Fission yeast switches mating type by a replication–recombination coupled process

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Fission yeast exhibits a homothallic life cycle, in which the mating type of the cell mitotically alternates in a highly regulated fashion. Pedigree analysis of dividing cells has shown that only one of the two sister cells switches mating type. It was shown recently that a site- and strand-specific DNA modification at the *mat1* locus precedes mating-type switching. By tracking the fate of *mat1* DNA throughout the cell cycle with a PCR assay, we identified a novel DNA intermediate of mating-type switching in S-phase. The time and rate of appearance and disappearance of this DNA intermediate are consistent with a model in which mating-type switching occurs through a replication–recombination coupled pathway. Such a process provides experimental evidence in support of a copy choice recombination model in *Schizosaccharomyces pombe* mating-type switching and is reminiscent of the sister chromatid recombination used to complete replication in the presence of certain types of DNA damage.

Keywords: imprinting/recombination/replication/
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Introduction

Mating-type switching in *Schizosaccharomyces pombe* involves a process of genetic recombination (Leupold, 1950; Egel, 1977; Egel and Gutz, 1981; Beach, 1983). The three mating-type loci, *mat1*, *mat2P* and *mat3M* (Beach, 1983; Kelly *et al.*, 1988), are located on the right arm of chromosome II. The loci are separated by the *L* (15 kb) and *K* (11 kb) regions (Beach and Klar, 1984). Two homology regions H1 (59 bp, distal) and H2 (135 bp, proximal) are common to all loci, whereas H3 (57 bp, proximal to H2) is present only at the *mat2P* and *mat3M* loci (Figure 1A).

Mating-type switching results from a genetic transfer of the silent loci, *mat2P* or *mat3M*, to the active locus, *mat1*. This transfer is highly regulated by a process called directionality (Figure 1), which restricts switching to the opposite mating type (Egel and Gutz, 1981; Klar and Bonaduce, 1991; Thon and Klar, 1993).

The transcriptionally active *mat1* cassette contains either the P or M alleles. Pedigree analysis of mitotic dividing

cells has shown that two consecutive asymmetric divisions are required to produce one switched cell among four cousins (Miyata and Miyata, 1981; Egel, 1984). Furthermore, the sister of a switched cell is competent for switching during the next division (Egel and Eie, 1987; Klar, 1987, 1990). In the strand segregation model proposed by Klar (1987), a semi-heritable chromosomal modification marks, in a strand-specific manner, one of the two sister chromatids at the first cell division, restricting the mating-type switching to only one of the two sister chromatids at the second cell division. Recently, it was shown that the *mat1* locus does not contain a site-specific, DNA double strand break (DSB) (Beach, 1983), but instead a fragile chromosomal site (Arcangioli, 1998). It was proposed that the fragility is due to a site-specific, DNA single strand break (Arcangioli, 1998) or an alkaline-labile (Dalgaard and Klar, 1999) DNA modification. This DNA modification is located at the junction of the *mat1*-specific alleles and the H1 region on the upper strand (Nielsen and Egel, 1989; Arcangioli, 1998), and is responsible for the DSB appearing during standard genomic DNA purification.

Cis- and *trans*-acting elements have been involved in the marking, or switching, process. Mutations in the SAS1 and SAS2 elements, located 140 and 70 bp away from the modification site, have a reduced DNA modification at *mat1* (Arcangioli and Klar, 1991; Klar *et al.*, 1991; Styrkarsdottir *et al.*, 1993) and might be responsible for a particular nucleosomal structure. Three classes of genes (defined as *swi* genes) when mutated exhibit a reduced rate of mating-type switching (Egel *et al.*, 1984; Gutz and Schmidt, 1985). Class Ia (*swi1*, -3 and -7) mutants might be required for the marking process. This hypothesis is supported by the finding that *swi7* encodes the catalytic subunit of the DNA polymerase α (Singh and Klar, 1993) involved in lagging strand DNA synthesis and associated with the primase activity. The latter activity is an excellent candidate in support of alkaline-labile modification, as a primer RNA left from the previous replication round (Dalgaard and Klar, 1999). Class Ib (*swi2*, -5 and -6) mutants have a normal rate of DNA modification at *mat1*, but switch less frequently. Class II (*swi4*, -8, -9 and -10 and *rad22*) mutants also have a normal rate of DNA modification, but produce a high proportion of heterothallic progeny containing extensive DNA rearrangements at the mating-type loci. Although the asymmetric distribution of the switching potential is well documented, little is known about how asymmetric mating-type switching leads to only one switched cell among two sisters.

Since the *mat1* locus is replicated preferentially from a centromere-distal origin (Arcangioli, 1998; Dalgaard and Klar, 1999), the leading strand synthesis should be stalled at the DNA modification site, producing an invading strand containing a 3'-OH. We reveal here a mechanism

Fig. 1. Schematic representation of the mating-type loci on chromosome II. (A) The *mat1* locus contains either the P (white box) or M (gray box) mating-type alleles, *mat2* contains the P and *mat3* the M loci located distal to the centromere (CEN II). The P and M DNA regions have no homology and are 1104 and 1128 bp, respectively. The *Bam*HI and *Eco*RI restriction sites in the three cassettes are shown. The H1 (59 bp) and H2 (135 bp) homology boxes are common to all cassettes, whereas the H3 (57 bp) box is common to only the silent *mat2P* and *mat3M* cassettes (Kelly *et al.*, 1988). The asterisk indicates the position of the DNA modification at *mat1*. The horizontal arrows, labeled P1-4 and P'1, indicate the position and the orientation of the PCR primers. The positions of the deletions in *mat1M*, *smt0* and *mat1M*, Δ *mat2-3* mutant strains are indicated. (B) Hypothetical intrachromosomal folding allowing *mat1P* switching to *mat1M*. The PCR primers are indicated and the gene conversion is represented by a cross connecting P1 with P3 or P4 primers. (C) Same as in (B) except that the hypothetical intrachromosomal folding allows *mat1M* switching to *mat1P*.

of gene conversion coupled with DNA replication, producing a switched chromatid and an unswitched chromatid that is marked for switching in the next generation.

Results

To detect mitotic gene conversion intermediates, we devised a PCR assay using yeast genomic DNA, prepared in solid agarose plugs, preserving the integrity of the DNA throughout the purification steps. Figure 1 shows the mating-type genomic region, two hypothetical chromosome foldings required for switching and the PCR primers used in this work. The PCR products obtained with the P1 and P2 primer pair are shown in Figure 2. Digestion with *Bam*HI or *Eco*RI restriction enzymes should discriminate between the P or M alleles (Figure 1A). Since the wild-type homothallic cell population is composed of 50% P and 50% M cell types, each of the enzymes should digest half of the initial fragment, as confirmed in Figure 2A, lanes 2-5. The P1 and P3 primers were devised to detect potential covalent DNA intermediates bridging the *mat1*-distal locus with *mat2P*- or *mat3M*-proximal loci (Figure 2A, lanes 6-9), while the P1 and P4 primers should detect only a DNA intermediate bridging *mat1P*-distal with *mat3M*-proximal loci (lanes 10-13). A similar

Primers	Enzymes	P (bp)	M (bp)
P1+P2	-	1284	1307
	+ <i>Bam</i> HI	332+952	1307
	+ <i>Eco</i> RI	1284	464+843
P1+P3	-	1425	1448
	+ <i>Bam</i> HI	473+952	1448
	+ <i>Eco</i> RI	1425	605+843
P1+P4	-	-	1497
	+ <i>Bam</i> HI	-	1497
	+ <i>Eco</i> RI	-	654+843

Fig. 2. Identification of a DNA species connecting *mat1* with *mat2P* or *mat3M* loci. (A) PCR amplification products using a wild-type homothallic genomic DNA as template, followed by endonuclease digestion and resolution by agarose gel electrophoresis. PCR primers and molecular weight markers are indicated. Twenty-five cycles of amplification were used with the P1-P2 primer pair and 30 cycles with the P1-P3 and P1-P4 pairs. (B) Expected size of the PCR products with and without enzymatic restriction.

strategy (see Materials and methods) has been used previously (White and Haber, 1990). Five additional amplification cycles were required to reveal the PCR products with the P1-P3 and P1-P4 primer pairs compared with the P1-P2 primer pair, indicating that the starting material is ~30 times less abundant than the *mat1* DNA. Similar results were obtained when the PCR assay was conducted with fewer cycles and labeled primers (data not shown and see below).

In order to relate the DNA intermediates observed with the process of mating-type switching, we analyzed genomic DNA isolated from two heterothallic (non-switching) mutant strains by PCR (Figure 3). The *mat1M*, *smt0* strain contains a deletion of 262 bp (see Figure 1), removing the *cis*-acting elements essential for the DNA modification at *mat1* (Arcangioli and Klar, 1991; Stykarsdottir *et al.*, 1993; Arcangioli, 1998; Dalgaard and Klar, 1999), and the *mat1M*, Δ *mat2-3* strain contains a deletion of the *mat2P* and *mat3M* loci (see Figure 1) but still retains the DNA modification at *mat1* (Klar and Miglio, 1986). Note that the P1 primer overlaps the *smt0* deletion and was therefore replaced by the P'1 primer located 240 bp distal from P1 (Figure 1A). In order to rule out potential artifacts due to abortive DNA amplifications and run-off products extending from the P'1 primer to the DNA modification site, genomic DNAs from

Fig. 3. Identification of a gene conversion intermediate. (A) Agarose gel electrophoresis analysis of PCR amplification products. The genomic DNAs were isolated from wild-type, *mat1M*, *smt0* and *mat1M*, $\Delta mat2-3$ strains, as indicated. The P1 primer was replaced by the P'1 primer (see Figure 1), and M indicates the molecular weight markers. Twenty-five cycles of amplification were used with the P'1-P2 primer pair and 30 cycles with the P'1-P3 and P'1-P4 pairs. (B) Native acrylamide gel electrophoresis analysis. The genomic DNA used is the same as above, and the P'1 primer was ^{32}P labeled to reduce the number of amplification cycles. Only 20 cycles were used with the P'1-P2 primer pair and 25 cycles with the P'1-P3 and P'1-P4 pairs.

both mutants were mixed and analyzed. P'1-P2 primers amplified the expected DNA fragments (Figure 3, lanes 1–4). The faster migrating PCR product obtained with the *smt0* genomic DNA is due to the 262 bp deletion. As expected, the P'1-P3 and P'1-P4 primers amplified a detectable PCR product after five additional cycles (Figure 3A, lanes 5 and 9). However, no product was obtained from both heterothallic mutant DNA templates (Figure 3, lanes 6–7 and 10–11), notably in the mixed reactions (lanes 8 and 12). In Figure 3B, PCR products were analyzed by native polyacrylamide gel electrophoresis. In this experiment, PCR primers were labeled and fewer amplification cycles were required. Under these conditions, the two alleles *mat1M* and *mat1P* could be separated, without further enzymatic restriction, the *M* allele migrating more slowly than the *P* allele (Figure 3B, lanes 1 and 5). Again no product was obtained with the P'1 and P3 or P4 primers and the DNA of non-switching mutants. Taken together, these data indicate that PCR products observed with the P'1-P3 and P'1-P4 primer pairs and DNA from the homothallic strain are most likely to reflect the existence of gene conversion DNA

intermediates. Since the P3 and P4 primers are located outside the H2 sequences, then the cellular DNA polymerases involved in the gene conversion process do not stop at the homologous H2 sequences of the silent loci, but go beyond the H3 homology box.

It is well established that bulky lesions in DNA cause the replication machinery to stall. However, DNA replication eventually resumes without the repair of the lesion (Friedberg *et al.*, 1995). It is presumed that a second intact DNA template will serve to bypass the lesion. We propose that in *S.pombe* the opposite silent mating-type locus provides, *in cis*, the undamaged template for gene conversion. The DNA break or alkaline-labile modification is stable during the entire length of the cell cycle, is located on the upper strand, at the junction of *mat1* and H1 DNA sequences, and only one of the two sister cells switches its mating type efficiently. Hence, we propose that the replication fork, coming from the *mat1*-distal side (Dalgaard and Klar, 1999), produces a 3'-OH-ended DNA strand when the leading strand DNA synthesis stalls at the DNA modification site (see Arcangioli, 1998), thereby initiating mating-type switching (Figure 6, step b). To investigate this possibility further, we analyzed the recombination intermediates throughout the cell cycle. To this end, a homothallic, *cdc25* conditional mutant strain (Russel and Nurse, 1986) was synchronized (see Materials and methods), and genomic DNA prepared as a function of time was analyzed by PCR. To ensure quantitative PCR amplification, the P1 primer was ^{32}P labeled and the number of amplification cycles was reduced. As before, three separate amplifications using the same three pairs of primers were carried out, and the PCR products were quantitated and plotted over time (Figure 4A and B). Cell cycle position and degree of synchrony were followed by flow cytometry analysis (Figure 4C) and microscopic observations (data not shown). The amount of P1-P2-generated products roughly doubled between 60 and 120 min, indicating the timing of *mat1* locus replication (Figure 4A), which takes place in S-phase observed by flow cytometry (Figure 4C). The P1-P3- and P1-P4-generated products were only transitory; an abundant product appeared and peaked at the 120 min time point and almost disappeared in early G₂-phase (Figure 4A and B), before mitosis (data not shown), which began after 220 min. Quantitation of the level of PCR products present at the 120 min time point indicated that the DNA intermediates represent ~20% of the total *mat1* DNA and correlates with the steady-state level observed in an asynchronous population (see above). Furthermore, 20% is the order of magnitude of the estimated proportion of switching cells in an exponentially growing population.

The disappearance of the switching DNA intermediates, in early G₂-phase, is likely to result from an efficient resolution of the gene conversion intermediates. Eleven genes have been implicated in mating-type switching. Depending on their functions they were assigned to different classes (Egel *et al.*, 1984). Importantly, none of these mutants abolish mating-type switching. The Ia and Ib classes exhibit a slow mating-type switching. The class II mutants are unstable and frequently produced stable heterothallic segregants. It was proposed that the heterothallic mutants originate from errors in the resolution of recombination intermediates during mating-type switching

Fig. 4. The gene conversion DNA intermediate appears at the end of S-phase and is resolved in early G₂-phase. **(A)** Autoradiogram of the PCR products resolved in a polyacrylamide gel under native conditions. The three primer pairs were described in Figure 1 and the genomic DNA templates were obtained from synchronized homothallic *cdc25* strain. The low level of DNA intermediate products (P1-P3 and P1-P4) found at all times during the cell cycle indicates the degree of synchrony of the *cdc25* cell population. Twenty PCR cycles were used with the P1-P2 primers, whereas 21 cycles were used with P1-P3 and 22 cycles with P1-P4. **(B)** The relative DNA concentration of the PCR product shown in (A) was plotted as a function of time. As expected, the P1-P2 PCR product roughly doubles during S-phase. The P1-P3 and P1-P4 PCR products appear at 120 min in the middle of S-phase and disappear in early G₂-phase. The P1-P2 PCR products were reduced 2-fold to facilitate the comparison. **(C)** DNA content of the synchronized *cdc25* population measured by flow cytometry. The cell population arrested in G₂-M is released at time 0 and enters anaphase at ~30 min and S-phase between 60 and 80 min (see the text for details). Septum formation is concomitant with S-phase and is followed by cytokinesis at the 140 min time point. The second mitosis begins after the 220 min time point (data not shown). The drifting of the 2C peak at 0 and 180 min is the result of the division of elongated cells.

Class	wt	Ia	Ib	II
<i>swi</i>	+	1 7	2 5 6	4 10
P1+P2				
P1+P3				
P1+P4				

Fig. 5. Analysis of the gene conversion intermediates in *swi* mutant strains. The panels show autoradiograms of the PCR products, resolved through a native polyacrylamide gel, of genomic DNA isolated from wild-type and the three classes of *swi* mutant strains, as indicated. Twenty cycles were used with the P1-P2 primer pair, which serves as internal control, and 25 cycles were used with the P1-P3 and P1-P4 pairs.

(Egel *et al.*, 1984). The steady-state level of the gene conversion intermediate was analyzed in the three classes of switching mutants. As shown in Figure 5, class Ia and Ib *swi* mutants exhibit a small but significant reduction in the amount of PCR products with the P1-P3 and P1-P4 primer pairs, compared with wild type, with the exclusion of the *swi6*⁻ mutant. The reduction in DNA intermediates correlates with the reduction of mating-type switching in these mutants. The behavior of *swi6*⁻ can be explained by a highly skewed switching ratio in favor of P-to-M events (Thon and Klar, 1994) as well as by a low spontaneous

frequency of chromosomal rearrangements (Lorentz *et al.*, 1992; see below). In contrast, the *swi4*⁻ and *swi10*⁻ mutants exhibit strong signals. A large body of evidence indicates that the class II genes (*swi4*, -8, -9 and -10 and *rad22*) are required for processing the gene conversion intermediates (Egel *et al.*, 1984; Gutz and Schmidt, 1985; Fleck *et al.*, 1992; Carr *et al.*, 1994; Paques and Haber, 1997; Rodel *et al.*, 1997). Unfortunately, we cannot distinguish between accumulation of gene conversion intermediates and spontaneous DNA rearrangements frequently occurring in the class II mutants. Furthermore, the *swi4*⁻ and *swi10*⁻ mutant strains produce ~10–20% elongated and dead cells (data not shown). This issue was not analyzed further and will await the construction of conditional *swi* mutant strains.

Discussion

In this study, we have revealed the existence of a covalent DNA molecule, bridging the acceptor *mat1* locus with the distal donor *mat2P* or *mat3M* loci mostly during S-phase. Such DNA intermediates are present in homothallic wild-type strains but are reduced or absent in slow or non-switching heterothallic mutant strains (except for the class II mutants). Taken together, we conclude that this novel DNA species is involved in the gene conversion event responsible for the mating-type switching during the DNA replication period in *S.pombe*.

The kinetics of the appearance and disappearance of

Fig. 6. Replication-recombination coupled model. Only the mating-type switches from P to M using the leading strand synthesis are represented for clarity. The H1, H2 and H3 boxes are indicated and the DNA modification on the *mat1* upper strand is symbolized by an asterisk. The modified *mat1P** locus in G₁-phase (a). The interrupted replication fork arriving from the right provided the 3'-OH invading strand (b). A double-stranded DNA end is formed transiently (c). Strand invasion in the H1 homology box of the opposite silent cassette, *mat3M* in this example (d). DNA synthesis is initiated and the newly synthesized strand is displaced from its template, forming a migrating D-loop (Ferguson and Holloman, 1996) (e). When copy synthesis has passed the H2-H3 boxes (DNA intermediates used as template in the PCR assay), an intramolecular secondary structure can be formed (f), recognition by the *swi4/8* gene products may stop DNA synthesis, followed by resolution mediated by the *swi9/10* gene products (see the text for details). Upon resolution, DNA synthesis can proceed, giving one unswitched chromatid (*mat1P**) capable of switching again during the next replication and one switched-intact chromatid (*mat1M*), which will be modified asymmetrically (*) during the next replication (z).

the gene conversion intermediates are consistent with a replication-recombination coupled process. In Figure 6, we propose that the leading strand replication complex, coming from the distal side of *mat1*, is stalled by the

DNA modification on the upper strand at the junction of the H1 region and the *mat1*-specific allele (Arcangioli, 1998; Dalgaard and Klar, 1999) and provides the 3'-OH-ended invading strand (Arcangioli, 1998; Figure 6, step b). The nature of the DNA modification (nick or RNA) at *mat1* is an important question, which is not addressed in the present study. However, it is assumed that DNA replication up to a single-stranded nick will produce a double-stranded DNA end (Arcangioli, 1998). Alternatively, a double-stranded DNA end can be produced upon DNA replication arrest (Michel *et al.*, 1997). The superimposition of recombination on replication is reminiscent of Belling's model known as the copy choice process, proposed many years ago as an alternative model for homologous recombination (Belling, 1933), a form of recombination-dependent DNA replication, somewhat similar to the break-induced replication mechanism (Mosig, 1987; Kogoma, 1997; Haber, 1999, and references therein).

The 30 min delay between the beginning of *mat1* replication and the appearance of the DNA intermediates may reflect the time required for the initial steps of mating-type switching. These steps include positioning of the opposite mating-type cassette next to the *mat1* locus, strand invasion by the newly synthesized 3'-OH-containing strand into the homologous H1 sequences, followed by polymerase assembly and priming (Figure 6, steps b-d). DNA synthesis of the opposite mating type occurs as a migrating D-loop (Ferguson and Holloman, 1996) (Figure 6, step e) or as a small replication bubble coordinating leading and lagging strand synthesis (Holmes and Haber, 1999). In the absence of silent loci (Klar and Miglio, 1986), it is assumed that another process involving breakage or reorganization of the replication fork will bypass the DNA modification. This may occur by recombination with the newly replicated DNA formed on the opposite strand brought about by the converging replication fork, which lacks the modification. This is a variation of the template-switching model proposed for *trans*-lesion DNA synthesis (Friedberg *et al.*, 1995), which is analogous to the type of sister chromatid exchange observed in bacteria, genetically in yeast and cytologically in mammalian cells (Wolff *et al.*, 1974; Formosa and Alberts, 1986; Kadyk and Hartwell, 1995; Zou and Rothstein, 1997).

The process of mating-type switching recruits many proteins (at least the products of the *swi* genes) that are normally required in other pathways. The class Ia genes are most likely to be required in the early steps of switching, preparing *mat1* DNA, one generation before gene conversion (Klar, 1987; Singh and Klar, 1996; Dalgaard and Klar, 1999). The class Ib genes seem to facilitate the assembly of the mating-type region into a higher order chromatin structure (Thon and Klar, 1993). Numerous studies have indicated that class II genes are involved in general DNA repair processes. The *swi4* and *swi8* genes share strong homology with the *MSH3* and *MSH2* subgroups, respectively, involved in the mismatch repair pathway (Fleck *et al.*, 1992; Rudolph *et al.*, 1999). The *swi9* and *swi10* genes share strong homology with the *RAD1* and *RAD10* genes, respectively, from *Saccharomyces cerevisiae* involved in the nucleotide excision repair pathway (Schmidt *et al.*, 1989; Schlake *et al.*, 1993; Tomkinson *et al.*, 1993; Bardwell *et al.*,

1994; Carr *et al.*, 1994; Rödel *et al.*, 1997). Finally, *rad22* is homologous to *RAD52*, which is indispensable in the recombinational repair of DSB in *S.cerevisiae*. However, *rad22* phenotypes do not match the severity of *rad52*, especially with regard to mating-type switching (Ostermann *et al.*, 1993). A hypothetical intramolecular secondary DNA structure between the H2 and H3 regions has been proposed recently (Rudolph *et al.*, 1999). Our data support the notion that the gene conversion event goes beyond the H2 and H3 sequences in order to allow the formation of this hairpin structure (Figure 6, step f). The generation of this secondary DNA structure might be facilitated by a looping out occurring when the gene conversion process is copying the H2 and H3 region and serves as a target for the *swi4/swi8* gene products, constituting a signal for correct termination of DNA synthesis. The resolution of the gene conversion intermediates might be carried out by the endonuclease activity of the products of the *swi9/swi10* genes (Figure 6). This last scenario is reminiscent of what is proposed for mating-type switching resolution in *S.cerevisiae* (Saparbav *et al.*, 1996; Sugawara *et al.*, 1997).

Depending on the switching direction and on the efficiency of gene conversion resolution, two types of DNA rearrangements were observed (Beach and Klar, 1984; Fleck *et al.*, 1990). When the cell switches from P to M (Figure 1B) and gene conversion does not terminate at the H2–H3 boundary (from *mat3M*), the 11 kb between *mat2* and *mat3* are duplicated in a direct configuration. On the other hand, when the cell switches from M to P (Figure 1C) and gene conversion does not terminate at the H2–H3 boundary (from *mat2P*), the 15 kb between *mat1* and *mat2* are also duplicated in a direct configuration, or produce an extrachromosomal *mat2:1°* circle. The frequency of DNA rearrangements increases in the class II mutants, and strains with up to seven cassettes in the mating-type region were found (Fleck *et al.*, 1990).

Zou and Rothstein (1997) have shown recently that Holliday junction recombination intermediates (xDNA) are detected in the rDNA repeats in *S.cerevisiae*, only in S-phase. The major consequences of this recombination event are the homogenization of the rDNA sequences, variation of the copy number and formation of extrachromosomal rDNA circles (ERCs). The appearance and accumulation of ERCs strongly correlate with aging (Sinclair and Guarente, 1997). Notably, the absence of the *FOB1* gene, which participates in the unidirectional replication fork block in the rDNA, decreases the number of ERCs in aging cells (Defossez *et al.*, 1999). The molecular basis of the generation of ERCs is still under investigation (Defossez *et al.*, 1999; Rothstein and Gangloff, 1999). Defects in the general DNA repair processes may increase the frequency of the spontaneous appearance of ERCs in *S.cerevisiae*, and decrease the efficiency of reintegration into the chromosomal rDNA cluster (Kim and Wang, 1989; Fleck *et al.*, 1990), together leading to the accumulation of ERCs (Rothstein and Gangloff, 1999). It is possible that in both systems, stalled replication forks might be involved in the formation of extrachromosomal DNA circles.

Our replication–recombination model proposes that the mating type of a switching cell does not replicate its sexual locus by a conventional DNA replication process,

although, as in *S.cerevisiae*, all the components necessary for genomic replication might be necessary for mating-type switching (Holmes and Haber, 1999). For clarity, we did not present the progression of the lagging strand synthesis in Figure 6. Finally, in every generation, ~20% of the cells delay the replication of the mating-type locus by 30 min in a cell cycle period that can formally be considered as a late S-phase (Figure 4B).

Despite growing understanding of asymmetric mating-type switching, generating only one switched cell among two sister cells, we still do not know how the DNA modification at *mat1* is asymmetrically distributed and maintained on only one of the two sister chromatids after each DNA replication cycle and how the cell can maintain two heterologous sequences at *mat1* during the G₂-phase. These remarks indicate the intriguing properties of this locus, since at each generation the asymmetry of the two sister chromatids seems to be undetected by the recombination–repair machinery.

Materials and methods

Strains

The *S.pombe* strains used were originally derived from Leupold's culture: 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). SP838 *h⁹⁰ swi1 ade6-M216*; EG371 *h⁹⁰ swi2-S73 lys1*; EG743 *h⁹⁰ swi4-1 ura4-Δ18*; EG372 *swi5-S39 lys1*; PG1 *h⁹⁰ swi6-115 leu1-32 ura4-Δ 18 ade6-M216*; SP112 *h⁹⁰ swi7- leu1-32 ade6-M216*; EG741 *h⁹⁰ swi10::ura4 ura4-D18*; gifts from Genevieve Thon, Richard Egel, Amar Klar, Henning Schmidt and Oliver Fleck.

Synchronized culture

The homothallic *h⁹⁰ cdc25* strain (Russel and Nurse, 1986; Arcangioli, 1998) was shifted to the restrictive temperature (36°C) for 4 h (arrested at the G₂–M transition) and re-incubated at 24°C, allowing a synchronous entry into the cell cycle. Samples of the cells were harvested at intervals and either treated for FACS analysis or genomic DNA was prepared in agarose plugs and analyzed by the PCR assay in which the P1 primer was ³²P labeled. The reactions were analyzed by 6% native polyacrylamide gel electrophoresis, dried and exposed to a phosphor screen (PhosphorImager 445SI) and quantified using ImagequantNT. Flow cytometry analysis was described earlier (Arcangioli, 1998), and the propidium iodide-stained cells were observed microscopically in parallel in order to distinguish G₂ and M phases unambiguously.

Genomic DNA preparation and PCR assay

Genomic DNA was prepared in agarose blocks as described earlier (Arcangioli, 1998), digested by the *HindIII* restriction enzyme, melted at 95°C and the resulting single-stranded genomic DNA fragments were extracted and purified using a QIAquick gel extraction kit (Qiagen). The strategy consisting of melting the double-stranded genomic DNA was used to avoid any preferential shearing of recombination intermediates during DNA manipulation. A similar strategy has been used previously with no success, presumably due to the DNA extraction procedure used (White and Haber, 1990). The PCR assay consisted of a first incubation of 5 min at 95°C, then *Taq* polymerase (Promega) was added, followed by the cycle steps (1 min at 95°C, 1 min at 52°C and 4 min at 72°C). The PCR products were resolved through agarose gel electrophoresis and stained with ethidium bromide. The primer sequences are: P1, CGAAGCAAATATCTCGTTAGAGG; P'1, ATGTAAAGAGTGGGT-AGATGCAT; P2, TTATATGTAGTTTATAATTGTTGTGTC; P3, TTGGCAGCTCGTAGGCTT; and P4, AGCAACTCTGATACATT-ACA. When P1 or P'1 were ³²P end labeled, the PCR products were resolved through a 4% native polyacrylamide gel, fixed, dried and exposed on a phosphor screen and the signals detected by PhosphorImager 445SI (Molecular Dynamics) and quantified using ImagequantNT. All PCR amplification experiments were conducted at least three times, using independent sources of genomic DNA.

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