The EMBO Journal vol.10 no.10 pp.3025-3032, 1991

A novel switch-activating site (SAS1) and its cognate binding factor (SAP1) required for efficient *mat1* switching in *Schizosaccharomyces pombe*

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Communicated by B.Dujon

The pattern of parental DNA strand inheritance at the mating type locus (*mat1*) determines the pattern of *mat1* switching in a cell lineage by regulating the formation of the site-specific double-stranded break (DSB) required for mating type interconversion in *Schizosaccharomyces pombe*. To study the molecular basis of this programmable cell type change, we conducted structural and functional analyses of the DNA sequence flanking the DSB at *mat1*. We have identified and characterized a DNA-binding activity that interacts with a specific sequence located 140 bp from the DSB site. Deletion analysis of DNA sequences located distal to *mat1* cassette revealed the presence of at least two switch-activating sites (SAS1 and SAS2), both of which are required for generating an efficient level of DSBs and consequently, for efficient switching. We found that SAS1 overlaps with the target site of the DNA-binding activity called SAP1 (for switch-activating protein). Point mutations generated in the SAS1 element that adversely affect binding to SAP1 protein *in vitro* were found to reduce the efficiency of switching *in vivo*, suggesting the requirement of SAP1 for switching. Pedigree analysis revealed that SAS1 is equally required for initial switching (one switch in four granddaughters of a cell) and for consecutive switching (where the sister of a recently switched cell switches again), indicating that the two developmentally asymmetric cell divisions required to generate a particular pattern of switching share the same molecular control mechanism.

Key words: double-stranded break/fission yeast/mating type switching/stem cell lineage/switch-activating protein/switch-activating site

Introduction

Haploid cells of the fission yeast *Schizosaccharomyces pombe* exist in two different and complementary mating types, called *P* (plus) and *M* (minus) (Leupold, 1950). In a wild-type homothallic strain (designated h^{90}), a spontaneous and efficient reversible genetic change between *P* and *M* mating types occurs (for recent reviews see: Egel, 1989; Klar, 1989). Consequently, a colony grown from a single cell of either *P* or *M* type contains nearly equal proportions of *P* and *M* cells. Under conditions of nutritional deprivation, cells of opposite mating types conjugate to form a temporary diploid, which undergoes meiosis to yield four haploid spores that will again repeat the switching cycle (Leupold, 1950).

When cellular growth was monitored at the single-cell level under conditions allowing mating, Miyata and Miyata (1981) observed that the pattern of switching in a cell lineage was not random. Among a pair of sister cells, one member grew to produce one switched and one unswitched progeny, while the other member never produced switched progeny. This observation implied that two consecutive asymetric cell divisions are required to restrict switching of one cell among four related (sister plus cousin) cells (the one-in-four granddaughters switching rule). Further pedigree analysis has shown that the sister of the recently switched cell will produce one switched and one unswitched daughter in the following cell division repeatedly in ~90% of the cases (the consecutive switching rule) (Egel and Eie, 1987; Klar, 1990). The switching appears to be formally a G_2 event, as only one of the two daughters of a switching-competent cell actually switches. The observed switching pattern is analogous to the mammalian stem cell lineage, where a cell produces one daughter like itself while the other daughter is advanced in the developmental programme (Klar, 1990).

Switching studies of diploid cells have suggested a chromosomal basis for the segregation of developmental asymmetry (Egel, 1984b). More recently, molecular and genetic results have been used to propose a specific strand-segregation model (Klar, 1987, 1990) for explaining the one-in-four and consecutive switching rules. This model proposes a DNA strand-specific imprinting event located within the 1450 bp *mat1* DNA sequence (contained in an *Sspl*−*Sspl* fragment). The imprinting event is suggested to be a precursory state required to generate a double-stranded break (DSB) of the chromatid that inherits the parental imprinted strand, probably during DNA replication.

Genetic and molecular analyses have shown that mating type phenotype is dictated by a complex locus contained in <50 kb of sequence located in the long arm of chromosome II. The mating type region consists of three components, *mat1*, *mat2-P* and *mat3-M* (Egel and Gutz, 1981; Beach, 1983; Egel, 1984a; Beach and Klar, 1984). The mating type of the cell is determined by the *mat1* locus; cells with the *mat1-P* allele exhibit the *P* mating type and those with the *mat1-M* allele exhibit the *M* mating type (Leupold, 1950; Beach, 1983; Kelly *et al.*, 1988). The *mat2-P* and *mat3-M* cassettes contain the same sequence present at *mat1*, but they always remain transcriptionally inactive (Kelly *et al.*, 1988). The *mat1* switching involves transposition of sequences from the *mat2-P* or *mat3-M* 'donor' locus to *mat1* by the process of unidirectional gene conversion (Egel and Gutz, 1981; Beach, 1983; Beach and Klar, 1984).

Southern analysis of DNA isolated from yeast cells revealed that a constant proportion (~30%) of the chromosome II molecules contain a DSB at *mat1* during the entire length of the cell cycle (Beach, 1983; Egel *et al.*, 1984). The DSB has recently been mapped by genomic sequencing and found to be near the junction of the *mat1* allele-specific and the H1 (homology region 1) regions

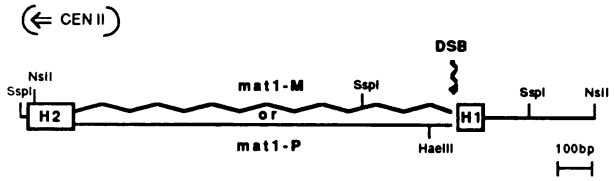


Fig. 1. Schematic representation of the *mat1* locus. H1 (59 bp) and H2 (135 bp) homology sequences (also present in the donor loci) are indicated by open boxes. *P*- (straight line) and *M*- (jagged line) specific sequences are shown. CEN indicates the position of the *mat1* locus relative to the centromere of chromosome II. The black arrow indicates the position of the double-strand break (DSB). Several restriction sites referred in the text are shown.

(Nielsen and Egel, 1989; see Figure 1). By analogy with the *Saccharomyces cerevisiae* system (Strathern *et al.*, 1982) and by employing several switching-defective mutants, it was suggested that the DSC constituted the recombination initiation event required for *mat1* switching (Beach, 1983; Beach and Klar, 1984; Egel *et al.*, 1984; Klar and Miglio, 1986).

Two classes of mutations which lower the frequency of switching by reducing the level of double strand breakage have been described. One class is comprised of *trans*-acting functions, while the other consists of *mat1 cis*-acting mutations and rearrangements. At least three *trans*-acting switch (*swi*) loci (*swi1*, *swi3* and *swi7*) are required for producing a high level of DSBs (Beach, 1983; Egel *et al.*, 1984; Gutz and Schmidt, 1985). The prototype of the second class is the mutation C13-P11 (also called *smt-s*, for slow-switching mating type), which was genetically mapped at the mating type locus (Gutz and Fecke, 1979; Beach, 1983). This mutation is a 27 bp deletion located just to the right of the *mat1* cassette (Klar *et al.*, 1991). Non-switching 'variants' maintaining very stable mating type are called heterothallic (Leupold, 1958; Gutz and Doe, 1973). These strains contain rearrangements at *mat1*, apparently resulting from aberrant resolution of switching intermediates, and create hybrid cassette fusions. It was shown by Southern blot analysis that only the hybrid cassettes containing *mat1*-distal sequences were cleaved *in vivo* (Beach and Klar, 1984; Egel *et al.*, 1984). Analysis of such *in vivo* fusions, combined with the fact that the 1450 bp *mat1*-containing sequence is switching competent, has suggested that sequences distal to *mat1* are essential for cleaving the DNA and consequently for switching. Furthermore, the pattern of switching appears to be under a *mat1 cis* control and not due to other cellular differences (Egel, 1984a; Egel and Eie, 1987; Klar, 1987, 1990). Therefore, it was of interest to undertake the functional analysis of the sequence located in the vicinity of the DSB.

Here, we describe the characterization of a DNA-binding activity (SAP1, for switch-activating protein 1) that interacts with a specific (SAS1, for switch-activating site 1 located 140 bp from the DSB site. Using a series of *Bal*31 mediated deletions, we showed that at least two *cis*-acting elements are required to efficiently produce the DSB and consequently for efficient mating type switching.

Results

Identification and characterization of a mat1-distal DNA-binding activity

Assuming that *mat1 cis*-acting sequences required for switching are located in the vicinity of the DSB and may

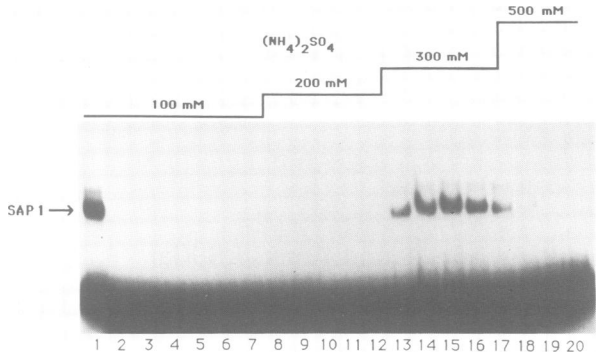


Fig. 2. Heparin–Ultrogel chromatography. Crude extract from *S.pombe* cells was chromatographed through a heparin–Ultrogel column as described in Materials and methods. DNA-binding activity was detected by a gel retardation assay using the 254 bp end-labelled *Hae*III–*Ssp*I DNA fragment bearing the DSB-flanking sequences (see Figure 1). Ammonium sulfate concentrations of each elution step are indicated. Lane 1 corresponds to the crude extract (S100); lanes 2–7 are flow-through fractions; lanes 8–20 are ammonium sulfate elution fractions.

function by interacting with *trans*-acting factors, we sought to identify those sequences and their cognate protein factor(s) by gel retardation analysis (Fried and Crothers, 1981; Garner and Revzin, 1981). For this purpose, the 254 bp *Hae*III–*Ssp*I DNA fragment of *mat1-P* (see Figure 1) was subjected to gel retardation assays. Using a crude protein extract from *S.pombe*, we detected a slowly-migrating protein–DNA complex (Figure 2, lane 1). The protein(s) responsible for the interaction was named SAP1 (for switch-activating protein 1).

For further characterization of the SAP1 activity, we fractionated the crude protein extract (S100) by passing it through a heparin column (see Materials and methods). The SAP1 DNA-binding activity, identified by gel retardation assays, eluted from the column in the 300 mM ammonium sulfate fraction (Figure 2, lanes 13–17). The DNA-binding experiment shown in Figure 2 indicated the presence of a second minor slower-migrating complex which coeluted with SAP1. Efficient competition of both activities occurred when the homologous DNA fragment was added as a competitor to the binding reaction (data not shown).

To map the DNA sequences that interact with SAP1 precisely, a DNase I protection experiment was carried out. The *Hae*III–*Ssp*I DNA fragment, 3′-end labelled at the *Ssp*I end (see Materials and methods), was incubated with the 300 mM ammonium sulfate-eluted fraction enriched for SAP1 activity. The DNA in the reaction was briefly treated with DNase I endonuclease and then fractionated by electrophoresis through a native polyacrylamide gel. The free labelled DNA and the DNA–protein complex were excised from the gel, eluted and analyzed in a sequencing gel according to the method of Arcangioli and Lescure (1985). The results shown in Figure 3A indicated that SAP1 DNA-binding activity protected two DNA regions, called α and β, with several accessible residues in between. The DNase I cleavages at some neighboring sites were increased relative to the free DNA control. The DNase I protection experiment conducted with the complementary strand showed a similar interaction (data not shown).

Figure 3B summarizes the DNase I protection data. SAP1 protected a region encompassing 29 or 30 bp, which we name SAS1 (switch-activating site 1). The site is situated

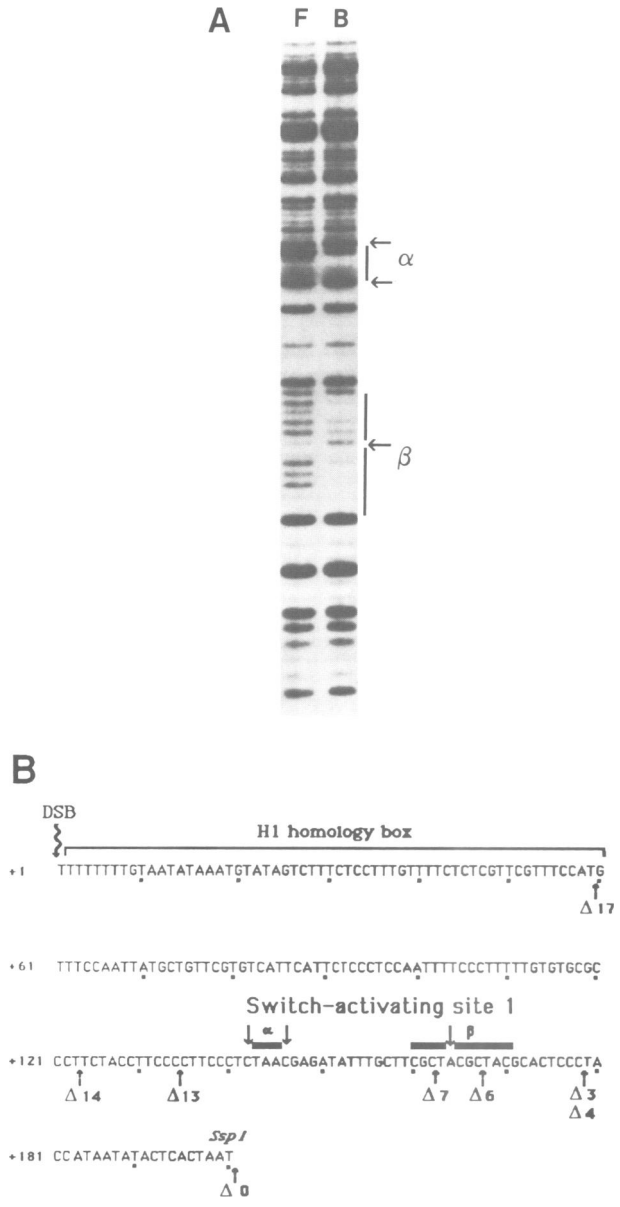


Fig. 3. Characterization of SAP1 binding site. (**A**) The *Hae*III−*Ssp*I *mat1-P* DNA fragment 3′-end labelled at the *Ssp*I side was used for DNase I protection experiments (see Materials and methods). Lane F, DNase I digestion of the free DNA; lane B, DNase I digestion of the DNA bound to the SAP1 activity. Protected nucleotides in the α and β regions are indicated. Arrows indicate DNase I hypersensitive sites. (**B**) Diagram representing SAP1 protected sequences of the upper strand of the DNA sequence located distal to *mat1*; position +1 refers to the DSB found at the *mat1-P* locus (Nielsen and Egel, 1989). The H1 homology sequence is boxed. Nucleotides protected by SAP1 against DNase I digestion are indicated by thick lines. α and β regions are shown. Bal31-deletion ends (Δ0−Δ17) used in this study are indicated.

140 bp and 30 bp from the DSB and *Ssp*I sites, respectively. These data allowed us to identify a strong DNA-binding activity, SAP1, in a DNA region previously hypothesized to be important for switching and for producing the DSB at *mat1* (Beach, 1983; Beach and Klar, 1984; Klar, 1987; Kelly *et al.*, 1988).

Identification of at least two switch-activating sites located distal to mat1

To investigate the role in switching of the identified SAS1 element, a series of *Bal*31 deletions were made *in vitro*, and the deleted constructs were reintroduced into the yeast chromosome. The *Bal*31 deletions were initiated at the unique *Xho*I site located 200 bp distal to the DSB site in the plasmid PL145 (see Materials and methods). The deletion end points were determined by sequence analysis and were referred to as Δ0, Δ3, Δ4, Δ6, Δ7, Δ13, Δ14 and Δ17 (see Figure 3B and Materials and methods for location of the deletion ends). Each plasmid construction was excised from the vector and introduced into yeast strains SP837 (h^{90}) and SP812 (*mat1-M smt-o*) (see Materials and methods for genotype) by DNA-mediated transformation selecting for leu$^+$ prototrophs. The *S.cerevisae LEU2* gene is known to complement the *S.pombe leu*1$^-$ mutation (Beach and Nurse, 1981). The *mat1-M smt-o* allele in strain SP812 is a deletion removing 26 bp of the distal end of the H1 region extending 262 bp distal to *mat1-M*; it fails to switch due to the absence of the DSB (Engelke *et al.*, 1987; O.Nielsen and R.Egel, personal communication; B.Arcangioli, unpublished results). Southern blot analysis of genomic DNA isolated from stable leu$^+$ transformants established the predicted structure of the deletions placed at *mat1* (see below).

Stable leu$^+$ transformants bearing these deletions were tested for their ability to switch by the iodine vapour staining procedure. In this assay, switching-competent colonies, as they contain spores, stain black, whereas switching-deficient colonies stain yellow since they lack spores (Gutz *et al.*, 1974). Δ0 and Δ3 deletions, when introduced into SP837, the wild-type (h^{90}) strain, showed iodine staining comparable to that of the parent strain (Figure 4B). The same constructs, when introduced into SP812, the *mat1-M smt-0* strain, changed its yellow staining (no switching) to an evenly black staining, restoring the homothallic phenotype characteristic of wild-type strains (data not shown). However, Δ6 and Δ13 deletions conferred the same speckled (streaky) staining phenotype, indicating reduced switching when introduced into either SP837 (Figure 4B) or SP812 (data not shown). When the larger A17 deletion was introduced into SP837 or SP812, their transformants consisted of non-staining colonies with a *P*-stable (heterothallic, non-switchable) phenotype.

These data allowed us to define at least two essential *cis*-acting elements located distal to *mat1* which are required for efficient switching. The first site corresponds to the SAS1-binding site as identified by DNaseI footprinting assays (see above) and by mutational analysis (see Δ3 versus Δ6). The second site, defined by Δ17, is roughly localized between nucleotides 123 and 59 (see Figure 3B) and was named SAS2. Interestingly, this SAS2 interval overlaps partially with the *smt-s* mutation. This mutation consists of a 27 bp deletion causing a reduction in the level of the DSB at *mat1*, and consequently, a reduced rate of mating type switching (Beach, 1983; Klar *et al.*, 1991).

SAS1 and SAS2 mutations reduce the level of the DSB at mat1

The observed streaky iodine staining of the mutant clones with Δ6 and Δ13 deletions (Figure 4B) showed that these deletions affect the rate of mating type switching. This phenotype is similar to that exhibited by a *mat1 cis*-acting (*smt-s*) mutation that reduces the rate of switching as a consequence of reducing the steady state level of the DSB (Beach, 1983; Klar *et al.*, 1991). Therefore, it was of interest

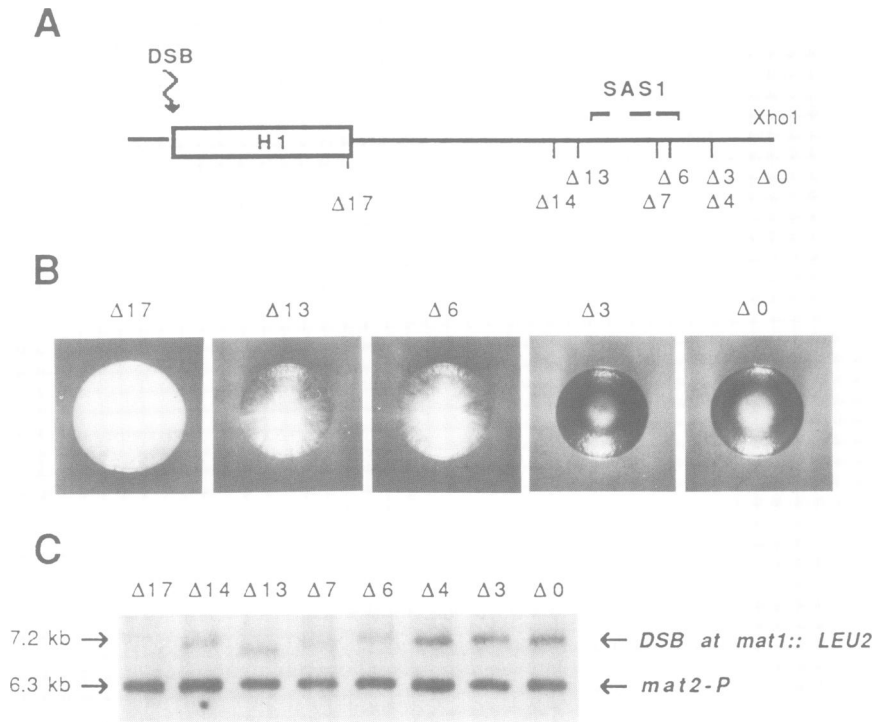


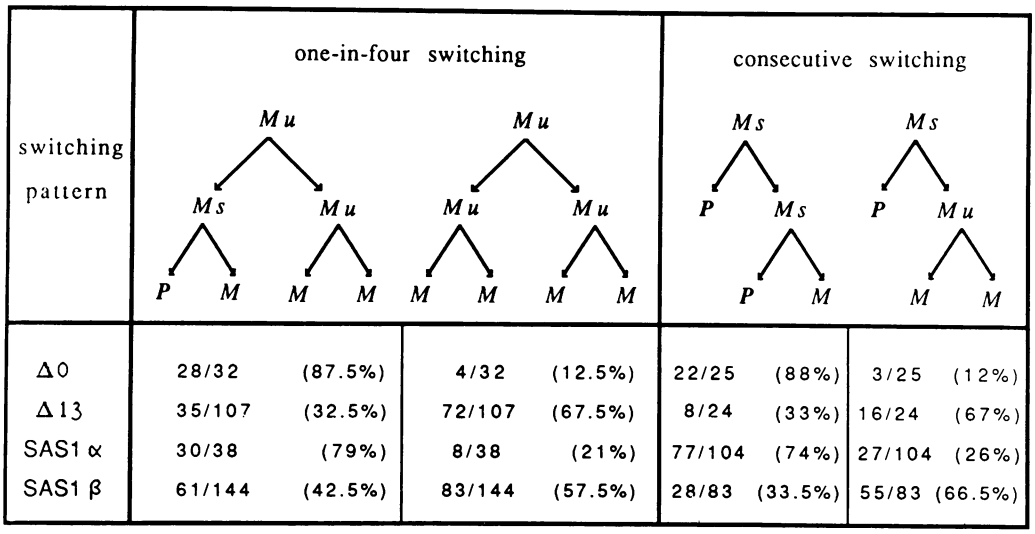
Fig. 4. DSB analysis of *mat1-P* deletion mutants. (**A**) Diagrammatic representation of the DNA located distal to *mat1*. The position of the DSB is marked by an arrow and the H1 homology sequence is boxed. The dotted line indicates the position of the switch-activating site 1 (SAS1). Bal31 deletions (Δ0−17) are also shown. (**B**) Iodine vapour staining of *S.pombe* colonies containing Bal31 deletions. Iodine specifically stains spore-containing colonies black; the level of staining reflects the extent of switching at the colony level (Gutz *et al.*, 1974). The glossy area in the middle of each colony is due to the camera lighting. (**C**) Southern blot analysis of the deletion mutants. Genomic DNA from different strains containing *mat1*-distal deletions (Δ0−Δ17) were digested with *Hin*dIII, Southern transferred (Southern 1975), and probed with a purified 3.8 kb *Nsi*I−*Nsi*I DNA fragment containing *mat1-P*::*LEU2* sequences (see Materials and methods). The 6.3 kb band corresponds to the *mat2-P Hin*dIII DNA fragment (Beach, 1983), while the upper band migrating around 7.2 kb reflects the level of the DSB as it contains the 5.0 kb *mat1*-distal DNA plus the 2.2 kb *LEU2* DNA fragment. Variations in the size of the 7.2 kb fragment are due to the extents of the bidirectional bal31 deletions from the *Xho*I site in both *mat1-P* and *LEU2* DNAs (see Materials and methods and text for details).

to assess the amount of the DSB in the deletion-containing strains.

Southern transfer of *Hin*dIII-digested DNA isolated from SP837 transformants was probed with a 5.2 kb *Nsi*I−*Nsi*I DNA fragment containing the *mat1-P*::*LEU2* sequence. As expected, this probe hybridized with fragments of sizes 12.6 kb (uncleaved *mat1*::*LEU2*), 6.3 kb (*mat2-P*) and 4.2 kb (*mat3-M*) and with the 5.4 kb (cleaved *mat1-proximal*) and 7.2 kb (cleaved *mat1*-distal::*LEU2*) (data not shown). The last two fragments result from the DSB at *mat1*. For comparison of the level of the DSB in deletion-containing strains, levels of hybridization to the 6.3 kb (*mat2-P*) silent cassette (as an internal control) and the 7.2 kb fragment resulting from the DSB at *mat1* are shown in Figure 4C. The 7.2 kb fragment corresponds to 5.0 kb of the cleaved *mat1*-distal DNA of the wild-type strain (Beach, 1983) plus 2.2 kb of the *LEU2* DNA sequence inserted into the *mat1*-distal *Ssp*I site. The mobility of the 7.2 kb fragment agrees with the exact extent of the *Bal*31 deletion determined by DNA sequencing (see Materials and methods for details). Deletions Δ3 and Δ4 show a level of cleavage comparable to that of the Δ0 (no deletion) construct when compared with the *mat2-P* fragment used as an internal control for the amount of DNA loaded in each lane. Deletions Δ6, Δ7, Δ13 and Δ14 show at least three-fold reduction in the amount of the DSB, while Δ17 does not have the 7.2 kb band (Figure 4C), thus showing the lack of the DSB at the *mat1* locus in this mutant strain.

Combinatorial effects of SAS1 deletion and swi mutations

Since SAS1 is required for efficient switching and overlaps with the specific binding site for SAP1, we next investigated whether SAP1 is the product of previously known *trans*-acting switching (*swi*) genes also required for generating the DSB at *mat1*. Two complementary approaches were used. First, we compared the DNA-binding profile of protein extracts prepared from wild-type, *swi1*$^-$, *swi3*$^-$ and *swi7*$^-$ mutant strains by gel retardation assays. No differences were observed (data not shown). All of the *swi* mutations analyzed so far are leaky; thus, in principle, these proteins may still show binding in our assay. Analyses of extracts prepared from strains containing chromosome disruptions of *swi* genes should be more definitive regarding whether SAP1 is encoded by one of these *swi* genes. Such deletion mutants are presently being constructed. Secondly, we used a genetic approach to determine the switching efficiencies of double mutants, containing the deletion Δ13 (see Figure 3B) along with *swi1*, *swi3* or *swi7* mutations. All double mutant combinations showed a cumulative reduction on iodine staining, indicating further reduction of switching of Δ13 in *swi* mutants (data not shown). These results taken together



Δ0	28/32	(87.5%)	4/32	(12.5%)	22/25	(88%)	3/25	(12%)
Δ13	35/107	(32.5%)	72/107	(67.5%)	8/24	(33%)	16/24	(67%)
SAS1 α	30/38	(79%)	8/38	(21%)	77/104	(74%)	27/104	(26%)
SAS1 β	61/144	(42.5%)	83/144	(57.5%)	28/83	(33.5%)	55/83	(66.5%)

Fig. 5. Pattern of *mat1* switching in a cell lineage, showing the efficiency of one-in-four and consecutive switching. Only the allele of the diploid strains that switched is presented. The numbers of pedigrees assayed and the percentage of switching are indicated. Lowercase *u* and *s* indicate the unswitchability and switchability of the *mat1-M* allele, respectively.

strongly suggest that the products of these *swi* genes do not solely interact with SAS1 and that their activities are distinct from SAP1.

SAS1 element affects the rate of first-time as well as consecutive switching

The work of Klar (1987, 1990) indicated that switching potential in *S.pombe* segregates in *cis* with the 1450 bp *mat1* DNA sequence contained within the *Ssp*I−*Ssp*I fragment (see Figure 1) and suggested an epigenetic, DNA strand-specific 'imprinting' event located in the vicinity of the DSB site. This imprinting event is thought to regulate the generation of the DSB at *mat1*; its inheritance causes developmentally asymmetric cell divisions required to generate the observed pattern of mating type switching in a cell lineage. It was therefore of interest to investigate the function of SAS1 in the regulation of mating type switching at the individual cell level.

The mating type switching of a strain containing Δ0 (used as the wild-type control) and Δ13 (SAS1 deletion) was followed microscopically by assaying the pattern of sporulation of single diploid cells in a cell lineage (see Materials and methods). To do this, we constructed diploid cells in which one homologue contained the non-switchable heterothallic *mat1-M smt-o* allele, while the other contained either *mat1* Δ0 or *mat1* Δ13 mutation. Diploid *mat1-M/mat1-M smt-o* cells fail to sporulate and will grow, but once the switchable *mat1-M* allele has changed to *mat1-P*, then that cell will form an ascus and will stop growing. By monitoring the pattern of sporulation of each cell in a pedigree, we can determine the switching pattern of the *mat1* Δ0 and Δ13 alleles.

The switching efficiency and the pattern of the control Δ0 (Figure 5) is the same as those described for the wild-type *mat1* locus in haploid cells (Miyata and Miyata, 1981) or in diploid cells (Klar, 1990). The unswitchable (*Mu*) cell produced two unswitched daughters, one of which was unswitchable (like her mother), while the other was switchable (*Ms*) in 87.5% of cell divisions. The switchable daughters (i.e. *Ms*) generated a switched and an unswitched pair of progeny, conforming to the one-in-four switching rule. This latter unswitched cell, like her mother, also produced a switched daughter in 88% of the consecutive cell divisions (Figure 5). Such a pattern of switching indicates that the presence of the *S.cerevisiae LEU2* gene next to the *mat1* locus does not interfere with the regulation of switching.

In contrast, results with Δ13 showed a significant decrease in efficiency both for the one-in-four (32.5%) and the consecutive (33%) switching (Figure 5). These data correlated with the low iodine staining reaction and the decreased level of the DSB observed in the Δ13 mutant strain (see Figure 4). Thus, SAS1 delection affects both the one-in-four and the consecutive switching efficiencies to the same extent, suggesting that both developmental asymmetrical divisions (i.e. *Mu* to *Ms* and *Ms* to *Ms*) occur by a common molecular mechanism requiring the SAS1 sequence.

SAS1 point mutations affecting SAP1 binding in vitro reduced mat1 switching

To determine whether the *cis*-acting SAS1 element corresponds to the SAP1 target sequence and whether the biological activity of SAS1 is due to binding to SAP1, we used a preliminary DMS interference result to identify nucleotides important for SAP1 interaction. We mutated those bases and analyzed their effect on the affinity of SAP1 binding *in vitro* and on *mat1* switching *in vivo*.

As expected, a 36 bp double-stranded oligonucleotide, containing the DNA sequence protected by SAP1 against the DNase I digestion (Figure 6B, Mo), showed a similar pattern of migration to the *Hae*III−*Ssp*I DNA fragment (Figure 6A, lanes A and B); while point mutations either in the SAS1α or SAS1β domains (Figure 6B, Mα or Mβ, respectively), showed a reduced level of affinity (Figure 5A, lanes C and D). These results indicated that stable SAP1 binding activity *in vitro* required simultaneous contact with both the α and β DNA sequences.

To determine the effect of SAS1 mutations on switching, we introduced mutations in the SAS1α or SAS1β domains into the yeast chromosome. The resulting strains, were tested both for their switching efficiency by the iodine vapour staining procedure and by pedigree analysis. When

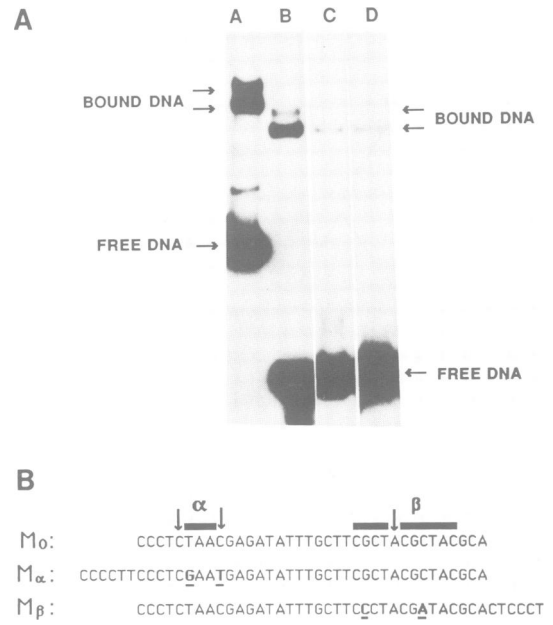


Fig. 6. Analysis of SAP1 binding activity using wild-type and mutant probes. (**A**) Crude extracts from *S.pombe* cells were incubated with the labelled 254 bp *Hae*III−*Ssp*I DNA fragment (lane A), or with the labelled 36 bp oligonucleotide (Mo) containing the wild-type SAP1 binding site (lane B), or with the labelled 42 bp oligonucleotide (Mα) containing two point mutations in the α domain of the SAP1 binding site (lane C), or with the labelled 42 bp oligonucleotide (Mβ) containing two point mutations in the β domain of the SAP1 binding site (lane D). After 10 min of incubation at room temperature, reaction mixtures were analyzed by gel retardation assay. (**B**) the 36 base (Mo) oligonucleotide containing the SAP1 binding site (nt −138 to −174, see Figure 3B), the 42 base (Mα) oligonucleotide and the 42 base (Mβ) oligonucleotide containing the mutated SAP1 binding sites are shown. The SAS1 α and β regions protected by SAP1 against DNase I digestion are indicated. Mutated nucleotides in Mα and Mβ are under lined.

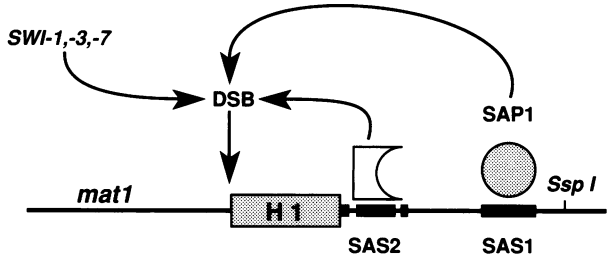


Fig. 7. Working model representing *cis*-acting elements and *trans*-acting factors required for producing the DSB. DNA regions flanking the DSB site are shown. SAS1 and SAS2 are indicated by thick lines, and the homology sequence H1 is boxed. SAP1 (circle) and a hypothetical SAS2 DNA-binding factor are designated above the SAS1 and SAS2 elements. *Swi-1*, *-3* and *-7* *trans*-acting products are also indicated.

introduced into the Δ17 strain, the yellow iodine staining (no switching) changed to a positive iodine staining, restoring the switching phenotype (see Materials and methods). Southern blot analysis of genomic DNA isolated from those transformants established the introduction of SAS1α and SAS1β mutations next to *mat1* (data not shown).

To investigate the switching defect in strains carrying SAS1 point mutations at the single cell level, pedigree analysis was conducted as described previously. Mutation in the SAS1β domain reduced the efficiency of switching to the level of the Δ13 deletion strain while mutation in the SAS1α domain exhibited only a marginal defect in switching (see Figure 5). The result, at least with the mutation in the SAS1β domain, is similar to the switching phenotype observed with the Δ6 deletion strain which has only 3 bp deleted from the distal end of the SAS1 element (see Figure 3B), and establishes a correlation between SAP1 binding *in vitro* and switching *in vivo*.

Discussion

Our study was aimed at investigating the molecular details of the mating type switching process. In particular, we wished to understand how switching potential and mating type switching are controlled by *cis*-acting events occurring at *mat1*. Since the DNA distal to the *mat1* cassette is thought to be required for mating type switching (Beach and Klar, 1984), and since it has been found that the 1450 bp *Ssp*I−*Ssp*I DNA fragment containing the *mat1* sequence contains all of the information needed for the *cis* regulation of the DSB (Klar, 1987), we undertook the functional analysis of the sequence located between the H1 homology box and the *mat1*-distal *Ssp*I site (Figure 1).

Using the *Bal*31 deletion procedure, we created a series of deletions in the DNA located distal to *mat1* and introduced them into the chromosome by homologous recombination. Two different classes of mutant phenotypes were observed on the basis of their iodine staining behaviour and the level of the DSB at *mat1* (Figure 4B and C). These data allowed us to define at least two essential *cis*-acting elements, SAS1 and SAS2, located within the 200 bp sequences present distal to *mat1* required for mating type switching.

The SAS1 site is defined by Δ6 behavior, which showed a streaky iodine staining reaction (Figure 4B) associated with a three-fold reduction in the level of the DSB at *mat1* (Figure 4C). Since Δ6 deleted only 3 bp of the SAP1-binding site (see Figure 3B), and point mutations SAS1α and SAS1β reduced SAP1 binding *in vitro* activity and reduced switching *in vivo*, it is tempting to suggest that SAP1 is required for the high steady state level of the DSB. Our results are consistent with the requirement of SAP1 for switching. However, a definitive conclusion must await cloning and mutational analysis of the SAP1 gene.

The characterization of the second site, SAS2, is incomplete, as it is based only on the Δ17 effect. However, Δ17 partially overlaps with the 27 bp deletion in the C13-P11 mutant which removed 7 bp of the distal end of H1 (Gutz and Fecke, 1979; Klar *et al.*, 1991). Since the SAS1 deletion (Δ13) showed a streaky iodine staining reaction, and SAS1 and SAS2 deletions (Δ17) caused a total absence of switching, it is unlikely that the 27 bp deletion of C13-P11 acted solely by reducing the distance between SAS1 and the DSB site.

Thus, mating type switching is controlled in *cis* by at least two sites, SAS1 and SAS2. These *cis*-acting elements appear to have redundant functions, as deletion of either one reduces the level of the DSB and simultaneous deletion of both sites causes a total lack of the DSB. During our DNA-binding study, we failed to find a specific SAS2 double-stranded DNA-binding activity from yeast extracts. We have not yet tested whether SAS2 binds to its cognate protein as a single-stranded molecule. We can also imagine that the SAS2 hypothetical binding factor contacts only weakly with its DNA target site, but may acquire a higher stability by

interacting with another protein such as SAP1. Thus SAS1 and SAS2 may constitute the target sites for two different regulatory proteins, each of them independently responsible for an intermediate level of the DSB. Alternatively, a single protein−DNA complex (containing SAP1) can act at both sites simultaneously. Also, we cannot exclude the possibility that additional sites are present between SAS1 and SAS2, and more analyses must be conducted to further our understanding of this critical regulatory region.

There seems to be a position-effect control for transcription and switching of *mat* cassettes. The expressed cassette at *mat1* switches, while the same sequences in the donor's unexpressed cassettes in *mat2* and *mat3* fail to switch (Kelly *et al.*, 1988). Certain cassette fusions between *mat1* and either *mat2* or *mat3* generate recombinant cassettes. It is known that when *mat1*-distal sequences are placed next to the donor loci (i.e. h^{+N} and h^{-U} rearrangements), then these loci exhibit the DSB and therefore switch, yet the hybrid cassettes are still unexpressed (Beach, 1983; Beach and Klar, 1984). The present study has defined these *mat1*-distal *cis*-acting sequences that are required in the switching control, and has shown that their deletion (Δ17) does not affect the expression at *mat1*. Thus, mating type gene expression and switching appear to be independently regulated in fission yeast. This situation can be contrasted with switching in *S.cerevisiae*, where the ability to switch is correlated with expression of the mating type loci (Klar *et al.*, 1981).

To characterize SAS1 function further, we studied the switching pattern of the SAS1 deletion mutant (Δ13) by pedigree analysis in diploid cells (Egel and Eie, 1987; Klar *et al.*, 1990). Our data showed (Figure 5) that the SAS1 deletion reduced the efficiency of the one-in-four switching and the consecutive switching equally. These results suggest that SAS1 (probably via SAP1) is required at each cell division producing one switchable daughter cell (i.e. *Mu* giving *Ms*+*Mu* and *Ms* giving *Ms*+*Pu*) and that both developmentally asymmetric divisions seem to follow the same molecular control mechanism for generating the DSB and switching. These data, taken together with the recent pedigree analysis of the *cis*-acting C13-P11 mutant, indicates that the DSB is sealed before DNA replication, and therefore must be cleaved again in consecutive cell divisions (Klar *et al.*, 1991).

It is worth noting that thus far we have been unable to detect any single- or double-stranded DNA cut at *mat1 in vitro* in either gel retardation or footprinting analysis. It is possible that the substrate DNA prepared from *E.coli* used in such assays may have lacked the DNA strand- and site-specific 'imprinting', which is postulated to be a prerequisite for the DSB to occur (Klar, 1987). In addition, an active process such as DNA replication by itself may be required to set up a complex machinery able to produce only one DSB among the two sister chromatids.

Our working model, shown in Figure 7, summarizes the functional relationship between *cis*- and *trans*-acting elements required to generate the DSB at *mat1*. Switching in *S.pombe* is formally a G_2 event producing one switched progeny among the two daughters of a switchable cell, a feature in contrast with the G_1 switching in *S.cerevisiae* which results in both progeny inheriting the switched phenotype (see Klar, 1989, for review). The complex *cis*-acting sites and the stringent *mat1 cis* regulation in *S.pombe* may be contrasted with the *MAT* switching in *S.cerevisiae* in which only a 24 bp sequence defines a DSB site *in vivo* and *in vitro* for the HO endonuclease (Nickoloff *et al.*, 1986). In addition, the pattern of switching in *S.cerevisiae* is strictly regulated through the expression of the site-specific endonuclease encoded by the unlinked *HO* gene, which is expressed in mother cells but not in daughter cells (Sternberg *et al.*, 1987; Nasmyth *et al.*, 1987). Stated another way, the pattern of switching in both yeasts is ultimately controlled through the generation of the DSB at the mating type locus. In *S.cerevisiae*, it is controlled by the regulated expression of the *HO* gene, while in *S.pombe*, the programmable regulation of mating type switching is controlled through the complex *mat1 cis*-acting elements and the inheritance of DNA chains by the progeny cells according to the classical model of semi-conservative DNA replication (Egel, 1984b; Klar, 1987, 1990). Further comparing and understanding details of both switching systems will enhance our understanding of how these systems have evolved.

Materials and methods

Yeast strains and media

S.pombe strains SP812 (*mat1-M smt-o, leu1-32, ade6-M216*) and SP837 (h^{90}, *leu1-32, ura4-D18, ade6-M216*) were used in this study. Standard conditions for cell cultures and iodine staining (Gutz *et al.*, 1974) were employed.

Preparation of yeast extract and heparin chromatography

Cells grown in rich medium were harvested by centrifugation, and extracts were prepared after breaking the cells with glass beads as described (Arcangioli and Lescure, 1985). The 100 000 *g* supernatant (S100) was diluted four times in order to reach a concentration of 0.1 M $(NH_4)_2$ SO_4 with buffer 0 (20 mM Tris pH 8, 1 mM EDTA, 7 mM 2-mercaptoethanol, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride) and applied to a heparin column equilibrated with buffer 100 (buffer 0 + 100 mM $(NH_4)_2$ SO_4). Proteins were eluted by a three-step salt elution of 0.2, 0.3 and 0.5 M $(NH_4)_2$ SO_4. Fractions were collected and tested for their DNA-binding activities. All operations were done at 4°C.

Gel retardation assay and DNase I protection experiments

The 254 bp *Hae*III−*Ssp*I restriction fragment (Figure 1) harboring the DSB-flanking sequences from *mat1-P* was cloned in the *Sma*I site of pUC18, digested with *Hin*dIII, end labelled with [α-^{32}P]dCTP in the presence of the Klenow fragment of DNA polymerase I, and then digested with *Eco*RI. The labelled DNA fragment was gel purified and used for the gel retardation assays and DNase I protection experiments as described (Arcangioli and Lescure, 1985).

Plasmids

PL145. This plasmid is a pUC19-based vector containing the *Eco*RI−*Eco*RI *mat1-P* DNA fragment (Beach, 1983; Kelly *et al.*, 1988). A *Xho*I linker was inserted at the *Ssp*I site located 200 bp distal to the DSB (see Figure 1). This *Xho*I site was subsequently used to insert the 2.2 kb *Xho*I−*Sal*I DNA fragment containing the *LEU2* gene of *S.cerevisiae*. A clone with the proper orientation (restoring the *Xho*I site at 200 bp of the DSB) was used to generate bidirectional *Bal*3 1 deletions.

PL145−Bal31 derivatives. The plasmid PL145 was linearized at the *Xho*I site, digested with Bal31 exonuclease and religated in the presence of *Xho*I linkers. The DNA sequence of each deletion used in this study was determined by the dideoxy chain terminations method (Sanger *et al.*, 1977). The extent of each deletion in the *mat1* distal region is shown in Figure 3B; deletions extending into *LEU2* are as follows: Δ0, no deletion; Δ3, no deletion; Δ4, 48 bp deletion; Δ6, no deletion Δ7; 111 bp deletion; Δ13, 193 bp deletion; Δ14, 49 bp deletion; Δ17, 122 bp deletion.

Point mutations in the SAP1α and SAS1β regions were introduced as follows: the 2.5 kb *Bam*HI−*Eco*RI DNA fragment containing the *mat1P*-distal sequences was introduced into M13 mp18. A clone with the proper orientation was used as a template in an oligonucleotide-directed *in vitro* mutagenesis reaction (Amersham), in which the 42 base oligonucleotides, containing specific mutations in the SAP1α or β domains (see Figure 6B

for sequence) were used as primers. The two constructions were then analyzed by the dideoxy chain termination method.

Construction of S.pombe mutants

Replacement of the wild-type *mat1* locus by the mutant alleles was accomplished using the strategy of Roshtein (1983). PL145 and Bal31 derivatives were digested with *Nsi*I and introduced into yeast by the lithium transformation procedure (Ito *et al.*, 1983) into either SP837 or SP812, which differ only in their *mat1* locus. Several leu$^+$ stable transformants were chosen for further analysis.

M13 mp18 recombinant clones containing SAS1α and SAS1β mutations were digested with *Bam*HI and *Eco*RI and introduced along with a vector containing *Ura4* gene into Δ17 strain by DNA mediated transformation. Among the ura$^+$ transformants, several leu$^-$ and iodine staining positive stable transformants were chosen for further analysis.

Southern blotting

Genomic DNA was isolated from 10 ml saturated cultures, 1 μg of DNA was digested with *Hin*dIII, subjected to electrophoresis through a 1% agarose gel and transferred to Genescreen membrane (Beach and Klar, 1984). The probe, consisting of the 3.8 kb *Nsi*I–*Nsi*I purified DNA fragment from PL145 containing the *mat1P::LEU2* sequences, was labelled by random priming using a kit from Pharmacia.

Pedigree analysis

Pedigree analysis was conducted as described (Klar, 1990).

Acknowledgements

We thank A.Arthur, S.Brown, P.Johnson and O.Nielsen for discussions or comments on the manuscript and R.Frederickson for the artwork. B.A. is the recipient of a long-term postdoctoral fellowship from the European Molecular Biology Organization. Research sponsored by the National Cancer Institute, DHHS, under contract No. NO1-CO-74101 with ABL. The contents of this publication do not necessarily reflect the views or policies of the department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsements by the US Government.

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Received on July 23, 1990; revised on May 24, 1991