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Fate of *mat1* DNA strands during mating-type switching in fission yeast

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The mating-type switching of the fission yeast, *Schizosaccharomyces pombe*, is highly regulated. Two consecutive asymmetric divisions are required to produce one mating-type switched cell among the four progeny. Using DNA density-gradient centrifugation we demonstrate that one-fourth of the *mat1* DNA is not replicated by the conventional semi-conservative mode, but instead both DNA strands are synthesized *de novo*. Our data are consistent with a gene conversion event, initiated by a site- and strand-specific DNA break (SSB). We further demonstrate that the virgin switched *mat1*-containing chromatid no longer contained the nick, while it is reintroduced during the lagging strand synthesis of the *mat1* locus on the sister chromatid. This finding establishes at the molecular level a firm experimental link between the phenotype and genotype in the process of asymmetric mating-type switching during mitotic divisions.

INTRODUCTION

Haploid cells of the fission yeast, *Schizosaccharomyces pombe*, exhibit a homothallic life cycle, in which the mating-type of the cell mitotically alternates (Figure 1A) producing a population of P (+) and M (–) cells (Leupold, 1950; Egel, 1977). The mating-type region contains three loci in the right arm of chromosome II: the expressed *mat1* locus and the two silent *mat2P* and *mat3M* loci (Egel and Gutz, 1981; Beach, 1983; Beach and Klar, 1984; Kelly *et al.*, 1988). In a process similar to gene conversion, the two silent *mat2P* and *mat3M* loci can donate their genetic information to *mat1* (Figure 1B). The efficiency of this process leads to a clonal haploid cell population containing roughly the same proportion of P and M cell types.

During vegetative growth, the mating-type locus is not expressed. Consequently the cells are genotypically different at the mating-type locus (either *mat1P* or *mat1M*) but phenotypically identical (formally sterile). When cells experience starvation, they arrest in the G_1 phase and the mating-type alleles (*P* or *M*) present in *mat1* are transcriptionally induced (Kelly *et al.*, 1988), allowing conjugation between cells of opposite mating-type followed by sporulation. The pattern of mating-type switching in the mitotic lineage has been determined in conditions approaching nitrogen starvation. These studies show (Figure 1A) that two consecutive asymmetric divisions are required, generating one switched and three unswitched cells (Miyata and Miyata, 1981; Egel, 1984). Furthermore, the sister of the switched cell is competent for switching during the next

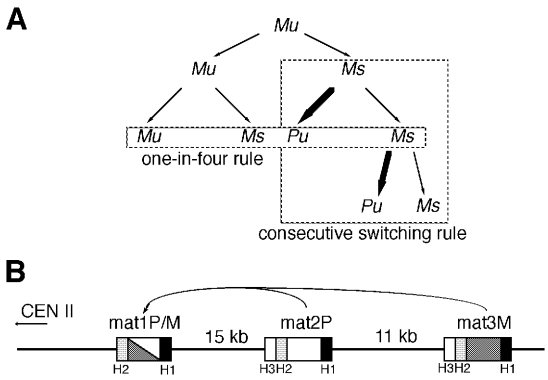

Fig. 1. Mating-type pedigree and loci of fission yeast. (**A**) *P* and *M* indicate the mating-type of the cell, the suffix *u* or *s* represents the unswitchable and switchable potential of the cell, respectively. For simplicity, only the *M* lineage is shown. The one-in-four and the consecutive switching rules are indicated. (**B**) The mating-type region on chromosome II. The *mat1* locus contains either the P (white box) or the M (grey box) alleles, and *mat2P* and *mat3M* are donors of genetic information. The H1 and H2 homology sequences are common to all cassettes whereas the H3 sequence is common to only the silent *mat2P* and *mat3M* loci (Kelly *et al.*, 1988).

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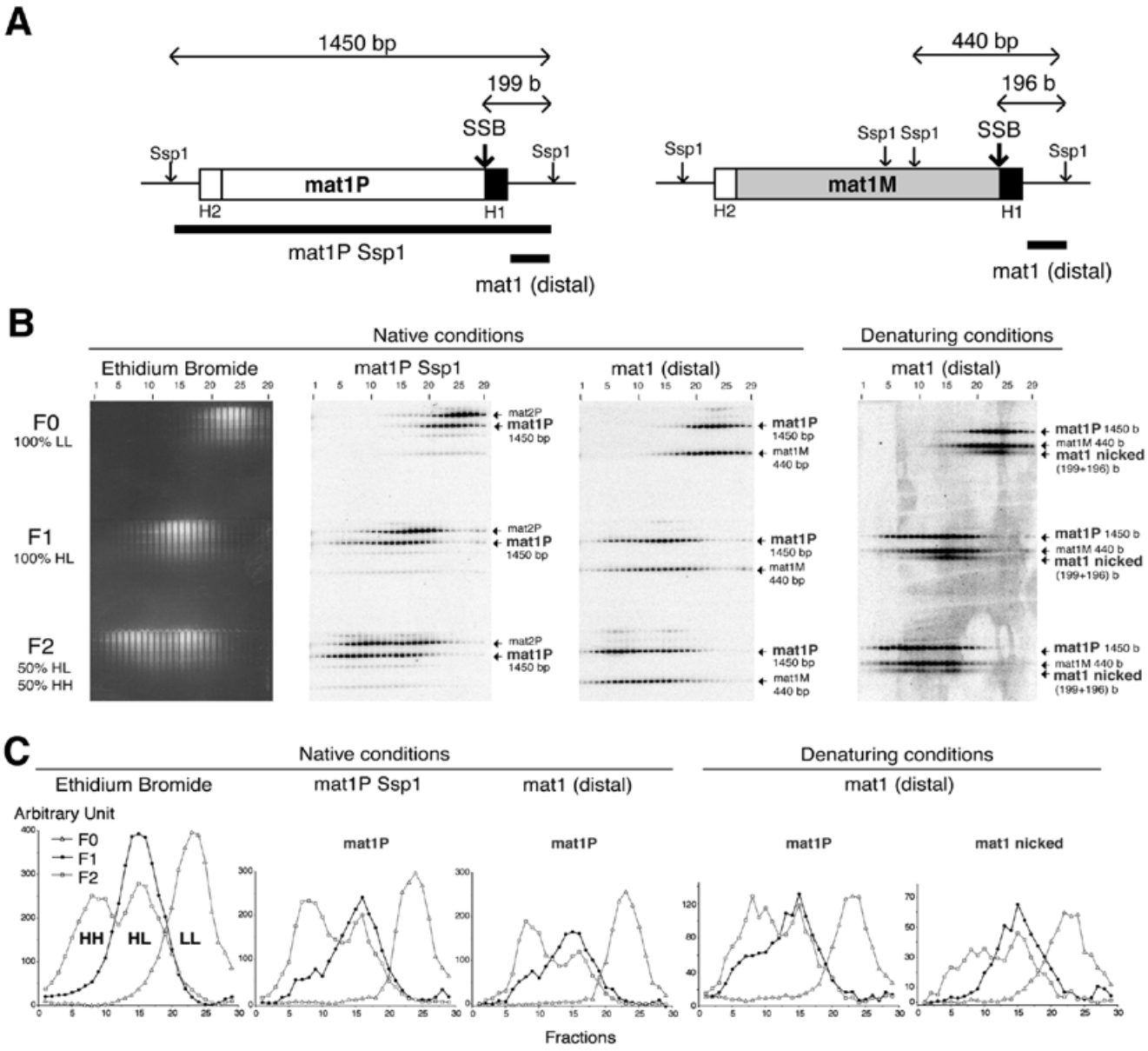

Fig. 2. Density distribution of the *mat1* locus. (**A**) The location of the *Ssp*I restriction sites at the *mat1* locus and their respective sizes are indicated. The position of the site- and strand-specific break (SSB) is shown by an arrow. 199 and 196 bases are the sizes of the *mat1P*- and *mat1M*-distal upper DNA strand, following *Ssp*I digestion. The H1 and H2 sequences are indicated and the two DNA probes used are shown at the bottom. (**B**) Distribution of the *mat1* locus analysed by DNA–DNA hybridization. DNA samples from the CsCl-eluted fractions were electrophoresed in native or denaturing conditions, as indicated. F_0, F_1, F_2, LL, HL and HH indicate the number of generations in heavy media and the double-stranded DNA density, respectively. The probes used (A) are double stranded and are indicated at the top of the autoradiogram. Note that the same probe, *mat1* (distal), is used for the Southern blots performed in native and denaturing conditions (right panel). The names and sizes of the DNA fragments are indicated by small arrows. The weak hybridization signals are due to the H1 and H2 sequences present at the three loci. (**C**) Quantification of the blots is shown above using the same nomenclature. The corresponding restriction DNA fragments are indicated in bold. The density distribution of the *mat1M* DNA fragments in denaturing conditions behaves similarly to *mat1P* and is not shown.

division cycle, forming a recurrent chain of asymmetric switching (Egel and Eie, 1987; Klar, 1987, 1990).

It was proposed that this switching pattern results from an imprinting event, marking one of the sister chromatids in a strand-specific manner at the *mat1* locus (Klar, 1987). Recent molecular experiments revealed the presence of a site- and strand DNA-specific break (Arcangioli, 1998) or DNA modification (Dalgaard and Klar, 1999) at the *mat1* locus, which prepares the switching event during the next round of DNA replication. The involvement of the orientation of the replication fork (Arcangioli, 1998; Dalgaard and Klar, 1999), together with the presence of a transitory gene conversion intermediate during S-phase, strongly supports the notion that DNA replication and recombination at *mat1* are coupled (Arcangioli and de Lahondès, 2000). This

process restricts mating-type switching to only one of the two sister chromatids during *mat1* DNA replication.

In an attempt to correlate the phenotypic switching pattern with the fate of the *mat1* DNA strands during growth, we used density-gradient centrifugation (Meselson and Stahl, 1958). We compared the density distribution of the *mat1* locus from switchable (h^{90}) and non-switchable strains. We also determined the density distribution of *mat1* DNA during mitotic divisions and examined the distribution of the broken *mat1* DNA strand. The results are interpreted in molecular terms that reveal the inheritance of *mat1* DNA strands during asymmetric mating-type switching.

RESULTS AND DISCUSSION

In the current study, we followed the inheritance of *mat1* DNA strands during mitotic divisions by performing Meselson–Stahl type experiments (Meselson and Stahl, 1958). A homothallic wild-type cell population grown in normal (light) minimum medium was enriched in small G_2 cells (see Methods). The partially synchronized cells were allowed to resume growth in an isotopically dense medium. Samples of cells were taken at time 0 and after one or two generations (F_0, F_1, F_2). Figure 2B (left panel) shows the density distribution of the *Ssp*I-digested genomic DNA in the CsCl-eluted fractions after electrophoresis in an agarose gel. DNA quantification (Figure 2C, left panel) is typical of the semi-conservative genomic DNA replication mode.

DNA from the agarose gel was transferred to a filter, and the density distribution of the mating-type loci was determined by quantitative hybridization. The *mat1P Ssp*I probe (Figure 2A) mostly detects the *mat1P* and *mat2P* loci (Figure 2B, second panel). The intensity of the *mat2P* signal is about twice the intensity of the *mat1P* since only 50% of *mat1* locus contains the P allele, while the other half contains the M allele. The density distribution of the *mat2P* DNA fragment in the three samples resembles the distribution of the genomic DNA (Figure 2B). However, the density distribution of the *mat1P* DNA fragment diverged from the *mat2P* and the total genomic DNA in several respects. In F_0, *mat1P* is slightly shifted compared with *mat2P* (Figure 2B). A similar difference in density was observed with *Ssp*I DNA fragments purified from *Escherichia coli* (data not shown) and reflects the intrinsic density imposed by the nucleotide sequence composition flanking the mating-type cassettes. In F_1, the *mat1P* DNA fragment is not only confined to the HL density fractions, but also spreads into the HH density fractions (Figure 2B, second panel in fractions 5–10). This demonstrates that both DNA strands have been synthesized *de novo*, in one generation, in a fraction of the cells. In F_2, *mat1P* is more abundant in the HH than in the HL density (Figure 2B). A very similar distribution pattern is observed when the membrane is hybridized with the *mat1* (distal) probe (Figure 2A), which detects both *mat1P* and *mat1M* (Figure 2B and C, third panels).

In order to confirm the intriguing density distribution of the mating-type locus we directly compared the *mat1* density distribution in F_1 of the wild-type h^{90} and the non-switchable, *mat1-Msmt0* mutant, strains. The *mat1-Msmt0* mutant contains a deletion of 262 bp (Figure 3A) next to the *mat1M* locus, abolishing mating-type switching (Arcangioli and Klar, 1991; Styrkarsdottir *et al.*, 1993). Wild-type and *mat1-Msmt0* mutant mixed culture

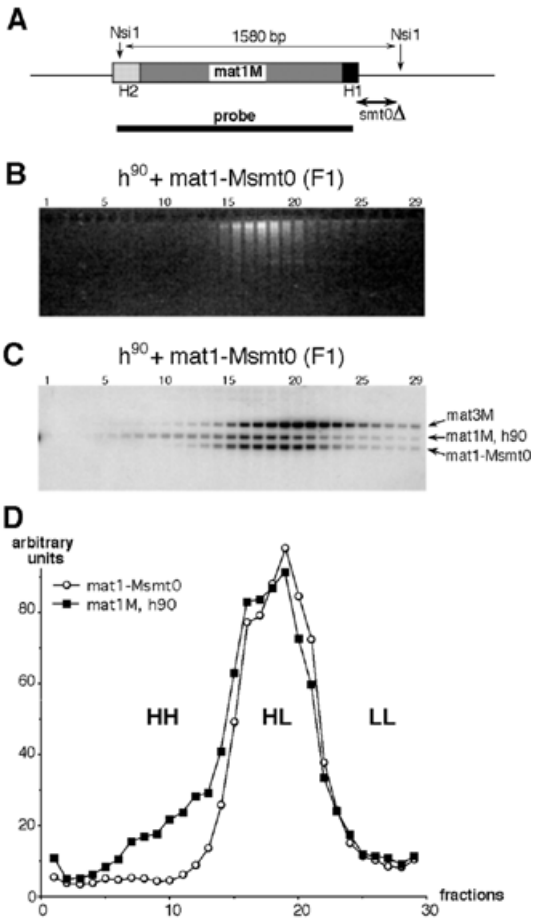

Fig. 3. Density distribution of the *mat1* locus from homothallic (h^{90}) and heterothallic strains in F_1. (**A**) Schematic representation of the *mat1M* locus on chromosome II. The *Nsi*I restriction sites flanking *mat1M* are indicated. The position of the deletions in the *mat1-Msmt0* mutant strain is shown as well as the probe used. (**B**) The co-culture of wild-type (h^{90}) and *mat1-Msmt0* strains (as indicated) was shifted from light to heavy medium and cells were collected after one generation (F_1). DNA samples from the CsCl-eluted fractions were separated by electrophoresis on agarose gel and stained with ethidium bromide. (**C**) Density distributions of the mating-type loci. DNA from the agarose gel was analysed by DNA–DNA hybridization. The names of the DNA fragments revealed by the *mat1M* probe (see above) are indicated. (**D**) Quantification of the hybridization signals. The *mat1M* signal from the wild-type strain (h^{90}) was multiplied by 1.8 for comparison with the *mat1-Msmt0* signal. HH, HL and LL indicate the density of the DNA fragments in the CsCl gradient.

grown in light media were shifted to heavy media [glucose (^{13}C) and $^{15}NH_4Cl$] for one generation. The genomic DNA from both strains was purified, digested with the *Nsi*I restriction enzyme and submitted to CsCl gradient centrifugation. Genomic DNA from the CsCl-eluted fractions was separated on an agarose gel (Figure 3B) and the density distribution of the *mat1* locus was analysed by DNA–DNA hybridization. Three DNA fragments (Figure 3C) are observed after hybridization with the *mat1M* probe (Figure 3A). The relative intensity of the signals corresponds to the number of loci. The *mat3M* locus is present in both strains. The *mat1M* signal from the wild-type strain is roughly

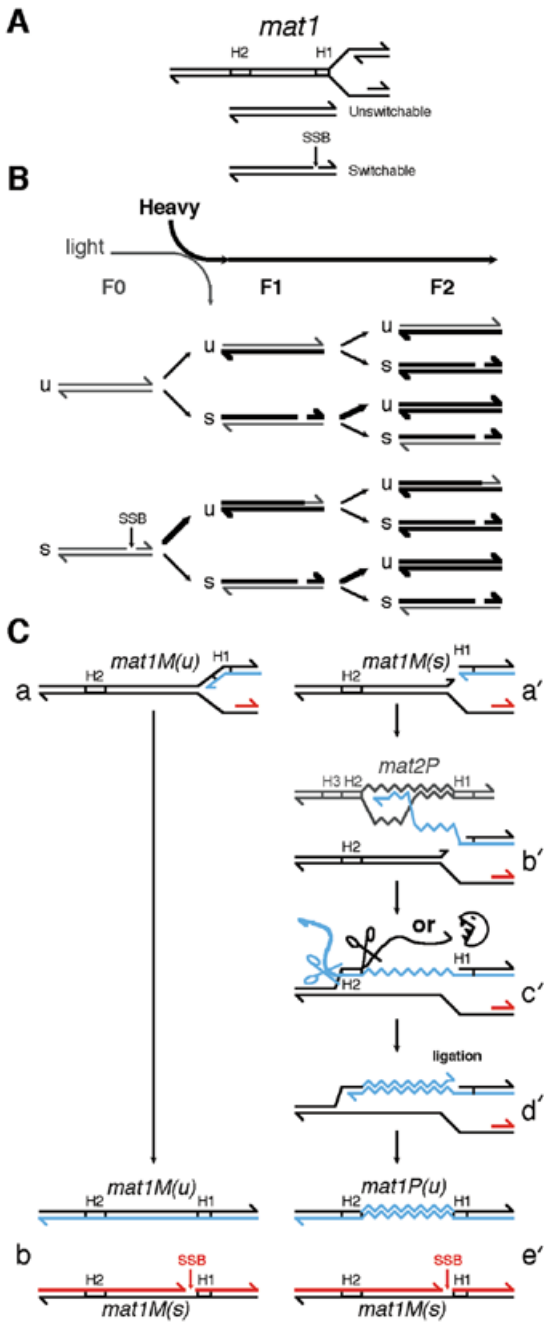

Fig. 4. Molecular pedigree of the *mat1* locus during mitotic divisions. (**A**) The top of the figure shows the two DNA strands (upper and lower) of the *mat1* locus and the direction of the replication fork. The intact and nicked (SSB) *mat1* loci are called switchable and unswitchable, respectively. The position of the nick is indicated on the upper DNA strand at the junction of the *mat1* specific allele (*P* or *M*) and the H1 homology box. (**B**) In a growing population, one half of the chromatids contain an intact *mat1* locus that is not competent for switching (*u* for unswitchable). The other half contains a nick, restricting mating-type switching (*s* for switchable) to only one of the two sister chromatids during the next round of DNA replication. Since the *P* lineage is the mirror image of the *M* lineage, only the suffixes *u* and *s* are emphasized for simplicity. The density of the medium and the generations are indicated. The thickness of the DNA strands indicates the density. (See text for details). (**C**) The replication fork progresses from the right side of *mat1M(u)* (a). Following replication, the newly replicated lagging strand is broken (SSB), producing an unswitchable *mat1M(u)* and a switchable *mat1M(s)* loci (b). The replication fork progresses from the right side of *mat1M(s)* and is blocked by the nick (a′). Strand invasion occurs at the H1 homology box of the opposite mating-type locus (*mat2P* in this example) and DNA synthesis is initiated forming a migrating D-loop (Ferguson and Holloman, 1996) or a small replication bubble that coordinates leading and lagging strand synthesis (Holmes and Haber, 1999) (b′). Following strand annealing in the H2 homology region (c′) both single-stranded DNAs are digested or clipped off, presumably by swi9p/swi10p (Rad1/Rad10 in *S. cerevisiae*) near the H2 homology sequences, providing 3′ ends for priming leading strand and filling-in DNA synthesis and ligation (d′). Ligation and the single strand break are probably coupled. Finally a switched ligated chomatid [*mat1P(u)*] and an unswitched chromatid [*mat1M(s)*] are formed following replication in G_2 (e′). The leading strand is coloured in blue and the lagging strand in red.

half the level of the *mat1-Msmt0* signal, since half of the wild-type *mat1* locus contains the P allele and the other half contains the M allele. The density distribution of the *mat1M* locus clearly spreads into the HH density fractions (Figure 3C, fractions 6–13), which contrasts with the *mat1-Msmt0* and *mat3M* loci. The quantification indicates that 20–25% (Figure 3D) of the *mat1M* locus have both DNA strands newly replicated in one generation. Finally, the observation that the copy transposition products are separated from the bulk of genomic DNA in F_1 by density centrifugation indicates that this approach can be used to isolate known or unknown recombinant sequences in a wide range of biological systems.

Quantification of three independent experiments shows that 20–25% of *mat1* is present in the HH density in F_1, and 60–65% in F_2. It is assumed that during mitotic growth, half of the cell population is competent for mating-type switching (Figure 4, the switchable chromatids). Since this gene conversion is restricted to only one of the two sister chromatids, it follows that 1/4 (25%) of the *mat1* locus should undergo gene conversion (Figure 4B, F_0 to F_1). This will replace the two *mat1* parental DNA strands by copying the opposite mating-type allele during DNA replication (Arcangioli and de Lahondès, 2000). A simple extrapolation of this rule to the next generation produces an HH density proportion of 5/8 (62.5%) in F_2 (Figure 4B). This result, together with the mating-type switching pedigree pattern, strongly supports the model shown in Figure 4B, which is an extension of the strand segregation model (Klar, 1990). Furthermore, this process preserves the nucleotide integrity of the donor of genetic information, *mat2P* (Figure 2B) and *mat3M* (Figure 3C), since their DNA strands followed the same density distribution as the genomic DNA. Consistently, the *mat1* DNA fragment reveals an informative shoulder at the HH side of the density profile after one generation, with no deviation in the LL side of the pattern (see Figures 2 and 3). This process, proposed more than 60 years ago by Belling (1933), is known as the copy-choice recombination model (for review see Kogoma, 1997; Haber, 1999).

The density transfer approach offers a unique opportunity to address the question of the fate of the DNA modification at *mat1* during growth. Genomic DNA in the CsCl-eluted fractions was denatured with formamide and formaldehyde, run on a formaldehyde gel, transferred to a filter and hybridized with the *mat1* (distal) probe (Figure 2A). The denaturing conditions allow for the observation of three DNA fragments, instead of the two observed under native conditions (Figure 2B, right panel). The fast migrating additional band corresponds to the denatured

mat1-distal upper strands of 199 and 196 bases (Figure 2A), melted from the complementary *mat1P* and *mat1M Ssp*I DNA fragments, respectively (Nielsen and Egel, 1989; Arcangioli, 1998). This result is more compatible with the presence of a nick at *mat1* (Arcangioli, 1998) rather than an alkali-labile DNA modification (Dalgaard and Klar, 1999). Such a DNA strand break will stall leading strand replication restricting mating-type switching to only one chromatid (Arcangioli, 1998; Dalgaard and Klar, 1999; Arcangioli and de Lahondès, 2000; Rothstein *et al.*, 2000).

In denaturing conditions, the additional fast migrating band is present in the HL fractions and absent in the HH fractions of the density gradient in F_1 (Figure 2B and C, right panels). If a nick were also present in the recently switched *mat1* (P or M) the additional band will also be observed in the HH fractions. This result demonstrates that the recently switched *mat1* locus, made of two neosynthesized DNA strands, does not contain the single strand DNA break. This virgin allele is not competent for switching during its next division. On the other hand, its sister inherits the competence for switching and hence the nick. The direction of the replication fork (Arcangioli, 1998; Dalgaard and Klar, 1999), together with the nicked *mat1* locus in the HL density in F_1, indicates that the switchable cells inherit the intact parental lower DNA strand. It follows that the old nicked strand must be removed and synthesized *de novo* (Figure 4C, step c') ruling out the possible conservation of the old nicked strand. This newly synthesized DNA strand must be cut again or remains unligated to the adjacent DNA strand. This probably occurs during lagging strand DNA synthesis (Singh and Klar, 1993) (Figure 4C) but does not exclude the participation of another replication fork arriving from the other side. The switching and cutting processes are tightly coupled in time since the steady state level of the nick appeared to be constant during S-phase (Arcangioli, 1998). However, the mechanism responsible for the nick is not coupled to switching *per se* since the same process occurs during replication of the unswitchable chromatid (Figure 4C, b and e' steps) and presumably requires the *cis*-acting sequences next to *mat1* (Arcangioli and Klar, 1991; Klar *et al.*, 1991).

We propose that this process transfers both newly synthesized DNA strands to the *mat1* locus during mating-type switching. This type of gene conversion process occurs during S-phase, is initiated at the nick in the H1 sequences and is resolved in the H2 sequences without crossing over (Arcangioli and de Lahondès, 2000). This replication/recombination coupled process is reminiscent of recombination-dependent DNA replication (RDR) in *E. coli* (Asai *et al.*, 1994; Kogoma, 1997) and synthesis-dependent strand annealing (SDSA) in *Drosophila*, Ustilago and the yeast *Saccharomyces cerevisiae* (Nassif *et al.*, 1994; Ferguson and Holloman, 1996; Haber, 1999). The present work establishes for the first time a firm experimental link between phenotype and genotype in the process of asymmetric mating-type switching in this organism. Such a link has previously been inferred indirectly according to criteria, based on mutant behaviour and cellular pedigrees, but direct positive evidence has been unavailable.

METHODS

Yeast growth conditions. Wild-type homothallic or *mat1-Msmt0* strains were grown at 32°C in 1 l of standard minimal medium (Moreno *et al.*, 1991) containing 1% glucose and 0.2% NH_4Cl. Cells were harvested by centrifugation (5 min at 5000 r.p.m.), resuspended in minimal medium, loaded onto a lactose gradient (7–30%) and centrifuged for 8 min at 1000 r.p.m. in a Jouan CR412 centrifuge (Barbet and Carr, 1993). The smallest cells at the top of the gradients were collected (5–10% of the starting material) and pelleted by centrifugation. One half was washed in 0.2 M EDTA, 0.01% azide at 4°C (the F_0 sample) and the other half was incubated in 100 ml of prewarmed minimal heavy media containing 1% glucose ($^{13}C_6$) and 0.2% $^{15}NH_4Cl$ (Advanced Research in Chemistry). In these conditions, the presence of both glucose and nitrogen is essential for cells to resume growth (data not shown). The cultures were followed by counting the cells, measuring the septation index and by flow cytometry, indicating 30–40% of cell synchrony (data not shown). Fifty milliliters of the culture were harvested after the first generation and 25 ml after the second, during the G_2 phases, referred to as the F_1 and F_2 samples, respectively. The G_2 phase is the longest cell cycle phase in *S. pombe*, occupying about three-quarters of the cell cycle.

DNA preparation and analysis. Genomic DNA was prepared and digested by *Ssp*I or *Nsi*I in agarose plugs (Arcangioli, 1998), purified (Quiagene kit) and the CsCl gradient centrifugation was performed essentially as described (McCarroll and Fangman, 1988). Collected fractions (0.2 ml) were isopropanol precipitated, resuspended in TE and samples of DNA fractions were analysed in agarose gels. In denaturing conditions, the DNA was mixed with loading dye to a final concentration of 15% formamide, 2% formaldehyde, 10 mM HEPES, 2 mM EDTA, 7% glycerol pH 7.8 and incubated for 5 min at 80°C. Electrophoresis was performed in a 1.4% agarose gel containing 6% formaldehyde for 3 h at 100 V. Separated DNA was transferred onto Hybond-N+ membrane (Amersham Pharmacia), hybridized, exposed to phosphor screen (Molecular Dynamics) and quantified using ImagequantNT.

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