a fast and efficient strand invasion into

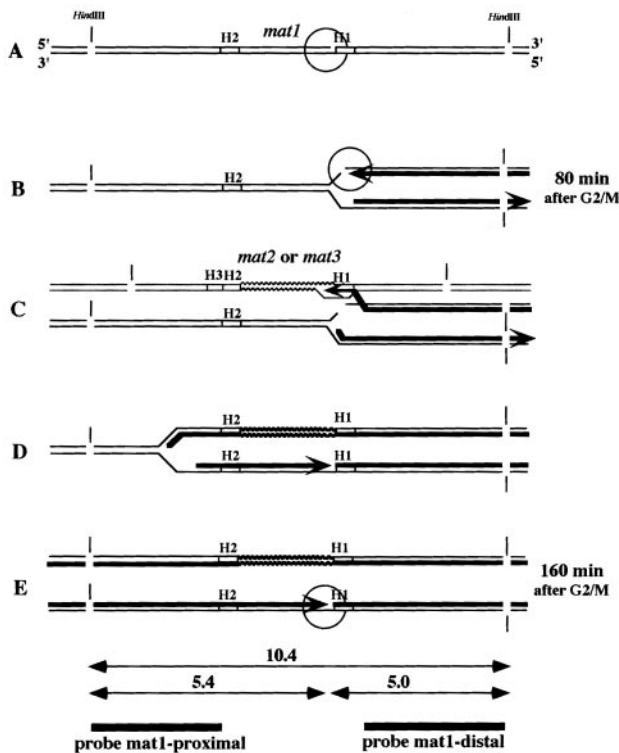


Fig. 8. Switching–replication coupled model. H1, H2 and H3 homology boxes flank the mating-type cassettes. The arrows indicate the direction of the replication. Thick and thin lines represent newly synthesized DNA and old DNA, respectively. The *mat2P* or *mat3M* silent loci are represented by wavy lines and *mat1* by dark lines. (A) Before replication a portion of the *mat1* locus contains a nicked upper strand. (B) Early in S phase, the fork of replication approaching from the distal region of *mat1* is interrupted (80 min after the G₂/M block release). (C) Strand invasion of the opposite mating-type locus allows DNA synthesis of the opposite mating-type. (D) Gene conversion–replication coupled process allows the *mat1* replication to proceed. (E) A switched-intact and an unswitched-nicked chromatid are produced upon replication allowing the switching cycle to begin again, next division. The probes used in Figure 7D and the size of the *Hind*III-digested *mat1* DNA fragments are indicated.

the opposite silent locus, allowing mating-type switching (unpublished data).

Additional work is needed to provide the molecular details of mating-type gene conversion, implicating the known *swi* genes. However, it is expected that mutations in the *swi1*, 3 and 7 genes previously required for the wild-type level of the DSB (Egel *et al.*, 1984) are in fact required for the SSB.

The data presented here do not explain how the newly switched chromatid is initially cleaved, preparing the chromatid for mating-type switching one cell cycle ahead. The strand segregation model (Klar, 1987) proposed that a strand-specific modification acted as a precondition for producing the cleavage at *mat1*, probably during replication one generation before switching. In this context it is interesting to note the migration properties of the *mat1M*-DNA fragment (running as a doublet) upon *Ssp*I-digestion, under native conditions (Figure 5A). The roles of the *cis*- and *trans*-acting elements causing and/or maintaining the nick on the upper strand of *mat1* are still not understood. Increasing evidence indicates that DNA replication plays an active role (Singh and Klar, 1993) in the steady-state

level of the SSB at *mat1*, and future work is needed to reproduce specific SSB formation at *mat1* *in vitro*.

The term ‘fragility’ used in this work is not related to the fragile sites that appear as non-staining gaps in chromosomes occasionally associated with *in vivo* chromosome breakage and rearrangements (Sutherland and Richards, 1995). However, it is conceivable that the nick at *mat1* might be accidentally transformed to a DSB along the progression of the cell cycle, especially around mitosis when compacting or pulling strength are applied to the chromosomes. Recently, the sequence between the silent *mat2* and *mat3* loci revealed that 4.3 kb share homology with centromeric repeat elements (Baum *et al.* 1994; Grewal and Klar, 1997). It is possible that these sequences have been selected as a functional accessory centromere, in addition to their proposed role in silencing (Grewal and Klar, 1996; Thon and Friis, 1997), to prevent mitotic loss of the distal part of chromosome II, if the nick at *mat1* has been accidentally broken.

Materials and methods

Strains and genomic DNA preparation

The *S.pombe* strains used are: SP62 h⁹⁰ *leu1-32 ura4-D18*; SP714 *mat1M mat2.3Δ::LEU2 leu1-32*; SP807 *mat1-Msmt0, leu1-32 ade6-210* (Klar and Miglio, 1986; Engelke *et al.*, 1987). Usually, 10⁷ *S.pombe* spheroplasts in 0.1 ml of 1 M sorbitol buffer were mixed with an equal volume of 1% low melting agarose kept in suffusion at 40°C and allowed to harden into sample molds. Solid samples were extensively digested for 48 h in 1 ml of 1% lauroyl sarcosine and 0.5 mg/ml proteinase K at 50°C, changed 4×. The plugs were rinsed overnight in a large volume of 50 mM EDTA pH 8, 100 mM NaCl, 1 mM PMSF and 3× 30 min in 1 ml of 10 mM Tris pH 8, 0.5 mM EDTA, 100 mM NaCl. For enzymatic digestion, the plug was incubated in 1 ml of enzyme buffer for 30 min and in 100 μl of fresh buffer together with the enzyme for 30 min at 4°C, then incubated at 37°C for 3 h. For the mung bean digestion, the optimum condition was obtained with an incubation at 50°C, allowing a better accessibility of the nicked DNA. The reaction was stopped by 20 mM EDTA, and rinsed as above before secondary treatment or loaded into the well of 0.8% agarose gel. After electrophoresis, the DNA was transferred to Hybond-N+ filters (Amersham) and cross-linked with UV Stratilinker (Stratagen). Following DNA–DNA hybridization the membrane was exposed on a phosphor screen and the signal detected by PhosphorImager 445SI (Molecular Dynamics) and quantified using ImagequantNT. When digested DNA was resolved into polyacrylamide gel, the reaction was frozen at –80°C and filtered by centrifugation through 0.22 μm cellulose acetate (Costar) and precipitated.

Probes synthesis and PCR extension

The probes were labeled with Random primed DNA Labelling Kit (Boehringer) and the unincorporated nucleotides were removed by gel filtration. The sequence of the oligonucleotide used were for M1: 5'-TAGTTCTAAGCACTGTAATGCCATA-3', for P1: 5'-CCAATTCCTT-CCTGTATATGTTATAC-3' and for S2: 5'-CGAAGCAAATATCT-CGTAGAGG-3'. Oligonucleotides were 5'-end labeled with [³²P]ATP and gel purified. Digested genomic DNA were mixed with one oligonucleotide and PCR amplified (Perkin Elmer). The linear amplification steps were, 95°C, 1 min; 50°C, 5 min and 72°C, 2 min for 10 cycles. The reaction was phenol extracted, ethanol precipitated and electrophoresed through a 6% polyacrylamide denaturing gel. The gel was then dried and exposed. Single-strand probes were prepared by PCR linear amplification using *mat1M* or *mat1P* DNA as template, one primer (M1, P1 or S2) and two (α³²P) labeled and two cold deoxynucleotides using the same steps as above. Then the probes were purified from the template and the unincorporated nucleotides through acrylamide gel and then eluted.

Synchronized culture

cdc25 wild-type for the mating-type locus was constructed, grown at 24°C in minimal medium, switched for 4 h at restrictive temperature

(36°C) and incubated at 24°C. Samples were taken every 20 min and monitored for septation index, cell number, DNA content and genomic DNA were prepared for further analysis.

Flow cytometry

Cells ($\sim 10^7$) were fixed in 3.6% paraformaldehyde for 30 min at room temperature, harvested by centrifugation, washed twice in 50 mM sodium citrate (pH 7.0), incubated for 1 min in the presence of 1% Triton-X at room temperature and washed again twice in 50 mM sodium citrate. The cells were resuspended in 50 mM sodium citrate and 50 µg/ml DNase-free RNase A (Boehringer Mannheim) for 1 h at 37°C. Then incubated 15 min with 5 µg/ml propidium iodide (Sigma) in the dark. The cell suspension was analyzed with an Epics XL Flow cytometer (Coulter) and the fluorescence of individual cells were plotted against cell numbers using the WinMDI Software (2.6).

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