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Localization and Properties of a Silencing Element Near the *mat3-M* Mating-Type Cassette of *Schizosaccharomyces pombe*

Geneviève Thon, K. Pernilla Bjerling and Inga Sig Nielsen

Department of Genetics, Institute of Molecular Biology, University of Copenhagen, DK-1353 Copenhagen K, Denmark

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ABSTRACT

Transcription is repressed in a segment of *Schizosaccharomyces pombe* chromosome II that encompasses the *mat2-P* and *mat3-M* mating-type cassettes. Chromosomal deletion analysis revealed the presence of a repressor element within 500 bp of *mat3-M.* This element acted in synergy with the *trans*-acting factors Swi6, Clr1, Clr2, Clr3, and Clr4 and had several properties characteristic of silencers: it did not display promoter specificity, being able to silence not only the *M* mating-type genes but also the *S. pombe ura4* and *ade6* genes placed on the centromere-distal side of the *mat3-M* cassette; it could repress a gene when placed further than 2.6 kb from the promoter and it acted in both orientations, although with different efficiencies, the natural orientation repressing more stringently than the reverse. Following deletion of this element, two semistable states of expression of the *mat3-M* region were observed and these two states could interconvert. The deletion did not affect gene expression in the vicinity of the *mat2-P* cassette, 11 kb away from *mat3-M.* Conversely, deleting 1.5 kb on the centromere-proximal side of the *mat2-P* cassette, which was previously shown to partially derepress transcription around *mat2-P*, had no effect on gene expression near *mat3-M.* A double deletion removing the *mat2-P* and *mat3-M* repressor elements had the same effect as the single deletions on their respective cassettes when assayed in cells of the *M* mating type. These observations allow us to refine a model proposing that redundant pathways silence the mating type region of *S. pombe.*

IN eukaryotes, specialized chromatin structures influence the availability of certain chromosomal regions to transcription. Thereby, two copies of the same gene occasionally display different levels of expression even though they are located within the same nucleus, as do, for example, allelic genes located in the female X chromosomes of mammals (for reviews, see Riggs and Porter 1996; Heard *et al.* 1997) and various imprinted genes (for review, see Ainscough and Surani 1996; Bartolomei and Tilghman 1997). Furthermore, the location and extent of the inactive chromosomal areas can vary from organism to organism and from cell to cell, causing variegated phenotypes (reviewed for Drosophila by Weiler and Wakimoto 1995). Such flexibility suggests that turning on or off large chromosomal regions that contain clusters of genes involved in a common process might be a means of regulating cellular differentiation and development. This type of regulation occurs, as best exemplified by the regulation of the Drosophila homeotic genes (reviewed by Paro and Harte 1996; Pirrotta 1996).

Where and how are inactive regions formed? The features that trigger transcriptional inactivation of specific regions appear diverse, as do the modes of subsequent inactivation. Inactivation does not always originate from a well-defined repressor element. For example, gene silencing in Neurospora and position effect variegation in Drosophila can be caused by repeats of a gene that is not silenced when present in single copy (for review, see Henikoff 1996; Russo *et al.* 1996). In other cases, specialized DNA elements or silencers are able to interact with nuclear proteins to create a zone of repression in the chromosomes of cells meeting specific criteria. The Polycomb response elements in Drosophila (reviewed by Paro and Harte 1996; Pirrotta 1996), and the *HML* and *HMR* silencers in *Saccharomyces cerevisiae* (reviewed by Holmes *et al.* 1996) are examples of such elements.

In the fission yeast *Schizosaccharomyces pombe*, position effects are observed near centromeres and telomeres and in the mating-type region (for review, see Allshire 1996). Prototrophic markers introduced at these locations are repressed in a variegated fashion. The repression is only partial in telomeric regions (Nimmo *et al.* 1994). It is tighter for some centromeric insertions (Allshire *et al.* 1994, 1995) and within the mating-type region (Thon and Klar 1992; Thon *et al.* 1994). The products of *swi6*, *rik1*, *clr1*, *clr2*, *clr3*, and *clr4* are required for the repression of transcription in these places, which indicates that the mechanisms of repression are related (Lorentz *et al.* 1992; Thon and Klar 1992; Ekwall and Ruusala 1994; Thon *et al.* 1994; Allshire *et al.*

Corresponding author: Geneviève Thon, Department of Genetics, Institute of Molecular Biology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark.
E-mail: gen@biobase.dk

1995; Grewal and Klar 1997). Swi6, Rik1, and Clr4 appear more important for centromeric silencing than the other three factors, and mutations in *swi6*, *rik1*, and *clr4* affect chromosome segregation in addition to transcription (Allshire *et al.* 1995). In the mating-type region, the six *trans*-acting factors repress not only transcription but also meiotic recombination (Egel *et al.* 1989; Klar and Bonaduce 1991; Lorentz *et al.* 1992; Thon *et al.* 1994). These pleiotropic phenotypes suggest an action at the level of chromatin structure.

The mating-type region comprises three linked loci in the right arm of chromosome II (Figure 1). The centromere-proximal locus, *mat1*, is expressed, whereas the two other loci, *mat2-P* and *mat3-M*, are not transcribed. In wild-type homothallic strains, designated h^{90}, *mat2-P* and *mat3-M* donate genetic information to *mat1* in an efficient process akin to gene conversion. This process leads to interconversion of *mat1* between two allelic forms, *mat1-P* and *mat1-M*, which determine, respectively, the *P* and *M* mating types of *S. pombe* (for review, see Klar 1992). The two mating-type genes present within *mat2-P* and the two mating-type genes present within *mat3-M*, as well as prototrophic markers introduced near the cassettes or in the 10.9-kb interval that separates them (K region), are subject to transcriptional silencing (Egel and Gutz 1981; Beach 1983; Ekwall *et al.* 1992; Thon and Klar 1992; Thon *et al.* 1994; Grewal and Klar 1997). The borders of the silenced region are not known. An essential gene is located between *mat1* and *mat2-P*, indicating the repression extends less than 10 kb on the centromere-proximal side of *mat2-P* (Michael *et al.* 1994). The first known gene on the centromere-distal side of *mat3-M* is *his2* and the distance between *mat3-M* and *his2* as inferred from a cosmid map (Hoheisel *et al.* 1993) is ~50 kb.

Sequences located within the K region, which separates *mat2-P* from *mat3-M*, are implicated in silencing (Grewal and Klar 1996; Thon and Friis 1997). The K region contains 4.3 kb of homology with centromeric repeats (Grewal and Klar 1997), suggesting that it interacts with the *trans*-acting factors shared by the mating-type region and centromeres. A deletion of 7.5 kb that removes the region with homology to centromeres causes the cells to interconvert between two epigenetic states: one similar to the wild type and one partially deficient for mating-type switching and transcriptional silencing (Grewal and Klar 1996; Thon and Friis 1997). The mutant state resembles the phenotype caused by mutations in the *trans*-acting factors *swi6*, *rik1*, *clr1*, *clr2*, *clr3*, and *clr4.* Furthermore, there is no cumulative effect when mutations in the *trans*-acting factors are combined with the 7.5-kb deletion in the K region. This is consistent with an element located within the deleted fragment, possibly the centromeric repeats, either catalyzing the assembly of the *trans*-acting factors in the wild-type mating-type region, or preventing loss of the silenced state.

Elements close to the *mat2-P* cassette are also important for silencing, as inferred from deletion studies (Ekwall *et al.* 1991; Thon *et al.* 1994). One or several elements on the centromeric side of the *mat2-P* cassette appear to act in a silencing pathway that is distinct from the pathway mediated by *swi6*, *rik1*, *clr1*, *clr2*, *clr3*, and *clr4* (Thon *et al.* 1994). The *esp1*, *esp2*, and *esp3* genes were proposed to act in that second pathway (Thon and Friis 1997).

We investigated whether elements similar to the element present near the *mat2-P* cassette were present near the *mat3-M* cassette. To this end, we introduced nested deletions in the chromosomal DNA flanking *mat3-M.* We determined the effect of these deletions on the expression of *mat3-M* and the region around it, as well as on gene expression in the *mat2-P* area. Conversely, we examined whether deletion of the *mat2-P cis*-acting element affected expression around *mat3-M.* We also tested whether cumulative effects were generated by combining deletions at the two loci or by combining deletions near *mat3-M* with mutations in the *trans*-acting factors *swi6*, *clr1*, *clr2*, *clr3*, *clr4*, and *esp3.*

MATERIALS AND METHODS

Plasmid constructions and DNA sequencing

***Escherichia coli* strains, plasmid preparation, and cloning techniques:** The *E. coli* strains DH5 (Hanahan 1983) and S1754 (*F⁻ lacI^Q metA endA hsdR17 supE44 thi-1 relA1 gyrA96*; a gift from Stanley Brown) were used for cloning. Plasmid DNA was prepared and purified in cesium gradient according to Sambrook *et al.* (1989). DNA fragments were isolated in agarose/TBE gels and electroeluted. Restriction enzymes (New England Biolabs, Beverly, MA), shrimp alkaline phosphatase (USB), and T4 DNA ligase (Pharmacia, Piscataway, NJ) were used as directed by the manufacturers. AmpliTaq (Perkin Elmer, Norwalk, CT) and Native Pfu (Stratagene, La Jolla, CA) were used for amplification by polymerase chain reaction (PCR). Nucleotides were purchased from Pharmacia.

***mat3-M* cassette with a deletion of the H3 homology box:** pSM10 consists of an *S. pombe* 4.2-kb *Hin*dIII genomic fragment containing the *mat3-M* cassette cloned in pUC119 (Beach 1983; Kelly *et al.* 1988). There are two *Eco*RI sites in pSM10: one in the polylinker, on the centromere-distal side of the cassette, and one within the cassette, which divides the *Hin*dIII genomic fragment in fragments of 2.5 kb (centromere-proximal) and 1.7 kb (centromere-distal; Figure 1). The 2.5-kb *Hin*dIII-*Eco*RI restriction fragment was cloned in M13mp19 (Yanish-Perron *et al.* 1985) and mutagenized using an *in vitro* mutagenesis system version 2 from Amersham (Arlington Heights, IL) and an oligonucleotide whose sequence (5′GCA TACAGAAAAATACTCGAGTGTAAAGTATCAGGA3′) was designed to replace the *mat3-M* H3 homology box with an *Xho*I recognition site. A mutagenized *Hin*dIII-*Eco*RI insert was partially sequenced and cloned in Bluescribe(−) (BSG43 construct; Stratagene). The *mat3-M* cassette was reconstituted but for the deletion of the H3 box by ligating the 1.7-kb centromere-distal *Eco*RI fragment of pSM10 in the *Eco*RI site of BSG43, creating pGT79. A large portion of pGT79 was subsequently sequenced (see below).

***mat3-M* proximal ExoIII deletions:** The double-stranded oligonucleotide produced by annealing 5′TCGAAGATCTCCAT GGCCATGGACGCGTGGGCCC3′ and 5′TCGAGGGCCCAC GCGTCCATGGCCATGGAGATCT3′ was cloned into the *Xho*I

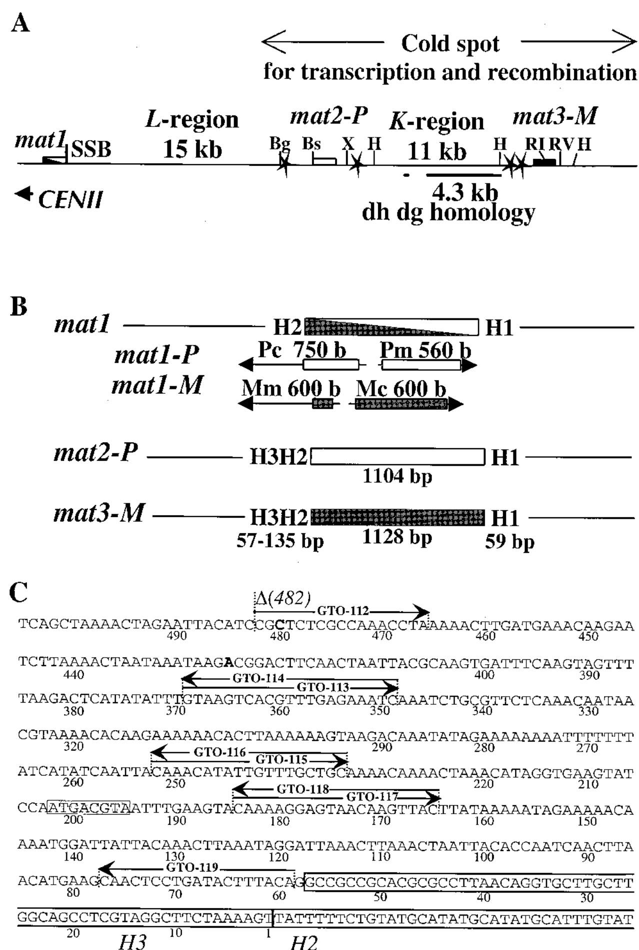


Figure 1.—Mating-type region of *S. pombe.* (A) General organization. The three mating-type cassettes of *S. pombe, mat1, mat2-P,* and *mat3-M,* are separated by a DNA segment where transcription and recombination can occur (*L*-region) and a segment where both transcription and recombination are inhibited (*K*-region). A single-strand break (SSB) is thought to initiate switching of the *mat1* allele (Arcangioli 1998, and references therein). The block to transcription and recombination extends outside of the *mat2-P-K-mat3-M* region for an unknown distance. The *K*-region displays homology with centromeric sequences [dh dg homology; Grewal and Klar (1997)]. Stars represent putative origins of replication: two near *mat2-P* allow plasmid replication (Olsson *et al.* 1993) and two near *mat3-M* match the core sequence of *S. pombe* ARS elements (Grewal and Klar 1997). Restriction sites mentioned in the text are indicated: Bg, *Bgl*II; Bs, *Bss*HII; H, *Hin*dIII; RI, *Eco*RI; RV, *Eco*RV; X, *Xba*I. Cells with the 7.5-kb deletion in the *K*-region (Grewal and Klar 1996; Thon and Friis 1997) lack DNA between the *mat2-P* centromere-distal and the *mat3-M* centromere-proximal *Hin*dIII sites. (B) Representation of the three cassettes. The three mating-type cassettes contain a *P*- or *M*-specific core flanked by short homology boxes. Two transcripts originate from each *mat1-P* and *mat1-M* (adapted from Kelly *et al.* 1988). (C) *mat3-M* flanking sequence. A portion of *mat3-M* flanking sequence is depicted, along with the H3 homology box and part of the H2 homology box. The *mat3-M* centromere proximal deletions presented in this study originated at the junction of H2 and H3 at the base pair labeled 1. The extent of the Δ*(482)* deletion is indicated, as well as the position and orientation of primers used to amplify and reintroduce DNA fragments in the Δ*(1185)* and Δ*(482)* deletions as shown in Figure 3. Boldface letters in the sequence represent differences between our clones and the published sequence (U57841; Grewal and Klar 1997). The sequence in the gray box is a putative Mts1/Mts2 (Atf1/Pcr1)-binding site.

site of pGT79. A clone in which the oligonucleotide was oriented with its *Apa*I site close to the *mat3-M* cassette was digested with the restriction enzymes *Bgl*II and *Apa*I, and deletions were introduced using ExoIII and S1 nucleases as described (Henikoff 1984). This resulted in clones with *mat3-M* proximal deletions starting at the junction between the H2 and H3 homology boxes, in which the deleted fragment was replaced with an *Xho*I site. The deletion series was used for sequencing and further cloning (see below). Clones with deletions of 482 bp (pGT79d482) and 1185 bp (pGT79d1185) were used to transform *S. pombe.*

mat3-M **distal ExoIII deletions:** pGT70 contains a 4.2-kb *Hin*dIII fragment with a modified *mat3-M* cassette in which an *Nco*I restriction site was introduced by a single bp substitution at the centromere-distal border of the cassette (Thon and Klar 1993). The double-stranded oligonucleotide produced by annealing 5′CATGAGATCTCCACCGCGGTGGACGCGTGGGCC3′ and 5′CATGGGCCCACGCGTCCACCGCGGTGGAGATCT3′ was cloned in the *Nco*I site of pGT70 with the *Apa*I recognition site close to the cassette. Nested ExoIII deletions were introduced after digestion with the restriction enzymes *Apa*I and *Bgl*II. A resulting plasmid with a deletion of 424 bp (pGT70d424) was used for further construction and to transform *S. pombe.*

Combination of *mat3-M* distal and proximal deletions: The 1.7-kb *Eco*RI fragment of pGT79d1510 (an ExoIII deletion product of pGT79) was replaced with the 1.3-kb *Eco*RI fragment of pGT70d424 to create a plasmid with a *mat3-M* proximal deletion of 1510 bp and a *mat3-M* distal deletion of 424 bp designated pGT181.

Cloning of PCR fragments in pGT79d482 and pGT79d1185: The oligonucleotides GTO-112: 5′CCACATGTCTCGAGCGCTCTCGCCAAACCTA3′, GTO-113: 5′CCACATGTCTCGAGGTAAGTCACGTTTGAGAAATC3′, GTO-114: 5′CCACATGTCTCGAGGATTTCTCAAACGTGACTTAC3′, GTO-115: 5′CCACATGTCTCGAGCAAACATATTGTTTGCTGC3′, GTO-116: 5′CCACATGTCTCGAGGCAGCAAACAATATGTTTG3′, GTO-117: 5′CCACATGTCTCGAGCAAAAGGAGTAACAAGTTAC3′, GTO-118: 5′CCACATGTCTCGAGGTAACTTGTTACTCCTTTTG3′, and GTO-119: 5′CCACATGTCTCGAGTGTAAAGTATCAGGAGTTG3′ can anneal near *mat3-M* as shown in Figure 1C, and the following pairs were used to amplify *mat3-M* flanking DNA by PCR: GTO-112 and GTO-119, creating the 424-bp fragment 1; GTO-112 and GTO-114, creating the 134-bp fragment 2; GTO-113 and GTO-116, creating the 136-bp fragment 3; GTO-115 and GTO-118, creating the 88-bp fragment 4; GTO-117 and GTO-119, creating the 126-bp fragment 5. PCR amplification was performed with Native Pfu (Stratagene) in reaction volumes of 80 μl using the buffer provided by the supplier, 1 μg of pSM10 as template in each reaction, primer concentrations of 750 nm, nucleotide concentrations of 250 μm, and five cycles of 1 min at 94°, 1 min at 54°, 2 min at 72°). The PCR products were cleaved with *Xho*I, purified from agarose gels, and cloned into the *Xho*I site of pGT79d482 either in their natural orientation relative to *mat3-M,* creating pGT132 with fragment 1, pGT124 with fragment 2, pGT125 with fragment 3, pGT126 with fragment 4, and pGT127 with fragment 5, or in the opposite orientation, creating pGT133 with fragment 1 and pGT131 with fragment 5. In addition, fragment 1 and fragment 5 were cloned in the *Xho*I site of pGT79d1185 in their natural orientation creating,

respectively, pGT142 and pGT137. The PCR inserts and insertion areas were sequenced.

***mat3-M(Eco*RV*)::ade6* allele:** The 4.2-kb *Hin*dIII fragment from pSM10 was filled in at the ends with the Klenow fragment of DNA polymerase I and ligated into the *Eco*RV site of pBluescript (Stratagene). The resulting plasmid was designated pPB16. The 3.0-kb *Spe*I-*Asp*700 fragment from pAS1 (Szankasi *et al.* 1988), which contains the *ade6* ORF, was filled in the ends with Klenow and cloned into the *Eco*RV site of pPB16, 150 bp away from the *mat3-M* cassette. A clone with the *ade6* gene inserted with its promoter near the *mat3-M* cassette was saved as pPB17.

Combination of Δ*(H3)mat3-M* with *(Eco*RV*)::ura4*: pGT77 contains the *mat3-M* 4.2-kb *Hin*dIII fragment with the *S. pombe ura4* gene inserted at the *Eco*RV site 150 bp distal to the cassette (Thon and Klar 1992). The 1.7-kb *Eco*RI fragment of pGT79 was replaced with the 3.5-kb *Eco*RI fragment of pGT77, to create a *mat3-M* cassette with a deletion of the H3 box and an *Eco*RV insertion of *ura4.* The resulting plasmid was designated pGT180.

DNA sequencing: DNA sequencing reactions were performed with an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA) and run with an ABI sequencing system model 377. The *mat3-M* proximal deletion series and synthetic primers were used to sequence *mat3-M* flanking DNA from the centromere-proximal *Hin*dIII site to the H2 box. Three differences were observed in a comparison with the published sequence (Grewal and Klar 1997): the G at position 10315 was missing in our clones, an additional C was found at position 10439, and an A was present instead of a G at position 10493. Our isolate of pSM10 had the same three differences with the published sequence.

S. pombe strain constructions and manipulations

Media: YES (Thon and Friis 1997) was used as rich, complete medium to propagate *S. pombe.* MSA (Egel *et al.* 1994) supplemented with 100 mg adenine, 100 mg uracil and 200 mg L-leucine per liter unless specified otherwise was used as sporulation medium. Drop-out media (AA-ura, AA-ade, AA-leu, AA-ade-ura; Rose *et al.* 1990) were used to identify and select prototrophic strains and FOA [AA-ura drop-out medium supplemented with 1 g 5-fluoroorotic acid (5-FOA) and 50 mg uracil per liter] was used to select *ura4* mutant cells. YE (5 g yeast extract, 2 g casamino acids, 30 g glucose per liter) was used to monitor *ade6* expression in colonies. Yeast extract, casamino acids, and yeast nitrogen base were purchased from Difco (Detroit). Amino acids and nucleotides were purchased from Sigma (St. Louis). Salts were purchased from Merck. 5-FOA was purchased from United States Biological.

Transformation: *S. pombe* cells were transformed using the lithium acetate protocol described by Heyer *et al.* (1986) with the modifications suggested by Moreno *et al.* (1991). The strains and plasmids used for the transformations are listed in Table 1. Chromosomal integrations in the mating-type region were obtained in mutant backgrounds (*swi6-115* or *clr2-E22*) that allow for, first, higher efficiency of recombination than the wild type and, second, positive and negative selections for prototrophic markers placed in the normally silenced region. All integrations were analyzed by Southern blot.

DNA preparation and Southern blot analysis: *S. pombe* DNA was prepared according to Moreno *et al.* (1991). The DNA preparations were digested with *Hin*dIII, *Hin*dIII + *Xho*I, and *Eco*RI and size fractionated in 0.7% agarose/TBE gels (Sambrook *et al.* 1989). The gels were blotted to Hybond-N nylon membrane as directed by the manufacturer (Amersham). Hybridization was performed overnight at 65° in 5× SSC, 5× Denhardt's, 1% SDS, 100 μg/ml sonicated salmon sperm DNA. The 4.2-kb *mat3-M Hin*dIII fragment from pSM10 was radiolabeled using a Promega (Madison, WI) Random Priming kit and 3000 Ci/mmol [α-^{32}P]dCTP from Amersham and used as probe. After hybridization, the blots were washed at 65° as follows: for 10 min in 2× SSC, 1% SDS; for 60 min in 2× SSC, 1% SDS; and for 30 min in 0.1× SSC, 1% SDS. They were autoradiographed on Agfa Curix X-ray films.

Genetic crosses: All strains listed in Table 1 as not originating from a transformation were obtained by tetrad dissection, with the exception of PG1, PG445, PG447, PG1049, PG1560, PG1562, PG1564, PG1566, PG1570, PG1615, PG1654, PG1655, PG1656, PG1657, PG1658, PG1659, PG1671, PG1672, PG1681, PG1682, PG1690, SP1122, SP1124, SP1125, SP1126, SP1138, and SP1151, which were obtained from random spore preparations.

RNA preparation and Northern blot analysis: Cells in liquid cultures were starved for nitrogen as described by Nielsen and Egel (1990). RNA was prepared according to Schmitt *et al.* (1990). A total of 5 μg of each sample was run in 1.5% agarose 2.2 m formaldehyde gels in 3-[*N*-morpholino]propanesulfonic acid buffer (Sambrook *et al.* 1989) and blotted onto Hybond-N membrane (Amersham) according to the manufacturer's instruction. Antisense RNA probes were prepared from a 665-bp *Xba*I-*Hin*dIII fragment of the *cdc2* gene (cdc2 probe; Hindley and Phear 1984; Nielsen and Egel 1990), a *mat1-M* 1016-bp *Bcl*I-*Taq*I DNA fragment, and a *mat2-P* 904-bp *Hin*PI-*Mlu*I DNA fragment (Mc and Pm probes; Kelly *et al.* 1988; Nielsen and Egel 1990) using a Riboprobe II core system (Promega) and 3000 Ci/mmol [α-^{32}P]UTP (Amersham). Hybridization was allowed to occur for 24 hr at 42° in 0.25 m $NaHPO_4$, pH 7.2, 0.25 m NaCl, 7% SDS, 1 mm EDTA, 50% formamide, 10% polyethylenglycol (4000), 5× Denhardt's solution, 100 μg/ml yeast RNA. Washes and autoradiography were as for the Southern blots.

RESULTS

Deletion analysis of the chromosomal region flanking *mat3-M*: We introduced nested deletions on both sides of the *mat3-M* cassette. The extent of the deletions and their effect on *mat3-M* expression in both *swi6*$^{+}$ and *swi6-115* background, where the integrations were obtained, are shown in Figure 2. Expression of *mat3-M* was monitored by the amount of haploid meiosis occurring in cells with the stable *mat1-P*Δ*17::LEU2* allele. This assay relies on the observation that coexpression of the *P* and *M* mating-type genes triggers meiosis, not only in zygotes or diploid cells where the situation naturally occurs, but also in haploid cells (Kelly *et al.* 1988). The amount of haploid meiosis observed in *mat1-P*Δ*17::LEU2* cells reflects the expression of the *M* genes from *mat3-M.* Haploid meiosis was monitored by exposing colonies to iodine vapors, upon which spore-containing colonies are stained darkly whereas colonies containing no spores are stained yellow (Bresch *et al.* 1968). According to this assay, deletion of 482 nucleotides on the centromere-proximal side of the *mat3-M* cassette increased expression of the *M* genes. Larger deletions that included these 482 bp had the same effect. A smaller deletion removing only the H3 homology box and a deletion distal to the cassette failed to derepress.

TABLE 1

Strains and their genotypes

Strain	Genotype	Source[a]
BP95	*mat3-P(EcoRV)::ura4 ura4-D18 ade6-delXB clr2-E22*	This study
BP100	*mat3-M(EcoRV)::ade6ori1 ura4-D18 ade6-delXB clr2-E22*	BP95 TW pPB17[b]
BP141	*mat3-M(EcoRV)::ade6ori1 leu1-32 ura4-D18 ade6-M210*	This study
BP181	*mat3-M(EcoRV)::ade6ori1 ura4-D18 ade6-M210 swi6-115*	This study
PG1	*leu1-32 ura4-D18 ade6-M216 swi6-115*	Thon and Friis (1997)
PG9	*mat3-M(EcoRV)::ura4 leu1-32 ura4-D18 ade6-M216*	Thon and Klar (1992)
PG355	*mat3-P(EcoRV)::ura4 leu1-32 ura4-D18 ade6-M210*	This study
PG445	*mat1-PΔ17::LEU2 leu1-32 ura4-D18 ade6-M216*	This study
PG447	*mat1-PΔ17::LEU2 mat3-M(EcoRV)::ura4 leu1-32 ura4-D18 ade6-M216*	Thon and Klar (1992)
PG587	*Δ(H3)mat3-M(EcoRV)::ura4 leu1-32 ura4-D18 ade6-M210*	PL12 × SP980
PG1049	*mat1-PΔ17::LEU2 mat3-M(EcoRV)::ura4 leu1-32 ura4-D18 ade6-M216 swi6-115*	This study
PG1063	*mat1-Msmt-0 mat2-P(XbaI)::ura4 ura4-D18 ade6-M210 swi6-115 esp3-1*	Thon and Friis (1997)
PG1141	*leu1-32 ura4-D18 ade6-M210*	This study
PG1165	*mat1-Msmt-0 Δ(BglII-BssHII)mat2-P(XbaI)::ura4 leu1-32 ura4-D18 ade6-M216 esp3-1*	This study
PG1174	*mat1-Msmt-0 mat2-P(XbaI)::ura4 ura4-D18 ade6-M216 esp3-1*	This study
PG1192	*mat1-PΔ17::LEU2 Δ(482)mat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT79d482[c]
PG1193	*mat1-PΔ17::LEU2 Δ(1185)mat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT79d1185[c]
PG1194	*mat1-PΔ17::LEU2 mat3-MΔ(424) leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT70d424[c]
PG1195	*mat1-PΔ17::LEU2 Δ(1510)mat3-MΔ(424) leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT181[c]
PG1357	*mat3-M(EcoRV)::ura4 leu1-32 ura4-D18 ade6-M210*	This study
PG1397	*mat1-PΔ17::LEU2 Δ(1510)mat3-MΔ(424) leu1-32 ura4-D18 ade6-M210*	PG1195 × PG1357
PG1398	*mat1-PΔ17::LEU2 Δ(1185)mat3-M leu1-32 ura4-D18 ade6-M210*	PG1193 × PG1357
PG1399	*mat1-PΔ17::LEU2 Δ(1185)mat3-M leu1-32 ura4-D18 ade6-M210 swi6-115*	PG1193 × PG1357
PG1401	*mat1-PΔ17::LEU2 Δ(482)mat3-M leu1-32 ura4-D18 ade6-M210 swi6-115*	PG1192 × PG1357
PG1403	*mat1-PΔ17::LEU2 Δ(482)mat3-M leu1-32 ura4-D18 ade6-M210*	PG1192 × PG1357
PG1404	*mat1-PΔ17::LEU2 mat3-MΔ(424) leu1-32 ura4-D18 ade6-M210*	PG1194 × PG1357
PG1412	*mat1-PΔ17::LEU2 Δ(482)mat3-M leu1-32 ura4-D18 ade6-M210 clr1-5*	This study
PG1418	*mat1-PΔ17::LEU2 Δ(482)mat3-M leu1-32 ura4-D18 ade6-M210 clr3-735*	This study
PG1420	*mat1-PΔ17::LEU2 Δ(482)mat3-M leu1-32 ura4-D18 ade6-M210 clr4-681*	This study
PG1421	*mat1-PΔ17::LEU2 Δ(482)mat3-M leu1-32 ura4-D18 ade6-M210 clr2-760*	This study
PG1435	*mat1-PΔ17::LEU2 Δ(482)mat3-M leu1-32 ura4-D18 ade6-M216 esp3-1*	PG1174 × PG1403
PG1526	*mat1-PΔ17::LEU2 Δ(1185)::(424)oriImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT142[c]
PG1527	*mat1-PΔ17::LEU2 Δ(482)::(424)oriImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT132[c]
PG1529	*mat1-PΔ17::LEU2 Δ(482)::(424)oriIImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT133[c]
PG1531	*mat1-PΔ17::LEU2 Δ(482)::(134)oriImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT124[c]
PG1534	*mat1-PΔ17::LEU2 Δ(482)::(136)oriImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT125[c]
PG1538	*mat1-PΔ17::LEU2 Δ(482)::(88)oriImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT126[c]
PG1541	*mat1-PΔ17::LEU2 Δ(482)::(126)oriImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT127[c]
PG1548	*mat1-PΔ17::LEU2 Δ(482)mat3-M(EcoRV)::ura4 leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1192 TW pGT77[d]

(*continued*)

TABLE 1

(Continued)

Strain	Genotype	Source[a]
PG1550	*mat1-PΔ17::LEU2 Δ(482)::mat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216*	SP1125 × PG1548
PG1554	*mat1-PΔ17::LEU2 Δ(482)::(126)oriIImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT131[c]
PG1557	*mat1-PΔ17::LEU2 Δ(1185)::(126)oriImat3-M leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1049 TW pGT137[c]
PG1560	*mat1-PΔ17::LEU2 Δ(H3)mat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M210*	PG587 × PG445
PG1562	*mat1-Msmt-0 mat2-P(Xba*I*)::ura4 mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210 swi6-115*	BP181 × SP1126
PG1564	*mat1-Msmt-0 Δ(Bgl*II*-Bss*HII*)mat2-P(Xba*I*)::ura4 mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210 swi6-115*	BP181 × SP1138
PG1566	*mat1-Msmt-0 leu1-32*	This study
PG1570	*leu1-32 ura4-D18*	This study
PG1579	*mat1-PΔ17::LEU2 leu1-32 ura4-D18 ade6-M210 clr3-735*	This study
PG1582	*mat1-PΔ17::LEU2 leu1-32 ura4-D18 ade6-M210 clr2-760*	This study
PG1584	*mat1-PΔ17::LEU2 leu1-32 ura4-D18 ade6-M210 swi6-115*	This study
PG1587	*mat1-PΔ17::LEU2 leu1-32 ura4-D18 ade6-M210 clr1-5*	This study
PG1594	*mat1-PΔ17::LEU2 leu1-32 ura4-D18 ade6-M216 clr4-681*	This study
PG1595	*mat1-Msmt-0 mat2-P(Xba*I*)::ura4 mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210*	PG1562 × SP1122
PG1596	*mat1-Msmt-0 Δ(Bgl*II*-Bss*HII*)mat2-P(Xba*I*)::ura4 mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210*	PG1564 × SP1122
PG1610	*Δ(H3)mat3-M(Eco*RV*)::ade6ori1 leu1-32 ura4-D18 ade6-M210 swi6-115*	PL1 TW pPB17[e]
PG1612	*mat1-PΔ17::LEU2 Δ(482)mat3-M(Eco*RV*)::ade6oriI leu1-32 ura4-D18 ade6-M210 swi6-115*	PG1041 TW pPB17[e]
PG1614	*mat1-PΔ17::LEU2 Δ(H3)mat3-M(Eco*RV*)::ade6ori1 leu1-32 ura4-D18 ade6-M210 swi6-115*	PG1399 TW pPB17[e]
PG1615	*mat1-PΔ17::LEU2 Δ(H3)mat3-M leu1-32 ura4-D18 ade6-M210 swi6-115*	PL1 × PG1049
PG1616	*mat1-PΔ17::LEU2 Δ(1185)::(424)oriImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1526 TW pGT77[d]
PG1617	*mat1-PΔ17::LEU2 Δ(482)::(424)oriImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1527 TW pGT77[d]
PG1619	*mat1-PΔ17::LEU2 Δ(482)::(424)oriIImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1529 TW pGT77[d]
PG1620	*mat1-PΔ17::LEU2 Δ(482)::(126)oriIImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1554 TW pGT77[d]
PG1622	*mat1-PΔ17::LEU2 Δ(482)::(126)oriImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1541 TW pGT77[d]
PG1624	*mat1-PΔ17::LEU2 Δ(1185)::(424)oriImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M210*	PG1616 × SP1125
PG1626	*mat1-PΔ17::LEU2 Δ(482)::(424)oriImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216*	PG1617 × SP1125
PG1629	*mat1-PΔ17::LEU2 Δ(482)::(424)oriIImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216*	PG1619 × SP1125
PG1630	*mat1-PΔ17::LEU2 Δ(482)::(126)oriIImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216*	PG1620 × SP1125
PG1632	*mat1-PΔ17::LEU2 Δ(482)::(126)oriImat3-M(Eco*RV*)::ura4 leu1-32 ura4-D18 ade6-M216*	PG1622 × SP1125
PG1646	*mat1-PΔ17::LEU2 mat2-P(Xba*I*)::ura4 leu1-32 ura4-D18 ade6-M210*	This study
PG1647	*Δ(H3)mat3-M(Eco*RV*)::ade6ori1 leu1-32 ura4-D18 ade6-M210*	PG1610 × SP1125
PG1649	*mat1-PΔ17::LEU2 Δ(482)mat3-M(Eco*RV*)::ade6oriI leu1-32 ura4-D18 ade6-M210*	PG1612 × SP1125
PG1650	*mat1-PΔ17::LEU2 Δ(1185)mat3-M(Eco*RV*)::ade6oriI leu1-32 ura4-D18 ade6-M210*	PG1614 × SP1125
PG1654	*mat1-Msmt-0 mat2-P(Xba*I*)::ura4 Δ(H3)mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210 swi6-115*	PG1610 × SP1126
PG1655	*mat1-Msmt-0 mat2-P(Xba*I*)::ura4 Δ(482)mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210 swi6-115*	PG1612 × SP1126
PG1656	*mat1-Msmt-0 mat2-P(Xba*I*)::ura4 Δ(1185)mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210 swi6-115*	PG1614 × SP1126
PG1657	*mat1-Msmt-0 Δ(Bgl*II*-Bss*HII*)mat2-P(Xba*I*)::ura4 Δ(H3)mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210 swi6-115*	PG1610 × SP1138
PG1658	*mat1-Msmt-0 Δ(Bgl*II*-Bss*HII*)mat2-P(Xba*I*)::ura4 Δ(482)mat3-M(Eco*RV*)::ade6oriI ura4-D18 ade6-M210 swi6-115*	PG1612 × SP1138

(*continued*)

TABLE 1

(Continued)

Strain	Genotype	Source[a]
PG1659	*mat1-Msmt-0 Δ(BglII-BssHII)mat2-P(XbaI)::ura4 Δ(1185)mat3-M(EcoRV)::ade6oriI ura4-D18 ade6-M210 swi6-115*	PG1614 × SP1138
PG1671	*mat1-PΔ17::LEU2 Δ(H3)mat3-M(EcoRV)::ade6ori1 leu1-32 ura4-D18 ade6-M210*	PG1647 × PG1646
PG1672	*mat1-PΔ17::LEU2 mat3-M(EcoRV)::ade6oriI leu1-32 ura4-D18 ade6-M210*	BP141 × PG1646
PG1675	*mat1-Msmt-0 mat2-P(XbaI)::ura4 Δ(482)mat3-M(EcoRV)::ade6oriI ura4-D18 ade6-M210*	PG1655 × PG1646
PG1680	*mat1-Msmt-0 Δ(BglII-BssHII)mat2-P(XbaI)::ura4 Δ(H3)mat3-M(EcoRV)::ade6oriI ura4-D18 ade6-M210*	PG1657 × PG1646
PG1681	*Δ(482)mat3-M(EcoRV)::ade6oriI leu1-32 ura4-D18 ade6-M210*	PG1649 × PG1141
PG1682	*Δ(1185)mat3-M(EcoRV)::ade6oriI leu1-32 ura4-D18 ade6-M210*	PG1650 × PG1141
PG1683	*mat1-Msmt-0 Δ(BglII-BssHII)mat2-P(XbaI)::ura4 Δ(482)mat3-M(EcoRV)::ade6oriI ura4-D18 ade6-M210*	PG1658 × PG1646
PG1685	*mat1-Msmt-0 Δ(BglII-BssHII)mat2-P(XbaI)::ura4 Δ(1185)mat3-M(EcoRV)::ade6oriI ura4-D18 ade6-M210*	PG1659 × PG1646
PG1686	*mat1-Msmt-0 mat2-P(XbaI)::ura4 Δ(H3)mat3-M(EcoRV)::ade6oriI ura4-D18 ade6-M210*	PG1654 × PG1646
PG1688	*mat1-Msmt-0 mat2-P(XbaI)::ura4 Δ(1185)mat3-M(EcoRV)::ade6oriI ura4-D18 ade6-M210*	PG1656 × PG1646
PG1690	*mat1-PΔ17::LEU2 Δ(H3)mat3-M leu1-32 ura4-D18 ade6-M210*	This study
PG1741	*mat1-PΔ17::LEU2 leu1-32 ura4-D18 ade6-M210 swi6-115 esp3-1*	PG445 × PG1063
PG1742	*mat1-PΔ17::LEU2 leu1-32 ura4-D18 ade6-M210 esp3-1*	PG445 × PG1063
PL1	*Δ(H3)mat3-M leu1-32 ura4-D18 ade6-M210 swi6-115*	PG355 TW pGT79[c]
PL6	*Δ(H3)mat3-M leu1-32 ura4-D18 ade6-M216*	PL1 × PG9
PL12	*Δ(H3)mat3-M(EcoRV)::ura4 leu1-32 ura4-D18 ade6-M216 swi6-115*	PG1 TW pGT180[c]
SP980	*his2 ade6-M210*	This study
SP1122	*mat1-PΔ17::LEU2 mat2-P(XbaI)::ura4 leu1-32 ura4-D18 ade6-M216*	This study
SP1124	*mat1-Msmt-0 mat2-P(XbaI)::ura4 ura4-D18 ade6-M216*	Thon *et al.* (1994)
SP1125	*mat1-Msmt-0 mat2-P(XbaI)::ura4 ura4-D18 ade6-M210*	This study
SP1126	*mat1-Msmt-0 mat2-P(XbaI)::ura4 ura4-D18 ade6-M210 swi6-115*	Thon *et al.* (1994)
SP1138	*mat1-Msmt-0 Δ(BglII-BssHII)mat2-P(XbaI)::ura4 ura4-D18 ade6-M210 swi6-115*	Thon *et al.* (1994)
SP1151	*mat1-Msmt-0 Δ(BglII-BssHII)mat2-P(XbaI)::ura4 ura4-D18 ade6-M216*	Thon *et al.* (1994)

[a]Strains were constructed by transformation with restricted plasmid DNA (TW), by genetic crosses of strains listed here (×) or by crosses involving strains not listed here (This study), in which case the complete genealogy is available upon request. Mutated and engineered alleles used for the strain constructions were described in the following studies: *mat1-PΔ17::LEU2*: Arcangioli and Klar (1991); *mat1-Msmt-0*: Engelke *et al.* (1987) and Styrkarsdottir *et al.* (1993); *mat3-M(EcoRV)::ura4*: Thon and Klar (1992); *mat3-P(EcoRV)::ura4*: Thon and Klar (1993); *mat2-P(XbaI)::ura4*: Thon *et al.* (1994); *ura4-D18*: Grimm *et al.* (1988); *ade6-M210* and *ade6-M216*: Gutz (1963); *ade6-delXB*: Zahn-Zabal *et al.* (1995); *swi6-115*: Gutz and Schmidt (1985); *clr1-5*: Thon and Klar (1992); *clr2-E22*: Ekwall and Ruusala (1994); *clr2-760, clr3-735, clr4-681*: Thon *et al.* (1994).

[b]Restricted with *Pst*I and *Sal*I.

[c]Restricted with *Hin*dIII.

[d]Restricted with *Pst*I and *Msp*AI.

[e]Restricted with *Eco*RI.

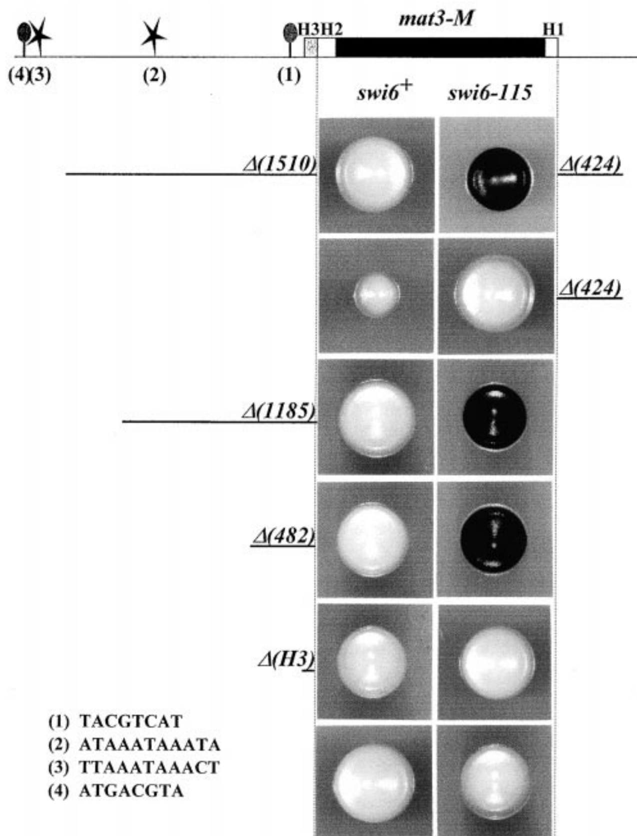


Figure 2.—Effect of *mat3-M* flanking deletions on the expression of *mat3-M.* Sporulated colonies of *mat1-P*Δ*17::LEU2* cells with the *mat3-M* flanking deletions represented by bars on the side of the photographs were stained with iodine vapors to monitor expression of *mat3-M* in both *swi6*$^+$ and *swi6-115* backgrounds as indicated. The intensity of staining reflects the amount of haploid meiosis occurring in the colonies. *swi6*$^+$ strains, from top to bottom: PG1397, PG1404, PG1398, PG1403, PG1690, and PG445; *swi6-115* strains, from top to bottom: PG1195, PG1194, PG1193, PG1192, PG1615, and PG1584. (1) and (4) represent two potential Mts1/Mts2 (Atf1/Pcr1)-binding sites. (2) and (3) represent two ARS consensus sequences.

In the cases where derepression was observed, the effect was small in a wild-type background but very pronounced in a *swi6-115* background.

Additional constructs were introduced in the chromosome to localize more precisely the element(s) responsible for the repression (Figure 3). In these constructs, PCR products representing portions of *mat3-M* flanking DNA were introduced in either the 1185- or 482-bp deletions. This analysis revealed the following. First, a 424-bp fragment representing the portion of DNA deleted in the 482-bp deletion except for the H3 box was sufficient to restore silencing in the 1185-bp deletion, indicating the remaining 761 bp were not required for silencing *mat3-M.* Second, part of the repression was mediated via an element located within 126 bp of the cassette. The 126-bp DNA fragment partially restored silencing when introduced in the large centromere-proximal deletion of 1185 bp or in the 482-bp deletion. An adjacent fragment of 88 bp also increased the repression although not as efficiently as the 126-bp fragment.

In summary, this series of experiments indicates that the H3 homology box does not exert a significant repression on the *M* genes contained in the *mat3-M* cassette and it also rules out an effect of the sequences immediately distal to *mat3-M.* In contrast, one or several DNA elements located proximally, within <500 bp of the H3 box, play an important role in silencing.

Combination of *cis*- and *trans*-acting mutations: Combining *trans*-acting mutations pairwise allows assignment of the silencing factors that repress transcription in the mating-type region to one of two groups. Factors whose mutations do not have a cumulative effect when combined are assigned to the same group. Thus, *swi6*, *clr1*, *clr2*, *clr3*, and *clr4* belong to one group (Thon *et al.* 1994) and *esp1*, *esp2*, and *esp3* to the second group (Thon and Friis 1997). The synergy between the *swi6-115* mutation and the *mat3-M* flanking deletion of 482 bp suggests that the deleted element acts in a pathway other than the one mediated by *swi6.* If the deleted element participates in the pathway containing the *esp* products, two predictions should be fullfilled. One prediction is that deletions flanking *mat3-M* should potentiate not only mutations in *swi6*, but also mutations in *clr1*, *clr2*, *clr3*, and *clr4.* The second prediction is that expression of *mat3-M* should not be increased when the deletion of 482 bp is introduced in *esp* mutant cells such as the *esp3-1* mutant. We have tested both predictions and found them to be true. First, combining the 482 bp deletion with mutations in *clr1*, *clr2*, *clr3*, or *clr4* caused a strong derepression of *mat3-M* leading to very high levels of haploid meiosis (Figure 4). Second, combining the 482-bp deletion with *esp3-1* did not enhance *mat3-M* expression: *Mc* transcripts were not detected in a Δ*(482) esp3-1* double mutant (Figure 5A). A low amount of *Mc* transcript was seen in RNA preparations from *swi6-115* cells, and much higher amounts were seen in *swi6-115* Δ*(482)* and *swi6-115 esp3-1* double mutants (Figure 5A), as expected from the sporulation phenotypes of these mutants (Figure 4 and data not shown).

Deletion of one or more *cis*-acting elements located on the centromere-proximal side of the *mat2-P* cassette in a 1.5-kb *Bgl*II-*Bss*HII fragment was previously reported to cause phenotypes similar to the phenotypes caused by the *mat3-M* flanking deletions reported here (Thon *et al.* 1994). The *Bgl*II-*Bss*HII deletion had only a small effect on its own but enhanced the effect of mutations in *swi6*, *clr1*, *clr2*, *clr3*, or *clr4.* We examined the amount of *Pm* transcript originating from *mat2-P* in single- and double-mutant backgrounds, as described above for the *mat3-M* cassette. As can be seen in Figure 5B, expression of the *Pm* gene was increased by combining a mutant *swi6* allele with either the *mat2-P cis*-acting deletion or a mutant *esp3* allele, but not by combining the *cis*-acting deletion with the mutant *esp3* allele. The

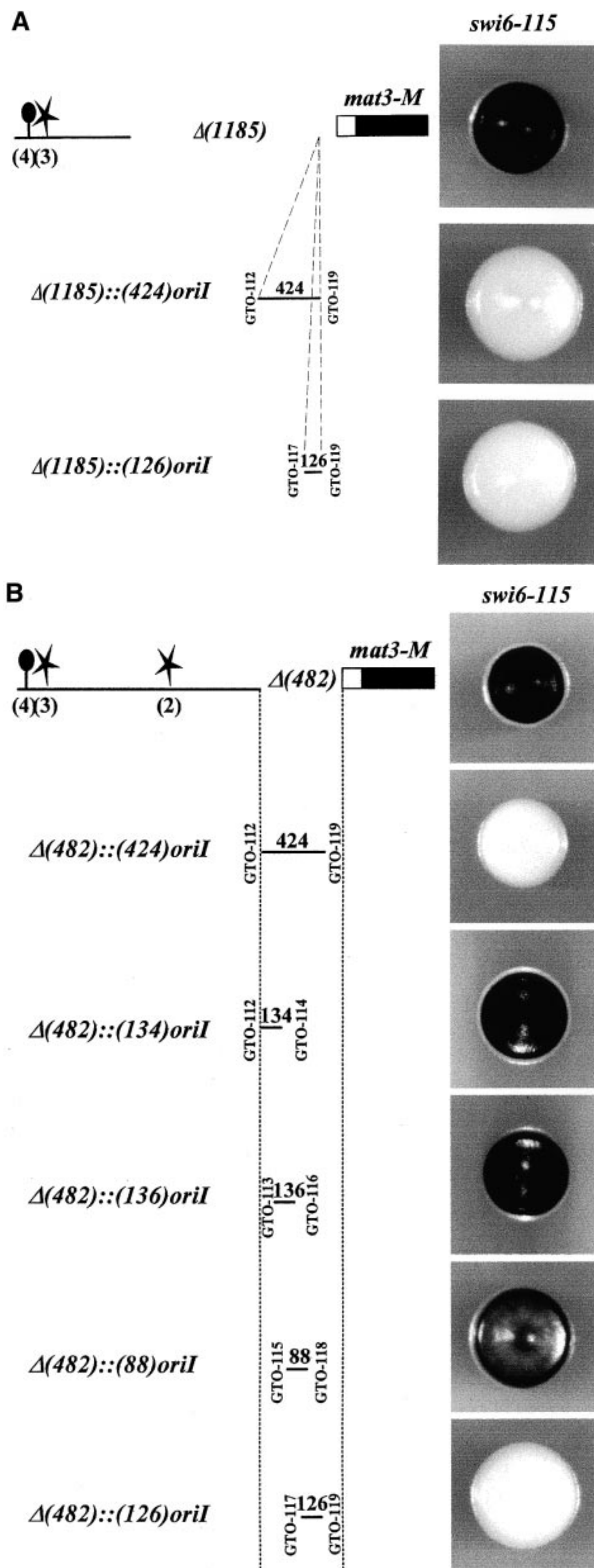


Figure 3.—Refined analysis of the centromere-proximal *mat3-M* flanking region. (A) Insertion of DNA elements in Δ*(1185)*. DNA fragments containing 424 or 126 nucleotides of *mat3-M* flanking DNA were produced by PCR using the indicated primers (GTO-112, -117, -119; sequences shown in Figure 1) and introduced in place of the Δ*(1185)* deletion in *mat1-P*Δ*17::LEU2 swi6-115* cells. Sporulated colonies were stained with iodine as in Figure 2. Δ*(1185)*: PG1399; Δ*(1185)::(424)oriI*: PG1526; Δ*(1185)::(126)oriI*: PG1557. (B) Insertions of DNA elements in Δ*(482)*. DNA fragments produced as in A were used to replace Δ*(482)* in *mat1-P*Δ*17::LEU2 swi6-115* cells. Δ*(482)*: PG1401; Δ*(482)::(424)oriI*: PG1527; Δ*(482)::(134) oriI*: PG1531; Δ*(482)::(136)oriI*: PG1534; Δ*(482)::(88)oriI*: PG1538; Δ*(482)::(126)oriI*: PG1541.

strongest derepression of *mat2-P* was caused by simultaneous impairment of the two *trans*-acting factors, *swi6* and *esp3*, which also led to the strongest derepression of *mat3-M* (Figure 5A).

Hence, the *mat2-P* and *mat3-M* flanking elements have similar genetic interactions with *trans*-acting factors important for silencing: a cumulative effect with mutations in *swi6*, *clr1*, *clr2*, *clr3*, and *clr4*, and no cumulative effect with a mutation in *esp3*.

Effect of *mat3-M* proximal deletions on the expression of the *S. pombe ura4* and *ade6* genes placed near *mat3*: The deletion analysis of the *mat3-M* flanking regions had revealed the presence of DNA sequences important for the repression of the mating-type genes contained within the cassette. We tested whether these sequences could silence other *S. pombe* genes placed on the centromere-distal side of *mat3-M*. The *S. pombe ura4* gene is repressed in wild-type backgrounds when placed at an *Eco*RV site located ~150 bp away from the *mat3-M* cassette (Thon and Klar 1992). We introduced the *ura4* gene at the *Eco*RV site in some of the strains with the *mat3-M* flanking deletions described above. As shown in Figure 6A, deleting the H3 box did not affect expression of *ura4* placed at the *Eco*RV site near *mat3-M*, whereas deleting 482 nucleotides led to an increased expression of *ura4*. The ability of cells to form colonies on FOA was not abolished by the deletion, but growth was greatly reduced, resulting in abnormally small colonies. These observations confirmed that one or more elements contained within the deleted fragment had a repressive effect on transcription and that the repression was not specific for the *M* genes located within *mat3-M* but could affect the promoter of another gene placed at a distance >2.6 kb from the *cis*-acting element.

Next, we introduced the *S. pombe ade6* gene at the *Eco*RV site previously used to integrate *ura4*. As shown in Figure 6B, *ade6* placed at the *Eco*RV site near *mat3-M* was repressed in wild-type cells and cells with the deletion of the H3 box. The *mat3-M* proximal deletions of 482 bp and 1185 bp caused an increased expression of *ade6*, allowing more cells to form colonies on a medium lacking adenine. In addition to their auxotrophic requirement, *S. pombe ade6* mutant strains form red colonies on media with low concentrations of adenine. Cells with no deletion or the deletion of the H3 box formed red colonies, consistent with their poor ability to grow in the absence of adenine. Cells with the deletions of 482 or 1185 bp displayed a variegated phenotype and formed both light red and white colonies. This variega-

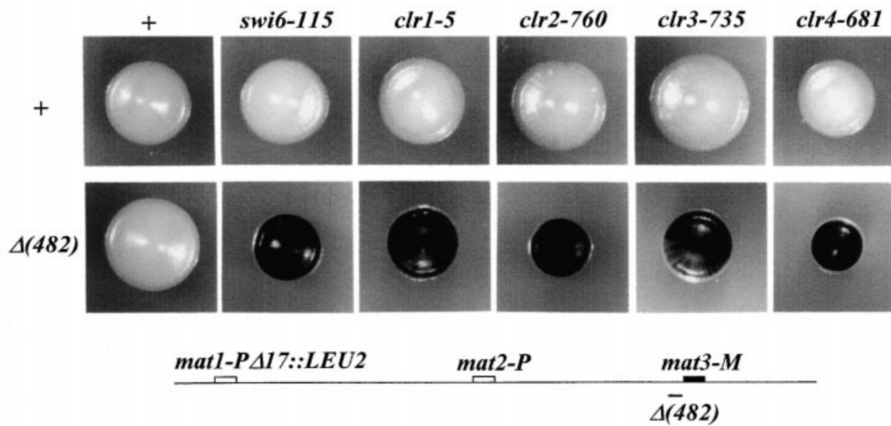


Figure 4.—Cumulative effects of *trans*-acting mutations in *swi6*, *clr1*, *clr2*, *clr3*, or *clr4* with a *mat3-M* flanking deletion. Sporulated colonies of *mat1-PΔ17::LEU2* cells with the indicated mutations were stained as in Figure 2. The strains were: +, +: PG445; +, *swi6-115*: PG1584; +, *clr1-5*: PG1587; +, *clr2-760*: PG1582; +, *clr3-735*: PG1579; +, *clr4-681*: PG1594; Δ*(482)*, +: PG1403; Δ*(482)*, *swi6-115*: PG1401; Δ*(482)*, *clr1-5*: PG1412; Δ*(482)*, *clr2-760*: PG1421; Δ*(482)*, *clr3-735*: PG1418; Δ*(482)*, *clr4-681*: PG1420.

tion indicated that the level of derepression of the *ade6* gene was not the same in all cells and that both the lower and higher levels of expression could be inherited for several generations.

We noticed a difference between h^{90} and *mat1-PΔ17::LEU2* cells in their ability to form white colonies on media poor in adenine (Figure 6C and data not shown). Expression of *ade6* was consistently higher in *mat1-PΔ17::LEU2* colonies than it was in h^{90} colonies. Expression of *ura4* at that same location was similar (data not shown): *ura4* was more expressed in *P* cells (*mat1-PΔ17::LEU2* or *mat1-P*) than it was in h^{90} cells or *M* cells (*mat1-Msmt-0* or *mat1-M*). This difference might be due to cell-type specific chromatin structures allowing a better accessibility of the *mat3-M* cassette in *P* cells. The deletions introduced near *mat3-M* did not diminish mating or the formation of zygotic asci in a wild-type background, indicating they did not affect mating-type switching (data not shown).

Variegation of *ade6* expression: Cells whose sole functional copy of *ade6* was placed near *mat3-M* formed both red and white colonies when plated on a low concentration of adenine. Higher frequencies of white colonies were observed with strains that had the *mat3-M* flanking deletion of 482 or 1185 bp than in colonies that had no deletion or a deletion limited to the H3 homology box. However, occasional white or sectored colonies arose also in these latter strains. We assayed the stability of the two phenotypes by isolating red and white colonies from these strains (Figure 6C). The two phenotypes were found to interconvert in all strains examined. Reversion to a repressed state was observed more frequently in h^{90} cells than in *mat1-PΔ17::LEU2* cells and, conversely, reversion to a derepressed state occurred more frequently in *mat1-PΔ17::LEU2* than in h^{90} cells. The two largest deletions, of 482 and 1185 bp, slowed the reestablishment of repression and facilitated conversion from the repressed to the derepressed state.

Epigenetic effects caused by a *cis*-acting element near *mat2-P*: Having observed that *mat3-M* flanking deletions conferred semistable repressed and derepressed phenotypes that could interconvert, we tested whether the *mat2-P* flanking deletion of 1.5 kb (Thon *et al.* 1994) also had such a property. We examined two phenotypes of strains having a *mat2-P* flanking deletion: the ability of such strains to express a *ura4* gene placed near *mat2-P* and the ability of unswitchable *mat1-Msmt-0* strains with the *mat2-P* flanking deletion to sporulate. Cells with the Δ*(Bgl*II*-Bss*HII*)mat2-P(Xba*I*)::ura4* allele express *ura4* weakly; few can form colonies on medium lacking uracil and many form colonies on medium containing FOA (Thon *et al.* 1994; Figure 7A). The stability of both Ura^+ and FOA^R phenotypes was assayed by spotting cells from, respectively, Ura^+ and FOA^R colonies onto selective plates (Figure 7A). Cells originating from Ura^+ colonies conserved the ability to grow in the absence of uracil and grew poorly on FOA plates, indicating the derepressed state was stable for many generations. Cells originating from FOA^R colonies gave the same growth pattern as cells originating from complete medium: nearly all cells could form a colony on an FOA plate, indicating *ura4* was repressed in these cells, and approximately one cell in a hundred could form a colony on medium lacking uracil, indicating the repressed state was reversible. The sporulation phenotypes, which reflect expression of the *mat2-P* genes, were consistent with there being fluctuations between an expressed and repressed state of the *mat2-P* region (Figure 7B). Most colonies grown under nonselective conditions were Spo^-, indicating that the *mat2-P* genes were repressed in these colonies. Occasional speckles and streaks of sporulation were observed, indicating transient derepression of the *P* mating-type information. On plates selecting for *ura4* expression, high levels of haploid sporulation were observed and, on nonselective sporulation plates, Ura^+ cells formed colonies containing more haploid asci than colonies originating from Ura^- cells. Hence, the *P* genes were expressed in a variegated manner and their expression covariegated with the expression of the *ura4* gene at the *Xba*I site.

Range of action of the *mat2-P* and *mat3-M* silencing elements: The *mat2-P* and *mat3-M* centromere-proximal

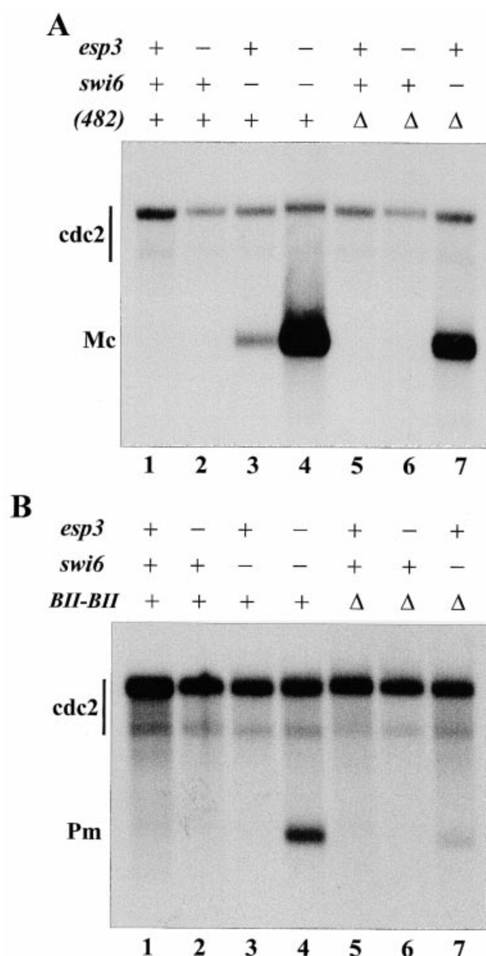
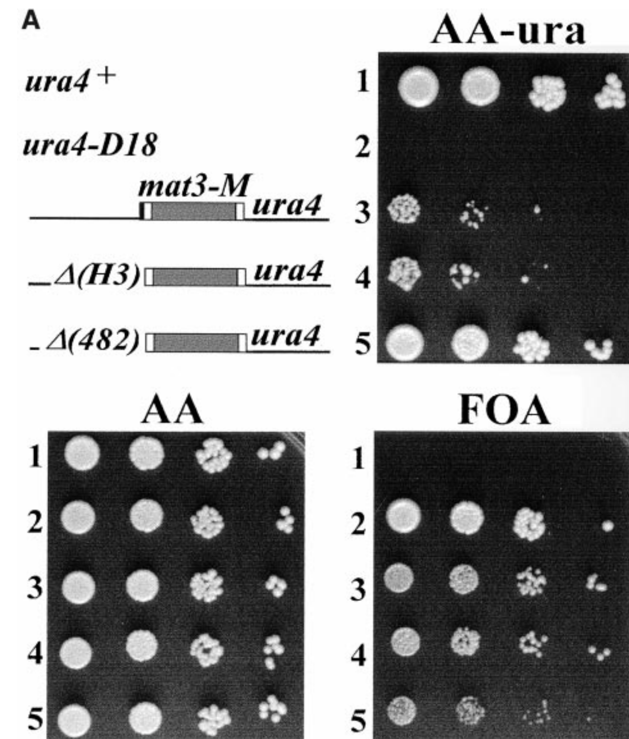


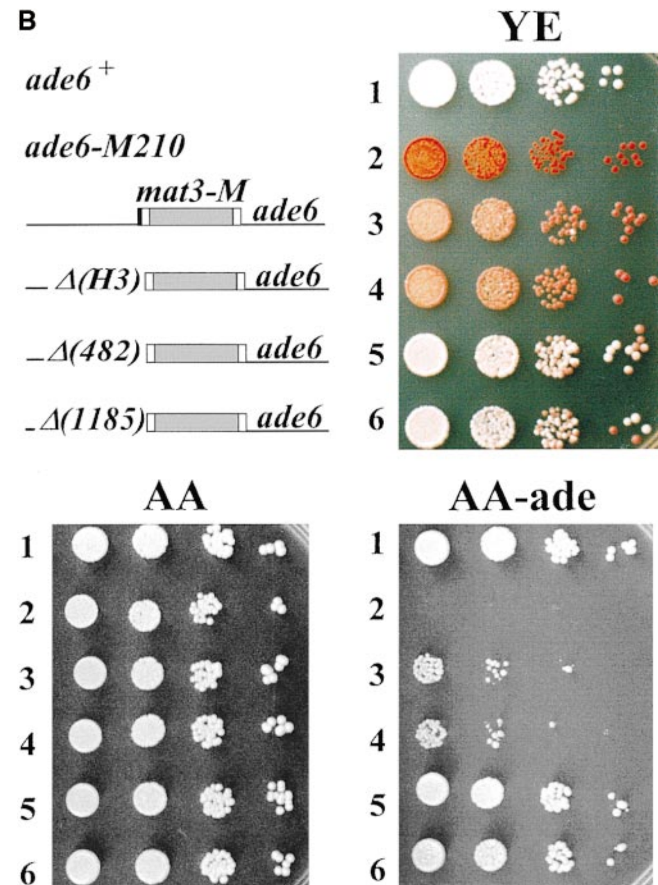
Figure 5.—Combined effects of mutations in *swi6*, *esp3*, and *cis*-acting deletions near *mat3-M* or *mat2-P*. RNA was prepared from nitrogen-starved cells with the indicated mutations and used for Northern blot analysis. The *Pm*, *Mc*, and *cdc2* probes are described in materials and methods. (A) Effects on *mat3-M*. All strains in A contain the *mat1-P*Δ*17::LEU2* allele. *(482)* represents the *mat3-M* centromere-proximal deletion of 482 bp. *Mc* transcripts originating from *mat3-M* were detected with an *M*-specific probe and the blot was reprobed with a *cdc2* probe to estimate the amount of RNA loaded in each lane. The *cdc2* probe recognizes several transcripts (Durkacz *et al.* 1986). The strains used were as follows: lane 1, PG445; lane 2, PG1742; lane 3, PG1584; lane 4, PG1741; lane 5, PG1403; lane 6, PG1435; lane 7, PG1401. (B) Effects on *mat2-P*. All strains used in this panel contain the *mat1-Msmt-0* allele, which allows detection of transcripts originating from *mat2-P* with a *P*-specific probe. The blot was reprobed with the *cdc2* probe as in A. *BII-BII* represents the *Bgl*II-*Bss*HII deletion on the centromere-proximal side of the *mat2-P* cassette. The strains used were: lane 1, SP1124; lane 2, PG1174; lane 3, SP1126; lane 4, PG1063; lane 5, SP1151; lane 6, PG1165; lane 7, SP1138.

silencing elements could both act at a distance on genes placed on the other side of, respectively, the *mat2-P* and *mat3-M* cassette. We tested whether these elements could act at an even greater distance by constructing strains with the *ura4* gene at the *Xba*I site near *mat2-P* and the *ade6* gene at the *Eco*RV site near *mat3-M*. All strains were of the *M* mating-type. In these strains, we tested whether deletion of the 1.5-kb *mat2-P Bgl*II-*Bss*HII proximal fragment increased expression of *ade6* near *mat3-M* and, conversely, whether deletions near the *mat3-M* cassette affected expression of *ura4* at the *mat2-P* distal *Xba*I site. We found that none of the deletions tested affected expression of the distant prototrophic marker (Figure 8). Combining the deletions near *mat2-P* and *mat3-M* within the same mating-type region did not increase expression of *ura4* near *mat2-P* or of *ade6* near *mat3-M*.

Because deletion of the *Bgl*II-*Bss*HII fragment near *mat2-P* caused variegated expression of the *ura4* gene at the *mat2-P* distal *Xba*I site and deletion of either 482 or 1185 bp near *mat3-M* caused variegated expression of *ade6* at the *Eco*RV site near *mat3-M*, we tested whether expression of the two prototrophic markers covariegated in strains that had both the *Bgl*II-*Bss*HII deletion and either the 482- or 1185-bp deletion. First, we spotted cells with the double deletions on medium lacking adenine, medium lacking uracil, and medium lacking both uracil and adenine. Many fewer colonies formed on medium lacking both uracil and adenine than on media lacking either supplement alone, indicating *ura4* and *ade6* were not expressed at the same time (Figure 8). In a second experiment, Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4* Δ*(482)mat3-M(Eco*RV*)::ade6* cells and Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4* Δ*(1185)mat3-M(Eco*RV*)::ade6* cells were propagated on, respectively, medium containing FOA, medium lacking uracil, and medium lacking adenine. As previously noted, cells that had been propagated in the absence of uracil remained able to grow well in the absence of uracil upon replating, whereas cells propagated in the presence of FOA grew poorly in the absence of uracil when replated. In contrast, the ability to grow on medium lacking adenine was not influenced by the absence of uracil or presence of FOA in the prior growth medium (data not shown). Conversely, cells that had been propagated in the absence of adenine had a better efficiency of plating than cells propagated under nonselective conditions when they were plated on medium lacking adenine, but not when they were plated on medium lacking uracil (data not shown). Hence, the expression of *ura4* near *mat2-P* and *ade6* near *mat3-M* appeared to be regulated independently in these strains where both the *mat2-P* and *mat3-M* silencing elements were deleted.

Orientation-dependence of the *mat3-M* silencing element: Silencing elements described in other systems repress transcription in either orientation (Brand *et al.* 1985; Mori *et al.* 1990; Kassis *et al.* 1991; Fauvarque and Dura 1993; Kallunki *et al.* 1995). We tested whether the element near *mat3-M* had such a property by reintroducing DNA fragments in a strain from which they had been removed in the orientation opposite to the wild-type. Two fragments were tested in this experiment: the centromere-proximal 424- and 126-bp fragments described above. In *swi6*$^{+}$ backgrounds, the 424-bp fragment was able to restore silencing of *ura4* equally





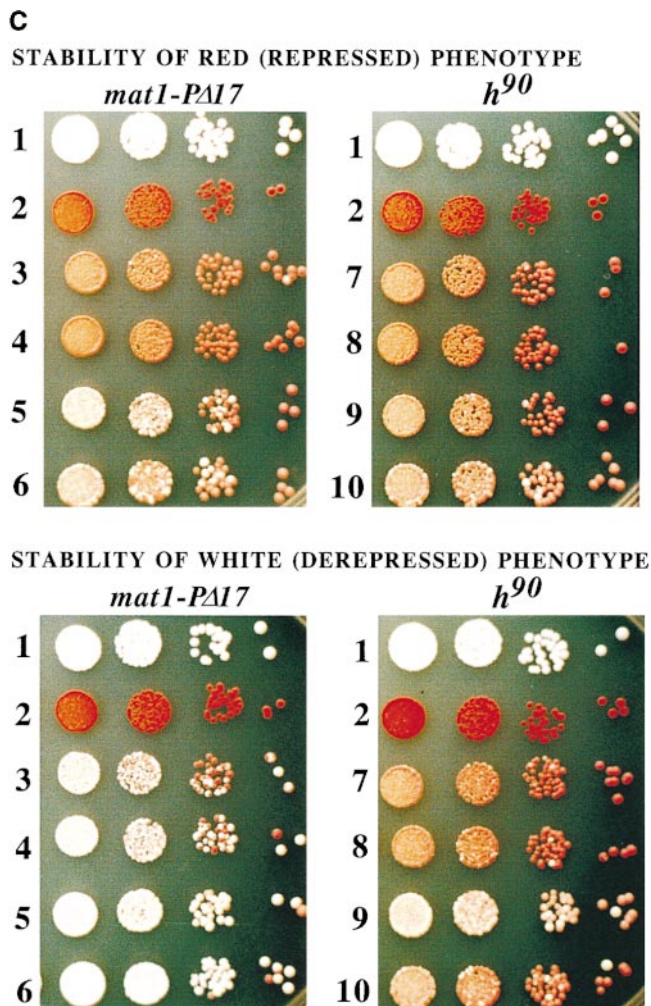
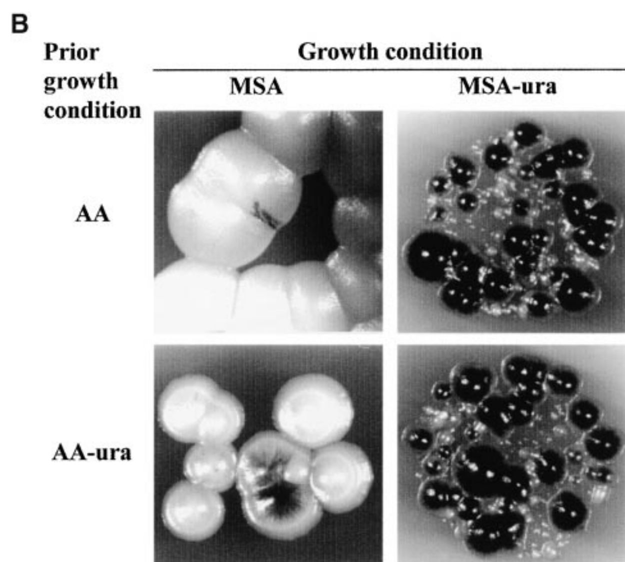


Figure 6.—Derepression of prototrophic markers caused by *mat3-M* flanking deletions. (A) Derepression of *ura4.* Cells containing the *mat3-M(Eco*RV*)::ura4* allele in combination with the indicated deletions were propagated under nonselective conditions (YES). Expression of *ura4* was monitored by spotting 10-fold serial dilutions of cell suspensions on medium lacking uracil (AA-ura), medium containing FOA (FOA), and complete medium (AA). Rows 1, PG1566; rows 2, PG1141; rows 3, PG447; rows 4, PG1560; rows 5, PG1550. (B) Derepression of *ade6.* Cells with the *mat3-M(Eco*RV*)::ade6* allele were propagated in YES medium. Tenfold serial dilutions of cell suspensions were plated on medium containing a low concentration of adenine (YE), on medium containing no adenine (AA-ade), and on complete medium (AA). Rows 1, PG1570; rows 2, PG1141; rows 3, PG1672; rows 4, PG1671; rows 5, PG1649; rows 6, PG1650. (C) Variegation of *ade6* expression in *mat1-P*Δ*17::LEU2* and h^{90} cells. The stability of the "Red on YE" and "White on YE" phenotypes of cells having their sole functional copy of *ade6* near *mat3-M* was assayed by isolating red and white colonies, allowing the cells to divide in rich medium supplemented with adenine (YES) for ∼10 generations, and replating them on YE. Cells with a stable *mat1-P* allele (*mat1-P*Δ*17::LEU2*; strains 3–6) or with a switchable *mat1* allele (h^{90}; strains 7–10) were used. Rows 1–6, same as in B; h^{90} strains: rows 7, BP141; rows 8, PG1647; rows 9, PG1681; rows 10, PG1682.

A

mat2-P allele	Prior growth condition	Growth condition: AA-ura	FOA	AA
Δ(*BglII-BssHII*)*mat2-P*(*XbaI*)::*ura4*	AA			
"	"			
"	"			
mat2-P(*XbaI*)::*ura4*	"			
"	"			
"	"			
Δ(*BglII-BssHII*)*mat2-P*(*XbaI*)::*ura4*	AA			
"	AA-ura			
"	"			
"	"			
"	"			
"	"			
"	AA			
"	FOA			
"	"			
"	"			
"	"			
"	"			

B



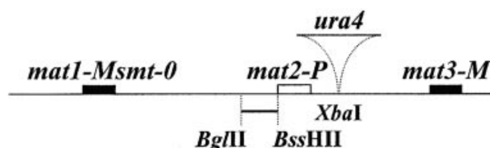


Figure 7.—Variegated phenotypes caused by a *mat2-P* flanking deletion. (A) Variegation of *ura4* expression in the Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4* allele. Expression of *ura4* placed at an *Xba*I site near *mat2-P* was assayed by spot tests in two strains: a strain with a *mat2-P* flanking deletion of ~1.5 kb [Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4*: SP1151] and a strain with no deletion [*mat2-P(Xba*I*)::ura4*: SP1124]. Tenfold dilutions of three independent cultures of each strain were spotted (top). The stability of the derepressed and repressed states was assayed by spotting on selective media independent cultures of Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4* cells (SP1151) that had been propagated in the absence of uracil (middle) or in the presence of FOA (bottom). (B) Variegation of sporulation phenotype. The SP1151 cells used above contain a stable *mat1-Msmt-0* allele. They were propagated in complete medium (AA) or medium lacking uracil (AA-ura) and plated on sporulation medium containing uracil (MSA) or lacking uracil (MSA-ura). Colonies were allowed to sporulate and stained with iodine to estimate the level of expression of *mat2-P.*

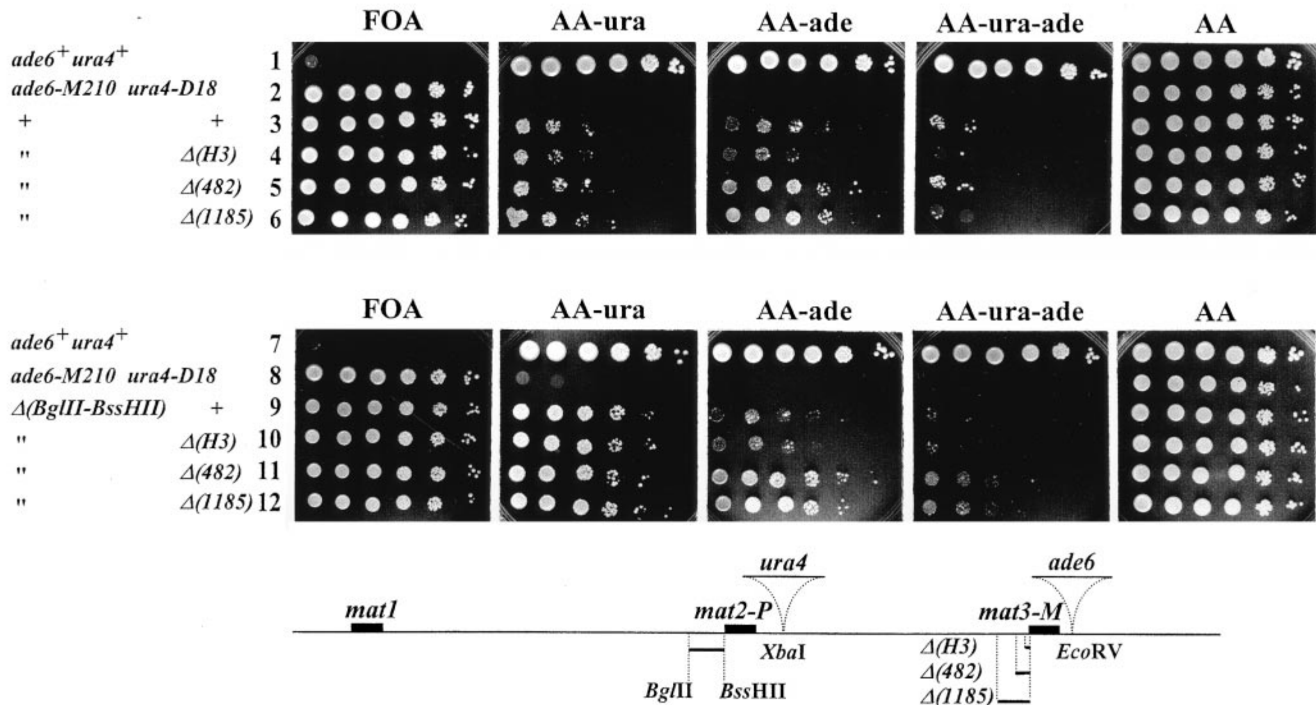


Figure 8.—Effect of the *mat2-P* and *mat3-M* silencing elements at a distance and in combination. Expression of *ura4* and of *ade6* was monitored by propagating the indicated strains under nonselective conditions (YES) and spotting 10-fold serial dilutions of cell suspensions on the indicated media. In both the top and bottom, the two first rows of spots are controls where neither *ade6* nor *ura4* is in the mating-type region. In all other rows, *ura4* is at the *mat2-P XbaI* site and *ade6* at the *mat3-M EcoRV* site. + on the left denotes a *mat2-P* allele with no deletion and + on the right denotes a *mat3-M* allele with no deletion. Rows 1 and 7, PG1566; rows 2 and 8, PG1141; row 3, PG1595; row 4, PG1686; row 5, PG1675; row 6, PG1688; row 9, PG1596; row 10, PG1680; row 11, PG1683; row 12, PG1685.

well in either orientation, to a level similar to that of *mat3-M(EcoRV)::ura4* in which no manipulation had been performed (Figure 9A). The 126-bp fragment also repressed *ura4* expression compared with strains that had the 482-bp deletion. It repressed equally well in both orientations, but not as tightly as the 424-bp fragment or as the wild-type element (Figure 9A). We also examined the influence of the orientation of the silencing element on the expression of the *M* genes from *mat3-M* by examining sporulation in *mat1-P*Δ*17::LEU2 swi6-115* cells with *mat3-M* alleles where the 482-bp deletion had been replaced with the 424-bp fragment in either orientation or with the 126-bp fragment in either orientation. In this assay, the DNA fragments having the orientation opposite to wild-type were able to exert a repression, but not as efficiently as when placed in the wild-type orientation (Figure 9B). We conclude from this set of experiments that the silencing elements located on the proximal side of *mat3-M* can act in both orientations although with different efficiencies, the orientation found in the wild type giving rise to the tightest repression of *mat3-M.*

DISCUSSION

We found that a DNA element adjacent to the *mat3-M* mating-type cassette of fission yeast participated in the repression of transcription of *mat3-M* and in the repression of prototrophic markers introduced near the cassette. Some of the properties of this element were similar to those of an element adjacent to the *mat2-P* cassette (Thon *et al.* 1994). Deletion of either the *mat2-P* or *mat3-M* flanking element increased expression locally in an area that comprised the cassette and chromosomal region close to the deletion, but it did not increase expression in the entire mating-type region. The local derepressions were markedly increased by combining the *cis*-acting deletions with mutations in the *trans*-acting factors *swi6*, *clr1*, *clr2*, *clr3*, and *clr4* but not by combining the *cis*-acting deletions with a mutation in *esp3.* Finally, simultaneous deletion of the *mat2-P* and *mat3-M* flanking elements in stable *M* cells did not increase expression further than each single deletion. We will discuss these phenotypes in the context of the current understanding of the mode of action of silencers in other organisms and in the context of models for silencing in the mating-type region of *S. pombe.*

Silencing and *mat3-M* flanking sequences: DNA elements close to the *mat3-M* mating-type cassette have recognizable sequence features that suggest a role in silencing. These elements are as follows: the H3 homology box, which is also found at the *mat2-P* silent cassette; two *S. pombe* ARS consensus sequences; and two potential binding sites for the *S. pombe* DNA-binding proteins

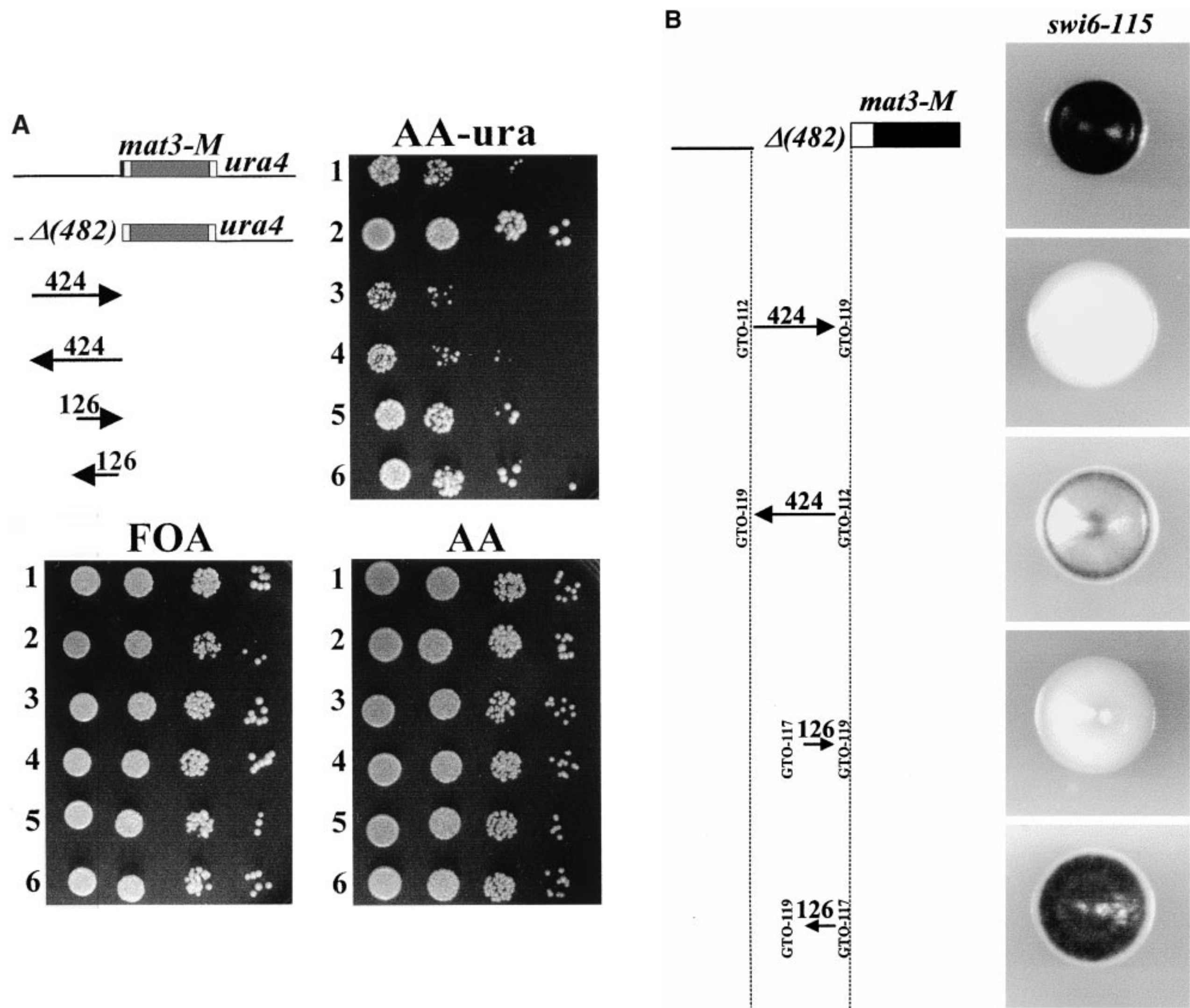


Figure 9.—Orientation dependence of *mat3-M* silencing element. (A) Effect of the orientation on *ura4* expression. Spot tests were performed as described in previous figures with cells having the *ura4* gene at the *Eco*RV site near *mat3-M* and the indicated deletions. Arrows represent *mat3-M* centromere-proximal DNA amplified by PCR as in Figure 3 and introduced in place of the 482-bp deletion in the wild type (rows 3 and 5) or reverse (rows 4 and 6) orientation. Rows 1, PG447; rows 2, PG1550; rows 3, PG1626; rows 4, PG1629; rows 5, PG1632; rows 6, PG1630. (B) Effect of the orientation on sporulation. Sporulated colonies of *mat1-P*Δ*17::LEU2 swi6-115* cells with the indicated replacements for the 482-bp fragment flanking *mat3-M* were stained with iodine vapors. The strains were, from top to bottom: PG1401, PG1527, PG1529, PG1541, and PG1554.

Mts1/Mts2. Our deletion analysis allows us to assess the likelihood that these elements participate in the repression of *mat3-M.*

Because it is present at *mat2-P* and *mat3-M* but not at *mat1*, the H3 homology box has been viewed as a potential silencing element (Kelly *et al.* 1988). A plasmid deletion analysis of the *mat2-P* flanking regions indicated the H3 box was not required for silencing *mat2-P* (Ekwall *et al.* 1991). Consistent with this previous study, we found that deleting the H3 box near *mat3-M* in the chromosome did not derepress the *M* genes, or other genes introduced near the *mat3-M* cassette. Because of the redundant nature of silencing, ruling out repression by H3, or any other *cis*-element, should be viewed with caution. However, deleting H3 did not potentiate the effect of a *trans*-acting mutation in the silencing factor Swi6, nor did it potentiate the effect of other *mat3-M* flanking deletions, further indicating that H3 is not a repressor element. Because of its position at the edge of the cassette, another possible function for H3 would be in resolution of *mat1* gene conversions.

ARS elements are part of the *HML* and *HMR* silencers of *S. cerevisiae* where they attract ORC proteins (Fox *et al.* 1997a and references therein). In *S. pombe*, both perfect and near matches to the sequence (A/T)(A/G)TTTATTTA(A/T) are consistently found within DNA segments allowing autonomous plasmid replication (Maundrell *et al.* 1988). Two 11-bp sequences matching the *S. pombe* ARS consensus are found on the centromere-proximal side of *mat3-M*, one 825 bp and the other 1525 bp upstream from the H2-H3 border (nucleotides 10082–10092 and 9372–9382, respectively,

in U57841; Grewal and Klar 1997). In addition, 3 10/11 matches and 21 9/11 matches to the consensus are found within 2 kb of the cassette. The 4.2-kb *Hin*dIII genomic fragment containing *mat3-M* can replicate as an extrachromosomal element (G. Thon, unpublished observations), supporting the notion that the putative ARS's are functional. We found that a deletion comprising the ARS consensus sequence closest to the cassette did not derepress *mat3-M* or flanking markers [Δ*(1185)::(424)oriImat3-M* and Δ*(1185)::(424)oriImat3-M(Eco*RV*)::ura4* alleles; Figure 3A and data not shown]. That deletion had no effect in the wild type nor in cells partially derepressed by either a mutation in *swi6* or by the deletion of 482 bp adjacent to *mat3-M.* A distinct possibility is that, if the ARS elements play a role in silencing, perfect or near consensus sequences can substitute for them after they are deleted. Hence, deletions larger than those introduced here, or deletion of *trans*-acting factors such as Abp1 or Abp2 (Murakami *et al.* 1996; Halverson *et al.* 1997; Sanchez *et al.* 1998), might be required to observe an effect.

The heptamer sequence ATGACGT and the overlapping consensus TGACG(T/A)(A/C) are protein-binding sites well characterized in *S. pombe* because of their occurrence in the *ade6-M26* allele and ability to bind the transcription factor Atf1, respectively. The *M26* mutation in the *ade6* gene increases meiotic recombination (Gutz 1971) by creating the sequence ATGACGT (Ponticelli *et al.* 1988; Szankasi *et al.* 1988; Schuchert *et al.* 1991). The ATGACGT heptamer also creates recombination hot spots when introduced by mutagenesis at various places and in either orientation in the *ade6* gene or in the *ura4* gene (Fox *et al.* 1997b), and this effect on recombination depends on the chromosomal context as shown by transplacements of the *ade6-M26* allele (Virgin *et al.* 1995). The Atf1/Pcr1 (Mts1/Mts2) heterodimer binds to the *M26* heptamer *in vitro* and is required for hot-spot activity (Wahls and Smith 1994; Kon *et al.* 1997). Atf1 binds *in vitro* to sequences matching the mammalian ATF-binding site TGACG(T/A)(A/C) (Jones and Jones 1989; Takeda *et al.* 1995) which overlaps with the *M26* heptamer. The Atf1 and Pcr1 proteins function as transcriptional activators of genes expressed during sexual differentiation and genes expressed in response to stress (Takeda *et al.* 1995; Kanoh *et al.* 1996; Shiozaki and Russell 1996; Watanabe and Yamamoto 1996; Wilkinson *et al.* 1996). However, Mts1 and Mts2 do not increase recombination at the *M26* site by increasing transcription, but rather by an unknown mechanism, indicating these proteins are multifunctional (Kon *et al.* 1997). Two sequences matching the ATF/*M26* consensus (ATGACGTA) are found near *mat3-M*, one 138 bp and one 1522 bp upstream from the H2/H3 junction. We found that an 88-bp fragment containing the consensus sequence closest to the cassette could partially restore silencing when introduced in a larger *mat3-M* flanking deletion [Δ*(482)::(88)oriImat3-M* allele; Figure 3B], indicating that Mts1/Mts2 may have a role in silencing in addition to activating transcription and recombination.

Orientation dependence of silencing elements: Two DNA fragments adjacent to *mat3-M*, a 482-bp and a 126-bp fragment, could exert a repression when placed at their natural location but in the reverse orientation compared to the wild type. In that reverse or the normal orientation, each fragment repressed the *ura4* gene placed distal to *mat3-M* as judged by our plating assay. However, in a *swi6-115* background, the wild-type orientation silenced the *M* genes more effectively than the reverse orientation. These different results could reflect properties of the markers used (*ura4 vs. M* genes) or an effect of the background (*swi6*$^+$ *vs. swi6-115*). Several propositions can explain the ability of a silencer to work in both orientations, yet preferentially in one. One possibility is that the element orients the nucleation of a protein complex that propagates preferentially in one direction. Another possibility is that the position or orientation of the silencer relative to other elements that were not inverted in the experiment is important, with the wild-type arrangement allowing optimal interactions between the factors attracted to the region. Silencing elements from other organisms can also exert a repression in both orientations although with different efficiencies (Brand *et al.* 1985; Shei and Broach 1995).

Redundancy of silencing in the mating-type region of *S. pombe*: Previous observations have suggested that more than one pathway of repression acts in the mating-type region. First, mutations and deletions in the class of *trans*-acting factors that include *swi6*, *rik1*, *clr1*, *clr2*, *clr3*, and *clr4* derepress transcription only partially (Thon and Klar 1992; Ekwall and Ruusala 1994; Allshire *et al.* 1995; Thon and Friis 1997). Mutations in *swi6*, *clr1*, *clr2*, *clr3*, and *clr4* combined pairwise do not cause a more pronounced derepression than any single mutation, indicating these factors work in a common pathway (Thon *et al.* 1994). Another class of *trans*-acting factors, encoded by the *esp* genes, acts in synergy with *swi6* (Thon and Friis 1997) and the four *clr* genes (G. Thon, unpublished observations) and thereby defines a pathway of silencing parallel to the pathway mediated by *swi6*, *clr1*, *clr2*, *clr3*, and *clr4.* The study of *cis*-acting elements, in particular the characterization presented here, also points to several silencing mechanisms. Neither the 7.5-kb deletion in the K region, nor deletion of the *mat2-P* proximal element nor deletion of the *mat3-M* element fully derepresses transcription in the mating-type region, indicating these elements can partially substitute for each other (Thon *et al.* 1994; Thon and Friis 1997; this study). Combining *trans*-acting mutations with the deletions of *cis*-acting elements gives some clues to the possible interactions between *cis*- and *trans*-acting factors. Strains that have both the 7.5-kb deletion between *mat2-P* and *mat3-M* and a mutation in *swi6*, *clr1*, *clr2*, *clr3*, or *clr4* display a partially derepressed

phenotype similar to the phenotype caused by the *trans*-acting mutations alone, whereas strains that contain the *cis*-acting 7.5-kb deletion in combination with *trans*-acting mutations in the *esp* genes are strongly derepressed (Thon and Friis 1997). In contrast, *mat2-P* or *mat3-M* flanking deletions have a pronounced cumulative effect with mutations in *swi6*, *clr1*, *clr2*, *clr3*, or *clr4* (Thon *et al.* 1994; this study), but not with the *esp* mutation whose effect was tested here (*esp3-1*). This suggests that the products of *swi6*, *clr1*, *clr2*, *clr3*, and *clr4* interact with the mating-type region via the K region whereas the *esp* products interact with the *mat2-P* and *mat3-M* flanking elements.

Epigenetic switches between repressed and derepressed states: A remarkable phenotype of cells in which *cis*-acting sequences have been deleted from the mating-type region is that they switch between two epigenetic states: one in which the mating-type region is partially derepressed and one in which a level of repression similar to the wild type is attained. This phenomenon was previously observed in cells with a 7.5-kb deletion between *mat2-P* and *mat3-M* (Grewal and Klar 1996; Thon and Friis 1997). We show here that similar phenotypic variations occur when other *cis*-acting elements are deleted. The *mat2-P* genes as well as a *ura4* gene placed near *mat2-P* were derepressed in a variegated manner in cells lacking the *mat2-P* flanking element. Similarly, expression of the *mat3-M* genes and of an *ade6* marker placed near *mat3-M* variegated in cells with *mat3-M* flanking deletions. A two-step model in which silencing is established by the interactions between *cis*-acting elements and *trans*-acting factors and subsequently maintained and inherited by a different process could account for these variegated phenotypes. Similar models have been proposed for other systems, in particular for silencing of the *HML* and *HMR* mating-type cassettes in *S. cerevisiae* (Pillus and Rine 1989; Mahoney *et al.* 1991; Sussel *et al.* 1993; Holmes and Broach 1996). In such models, two types of mutations could cause switches between a repressed and derepressed epigenetic state: mutations decreasing the rate of establishment and mutations decreasing the fidelity of inheritance of the silenced state. We propose an extension to this model that accommodates the existence of two silencing pathways and indicates how the loss of each *cis*-acting element could diminish the rate of establishment without preventing complete silencing from occurring. In this model, protein-protein interactions could substitute for protein interactions with *bona fide* silencing elements to attract the components required for efficient silencing, albeit in an inefficient fashion. Cells containing only some of the *cis*-acting elements could remain for several generations in a partially derepressed state where the interactions occurring normally via the *cis*-acting elements present would have been established, but not all interactions required for full silencing. Occasionally, and not as often as in the wild-type, the other *trans*-acting factors required for full repression would be recruited and establish the wild-type level of repression. An alternative to the recruitment by protein-protein interactions is a recruitment by weak DNA-binding sites. The two 7.5-kb deletions introduced in the K region (Grewal and Klar 1996; Thon and Friis 1997) both left ~100 bp of homology with centromeric repeats in the mating-type region. This short stretch of DNA might be able to occasionally nucleate a silencing complex normally nucleated by the 4.3-kb centromeric repeat in the wild type. The ARS consensus sequences located near *mat3-M* might also be able to substitute for centromeric sequences to attract proteins because in *S. pombe* ARS- and centromere-binding proteins appear to have broad and overlapping binding specificities (Murakami *et al.* 1996; Halverson *et al.* 1997; Lee *et al.* 1997; Sanchez *et al.* 1998).

Independent regulation of *mat2-P(Xba*I*)::ura4* and *mat3-M(Eco*RV*)::ade6* expression in strains with double silencer deletions: Fluctuations between repressed and derepressed states were observed in strains with double deletions of *mat2-P* and *mat3-M* flanking sequences. In these strains, the *mat2-P* and *mat3-M* regions were rarely coexpressed. Because an element located between the cassettes is important for silencing, the fluctuations might represent a property of silencing mediated by that element. For example, the element might nucleate a region of silenced chromatin that would not always expand with an equal efficiency toward *mat2-P* and *mat3-M.* In some cells, *mat2-P* and *mat3-M* would both be engulfed in silencing whereas in others only one of the cassettes would be affected. In wild-type cells, the *mat2-P* and *mat3-M* flanking elements would facilitate the spreading of silencing by themselves attracting *trans*-acting factors.

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