Mutations in *rik1*, *clr2*, *clr3* and *clr4* Genes Asymmetrically Derepress the Silent Mating-Type Loci in Fission Yeast

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ABSTRACT

In *Schizosaccharomyces pombe* the mating-type information is stored at two transcriptionally silent loci (*mat2* and *mat3*). The region between these sites (*K* region) is inert for meiotic crossing over. The mating-type genes (*M* or *P*) are expressed only when present at a third, active locus (*mat1*). We have earlier shown that the positional regulation of *P* genes is based on repression at the silent site, caused by elements in the flanking DNA sequences. In this study we have mutagenized a sterile *mat1* deleted strain and selected for cells that are able to conjugate. Recessive mutations of this type should define genes encoding *trans*-acting factors involved in repression of the silent mating-type loci. Before this work mutations in two genes, *clr1* and *swi6*, had been shown to allow both expression of the silent loci and recombination in the *K* region. The sensitivity of the present selection is demonstrated by the isolation of new mutations that derepress one or both of the silent loci (M-mating or bi-mating). The frequency of M-mating mutants was almost two orders of magnitude higher than that of bi-mating mutants and in all mutants analyzed *mat3-M* expression was significantly higher than *mat2-P* expression. The mutations define three new genes, *clr2*, *clr3* and *clr4*. In addition we show that the *rik1* mutant previously known to allow recombination in the *K* region also derepresses the silent loci.

MANY examples in eukaryotes show that the position of a gene in the genome influences its expression. In mutant Drosophila cells translocation of euchromatic genes to centromeric heterochromatin causes strong repression, which is most dramatically exemplified by partial disappearance of the normal red eye color (HENIKOFF 1990). In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* the mating type genes are subject to position effects. Both yeasts have two different unexpressed loci of mating type genes, which are expressed only after transposition to a third, transcriptionally active, locus (reviewed in KLAR 1989). Here the position effect is crucial for unambiguous expression of the mating-type.

Many observations both from Drosophila and *S. cerevisiae* support the idea that long range control of chromatin structure is important for these position effects (RIVIER and RINE 1992). In Drosophila the coincidence of transcriptional inactivity and the compact heterochromatin of the salivary gland chromosomes can be observed directly in the microscope (HAYASHI *et al.* 1990) and in *S. cerevisiae* mutations in histone H4, one of the four protein constituents of nucleosomes influence the mating type gene repression (GRUNSTEIN 1990). In Drosophila 120 factors are estimated to affect position effects and many of them have been defined as mutations suppressing or enhancing the severity of the position effect (SHAFFER, WALLRATH and ELGIN 1993). In *S. cerevisiae* silent information regulators *SIR1-4* were found as mutations derepressing the silent mating type loci (IVY, KLAR and HICKS 1986; RINE and HERSKOWITZ 1987). To date 18 genes have been shown to influence *S. cerevisiae* silencing (LAURENSON and RINE 1992). The large number of components in these systems suggests that the position effects depend on rather elaborate mechanisms.

We have started to investigate position effects at the silent mating type region in the fission yeast, which is genetically tractable and in evolutionary terms very distant from both fruit fly and budding yeast. We hope to find conserved components between the different organisms, which with the possible exception of the histone genes, have not been found between Drosophila and *S. cerevisiae* (GRUNSTEIN 1990; MOORE, SINCLAIR and GRIGLIATTI 1983). Most importantly, fission yeast offers in one organism an opportunity to compare the components involved in centromeric position effects with those engaged in silencing of the mating-type genes. Centromeric repression has not been reported for the relatively small centromeres of *S. cerevisiae*. In contrast, the centromeres in fission yeast are at least two orders of magnitude larger, consist of repeated sequences like centromeres in higher eukaryotes and cause alternate transcriptional states on genes placed inside the region (R. ALLSHIRE, personal communication) very much as seen in Drosophila.

We have earlier shown that the position effect at the silent *P* locus in fission yeast is caused by four

repressive DNA elements in the flanking sequences (EKWALL, NIELSEN and RUUSALA 1991). Autonomously replicating sequences were shown to physically overlap with these elements, suggesting a role for DNA replication in repression (OLSSON, EKWALL and RUUSALA 1993). *Trans*-acting factor genes *mrf1* and *mrf2* were identified as mutations derepressing a plasmid-borne *mat2* locus (EKWALL, OLSSON and RUUSALA 1992). However, these mutations did not derepress the chromosomal *mat2* locus. Recently mutations in the mating type switching gene, *swi6* (LORENTZ, HEIM and SCHMIDT 1992), and in the cryptic loci regulator gene, *clr1*, were shown to allow expression from the silent loci (THON and KLAR 1992).

Here we employ a selection system for *S. pombe* mutants that are able to conjugate in the absence of the active mating type cassette. A similar approach has earlier been used in *S. cerevisiae* where the *sir1-1* mutant was isolated as a suppressor of the mating-type defect of *mat*α*1-5* (RINE *et al.* 1979). The requirement for our procedure is that only one (*Pc* or *Mc*) of the two genes in the silent *P* or *M* mating type cassettes becomes active (KELLY *et al.* 1988). Because we are directly testing for mating type expression this selection could potentially cover all genes involved in this particular position effect without a requirement for a secondary phenotype that was a prerequisite for detecting mutations in *swi6*. We report the finding of mutations in three new complementation groups, *clr2*, *clr3* and *clr4*. Moreover we show that the *rik1* mutation, previously shown to relieve the meiotic recombination block between the silent loci (EGEL, WILLER and NIELSEN 1989), also derepresses the silent *loci*.

MATERIALS AND METHODS

***S. pombe* strains and media:** All the strains used in this study are listed in Table 1. Strains were grown on rich medium YE, sporulation medium ME or minimal medium with ammonium as nitrogen source (MORENO, KLAR and NURSE 1991).

Genetic techniques: Standard *S. pombe* genetic procedures were used for genetic crosses, tetrad dissection, random spore analysis, mutagenesis with ethyl methanesulfonate, iodine staining and isolation of diploid strains using *ade6-M210/ade6-M216* complementation (MORENO, KLAR and NURSE 1991). Protoplast-fusions were performed according to NADIN-DAVIS and NASIM (1990). Cells were haploidized by streaking them on minimal medium plates containing *meta*-fluorophenylalanine, *m*-FPA (Sigma, F 5126) at a concentration of 500 mg/liter, and supplements to ensure the growth of all segregants (KOHLI *et al.* 1977). The mechanism of action of *m*-FPA is unknown, but the isomer *para*-fluorophenylalanine, *p*-FPA, was studied using pedigree analysis of *p*-FPA grown cells (FLORES DA CUNHA 1970) and is thought to induce progressive loss of chromosomes possibly by misincorporation of *p*-FPA into proteins of the mitotic apparatus.

Quantitative conjugation tests: Overnight cultures in 2 ml supplemented YE medium (YES) were mixed with recipient cultures KE05 (*M* cells) and KE06 (*P* cells), respectively (50 μl of the tested strain and 100 μl of recipients). A 10-μl drop of this mixture containing about 5×10^5 cells was placed on a ME plate, and incubated 2 days at 25°. Cells were resuspended in sterile water to about 5×10^7 cells/ml and then serially diluted in steps of fivefold dilutions, using a multichannel pipette and round bottomed 96-well microtiter plates (Corning, New York). Clumping of cells was minimized by resuspending before each dilution step. Diluted cells were spotted in 10-μl drops onto minimal medium plates supplemented either with adenine (scores the number of conjugating cells), leucine, lysine, adenine (scores the total number of tested cells) or uracil, adenine (scores the number of recipient cells). After 3 days at 30° plates were photographed and scored. The fraction of conjugating cells was determined for each culture. This mating test resulted in 90–100% conjugation levels using heterothallic h^+ and h^- control strains after mixing with KE05 and KE06 tester strains respectively, showing that it quantitatively measures the mating efficiency of the tested strains. One example of such a test is shown in Figure 3.

Northern analysis, construction of the KE46 strain and Southern analysis: Extraction of *S. pombe* RNA was done by the glassbead/phenol method as described previously (EKWALL, NIELSEN and RUUSALA 1991). The cultures were starved for nitrogen for 17 hr before harvesting and RNA preparation. Northern blotting was performed using formaldehyde denaturing gels according to SAMBROOK, FRITSCH and MANIATIS (1989). 50 μg RNA was used for each sample. Single stranded RNA probes for *Pc* and *Mc* genes were made as described by NIELSEN and EGEL (1990). The strain Eg380 containing a *mat1Δ::ura4*$^+$ allele was kindly provided by MARTIN WILLER, Copenhagen. The deletion extends from about 600 bp proximal of the *H2* box to about 400 bp distal of the *H1* box and contains a *Bgl*II linker. The *ura4*$^+$ gene carried on a 1.8-kb *Bam*H1 fragment is inserted into this *Bgl*II site. Eg380 was mutagenized and an auxotrophic cysteine requiring strain was isolated and the presence of the *mat1* deletion was verified by Southern blotting (SAMBROOK, FRITSCH and MANIATIS 1989). About 20 μg of *Hin*dIII digested DNA was separated on a 0.7% agarose gel, and transferred to Hybond C filters (Amersham). Hybridization with *P* or *M* specific probes each revealed only one band with the expected sizes of 6.3 kb and 4.2 kb, respectively, confirming that the *mat1* locus was deleted. The cysteine requiring strain was then fused by the protoplast method with Eg242, haploidized and a *leu1,mat1Δ::ura4*$^+$, *ura4* recombinant was selected. This strain was then fused to Eg320 and haploidized to produce the *lys1,leu1, mat1::ura4*$^+$, *ura4* strain KE46.

cDNA and polymerase chain reactions (PCR): cDNA was made from 10 μg of total RNA preparations using the K3 primer (see below) and 12 units of AMV reverse transcriptase (Pharmacia) for each reaction according to IKONEN *et al.* (1992). cDNA was diluted 10-, 100-, and 1000-fold and subjected to PCR. The K2 primer 5′-ATGGATCCAAGATTAAGAGCA-3′ and the K3 primer 5′-GAATATAGTATGCGCTCTAAC-3′ were designed, using the *Pc* sequence (KELLY *et al.* 1988), to amplify a 311-bp internal fragment from the *Pc* cDNA. The PCR was carried out for 29 cycles each at 95° for 1 min, 50° for 2 min and 72° for 1 min. The PCR product was separated from excess of primers on a 1.1% agarose gel.

RESULTS

Isolation of mutants that express the silent mating type loci: A sterile *S. pombe* strain deleted for the *mat1*

TABLE 1

Strains used in this study

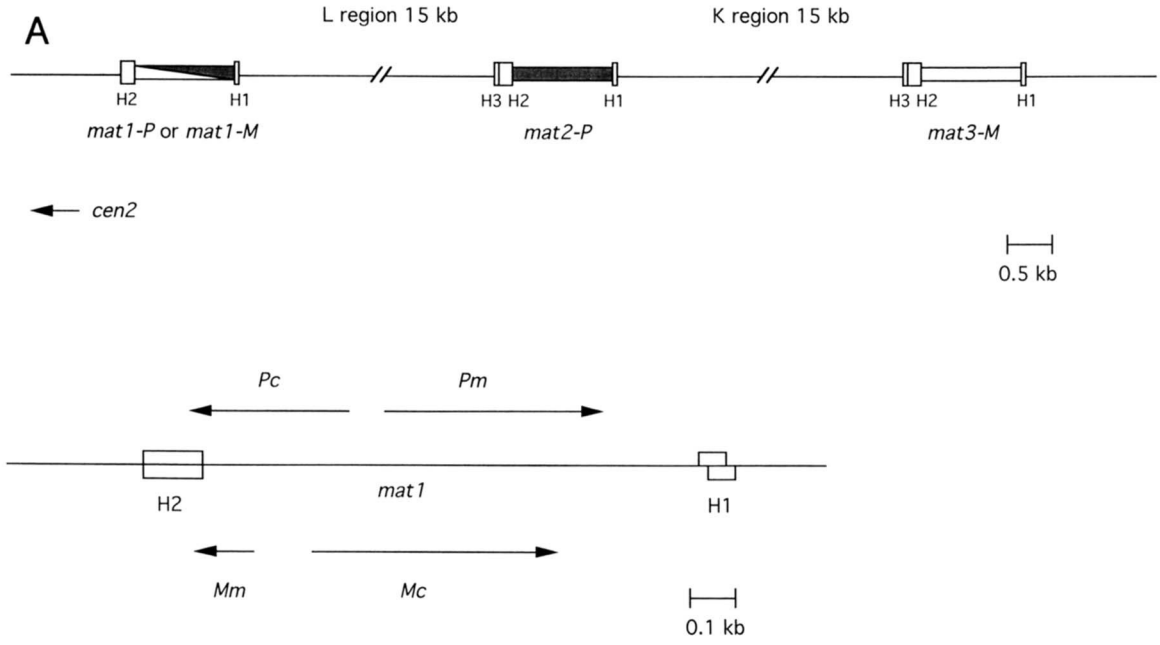
Strain	Genotype	Source/Reference
Eg242	*h^{90}, leu1, ura4*	R. EGEL, Copenhagen
Eg320	*mat2-B102, lys1, ura4-294*	R. EGEL, Copenhagen
Eg363	*h^{90}, ade6-M216*	R. EGEL, Copenhagen
Eg373	*h^{90}, swi6-S115, ade6-M210*	R. EGEL, Copenhagen
Eg380	*mat1Δ::ura4$^+$, ura4-D18*	M. WILLER, Copenhagen
Eg388	*h^{90}, leu1-32, ura4-D18, rik1-304*	R. EGEL, Copenhagen
PG9	*h^{90}, mat3-M::ura4$^+$, leu1-32, ura4-D18, ade6-M216*	THON and KLAR (1992)
PG339	*h^{90}, his2, clr1-6, ura4-D18, ade6-M210*	THON and KLAR (1992)
PG404	*h^{90}, leu1-32, his2, clr1-1, ura4-D18, ade6-M210*	THON and KLAR (1992)
HE307	*h^{90}, leu1-32, swi1-1*	H. SCHMIDT, Braunschweig
HE308	*h^{90}, leu1-32, swi2-3*	H. SCHMIDT, Braunschweig
HE309	*h^{90}, leu1-32, swi3-1*	H. SCHMIDT, Braunschweig
KE05	*mat1-M, mat2,3Δ::LEU2$^+$, ura4-D18, ade6-M216*	EKWALL, OLSSON and RUUSALA (1992)
KE06	*mat1-P, mat2,3Δ::LEU2$^+$, ura4-D18, ade6-M210*	EKWALL, OLSSON and RUUSALA (1992)
KE46	*lys1, leu1, mat1Δ::ura4$^+$, ura4*	This study
KE50	*leu1, mat1Δ::ura4$^+$, ura4, ade6-M210*	This study
KE51	*leu1, mat1Δ::ura4$^+$, ura4, ade6-M216*	This study
KE54	*leu1, mat1Δ::ura4$^+$, clr1-6, ura4, ade6-M210*	This study
KE55	*swi6-S115, leu1, mat1Δ::ura4$^+$, ura4, ade6-M210*	This study
KE61	*leu1, mat1Δ::ura4$^+$, clr1-1, ura4, ade6-M216*	This study
KE73	*leu1, mat1Δ::ura4$^+$, ura4, rik1-304, ade6-M216*	This study
KE76	*h^{90}, swi6-E45, ura4, ade6-M210*	This study
KE78	*h^{90}, clr2-E22, ura4, ade6-M210*	This study
KE81	*h^{90}, clr3-E36, ade6-M216*	This study
KE85	*swi6-S115, leu1, mat1Δ::ura4$^+$, ura4, ade6-M216*	This study
KE88	*leu1, mat1Δ::ura4$^+$, clr2-E22, ura4, ade6-M210*	This study
KE89	*leu1, mat1Δ::ura4$^+$, clr2-E22, ura4, ade6-M216*	This study
KE90	*lys1, leu1, mat1Δ::ura4$^+$, clr3-E36, ura4, ade6-M210*	This study
KE91	*lys1, swi6-E45, leu1, mat1Δ::ura4$^+$, ura4, ade6-M210*	This study
KE102	*lys1, leu1, mat1Δ::ura4$^+$, clr4-S5, ura4, ade6-M210*	This study
KE108	*h^{90}, clr4-S5, ura4, ade6-M216*	This study

locus (KE46) was constructed (see MATERIALS AND METHODS). This strain is unable to conjugate with strains of either *M* or *P* mating type. It is also auxotrophic for lysine and leucine and therefore cannot grow on minimal medium. We employed a selection for mutant KE46 cells being able to form prototrophic diploids with *P* or *M* cells that are auxotrophic for adenine and uracil. The selection requires that either *Pc* or *Mc* gene in the silent mating type loci are expressed (Fig. 1).

The appearance frequency of M-mating mutants (cells able to conjugate with the *P* tester strain) was after mutagenesis (34% survival) 8×10^{-7} whereas P-mating mutants (cells able to conjugate with the M tester strain) appeared at a frequency of 1×10^{-8}. The selected diploid cells were restreaked and subjected to iodine staining (Fig. 2). We reasoned that recessive mutations would be complemented by the wild type allele present in the recipient strain and should produce nonsporulating diploids. The tester cells lack the donor loci for switching and therefore are stable *M* or *P* cells. In contrast, dominant mutations should give sporulating colonies due to the simultaneous expression of *P* and *M* genes. Both classes of mutations were found. About 95% of the mutants selected for M-mating were nonsporulating or very weakly sporulating, whereas only seven out of 21 mutants selected for P-mating were of this class. Only the weakly sporulating and nonsporulating mutants were studied further.

The nonsporulating diploids that resulted from M-mating KE46 cells were haploidized and out of 36, 21 were discarded because in retesting they were sterile or displayed very weak conjugation phenotypes. Fourteen mutants conjugated only with *P* cells and one (*E36*) with both *P* cells and *M* cells (a bi-mating phenotype). Out of seven weakly sporulating or nonsporulating diploids that were selected as P-mating KE46 cells only one was successfully haploidized. This mutant (*E22*) also showed a bi-mating phenotype.

Three new complementation groups of mutants: The 16 haploid mutant strains with a deleted *mat1* cassette were fused as protoplasts to an identically deleted nonmutated strain using *ade6-M210/ade6-M216* selection for diploid formation. These diploids were tested for conjugation and in all cases the recessive nature of the mutations could be confirmed (Figure 3 and data not shown). Homozygous diploids



B SELECTION SYSTEM BASED ON CONJUGATION

	I	*II*		*III*	
mutant	*lys1*	*leu1, mat1Δ::ura4+, mat2-P, mat3-M*		+	*ura4*
tester *P*	+	+	*mat1-P, mat2,3Δ::LEU2+*	*ade6, ura4*	
tester *M*	+	+	*mat1-M, mat2,3Δ::LEU2+*	*ade6, ura4*	

1) Mutagenize and mix with tester strains
2) Select prototrophic diploids and analyze by iodine staining dominant (spo+) or recessive (spo-)
3) Haploidize, select ura+ haploids and check for conjugation phenotype
4) Map the mutants to one of the three chromosomes using *lys1*, *leu1* and *ade6* markers
5) Arrange into complementation groups by constructing *mat1Δ* diploids
   Complementation gives sterile diploids
   Non-complementation gives *M* and/or *P* -mating diploids

FIGURE 1.—Mating-type region, mating type genes and selection system for *clr* mutants. (A) Schematic representation of the mating-type region and the mating-type genes in *S. pombe* [compiled from THON and KLAR (1992), EGEL (1989) and KELLY *et al.* (1988)]. The mating-type region is located on chromosome *II* and consists of three subloci. The active locus, *mat1*, is closest to the centromere. The homology regions H1 (59 bp) and H2 (135 bp) are common to all three loci and H3 (57 bp) is shared between the two silent loci: *mat2* and *mat3*. There are four mating-type genes. The direction of transcription and the size of the genes are indicated by arrows. The *P* cassette contains the *Pc* (*c*onjugation) gene of 118 amino acids and the *Pm* (*m*eiotic) gene of 159 amino acids. The *M* cassette contains the *Mm* (*m*eiotic) gene of 42 amino acids and the *Mc* (*c*onjugation) gene of 181 amino acids. (B) Selection system based on conjugation. The genotype of the KE46 strain used for mutagenesis (mutant) and the two recipient strains KE06 (tester *P*) and KE05 (tester *M*) are presented. Chromosomes are indicated by *I*, *II* and *III*. The isolation procedure is schematically presented in five steps.

showed conjugation levels comparable to mutant haploids, indicating that this test was not significantly dependent on ploidy and could thereby be used to divide the mutants into complementation groups. Full complementation should give sterile diploid cells whereas non-complementation should be revealed as a P-mating and/or M-mating diploid. Partial complementation or semi-dominance that has been reported for *clr1* (THON and KLAR 1992) results in reduced mating in the heterozygous strain as compared to the strain homozygous for the mutation.

As positive controls, the previously isolated *clr1* (THON and KLAR 1992) and *swi6* (LORENTZ, HEIM and SCHMIDT 1992) mutants were used. These muta-

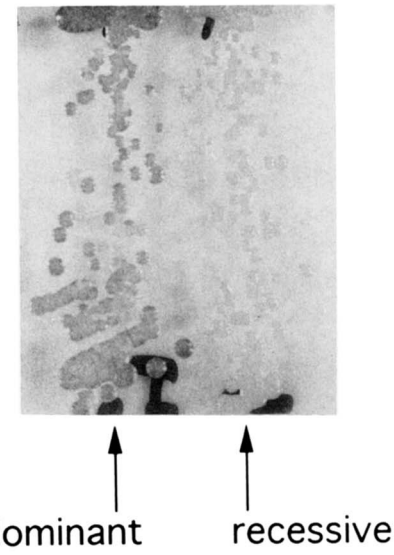


FIGURE 2.—Classification of dominant and recessive mutations. One example showing the result of iodine staining of colonies from two different prototrophic diploids. Sporulation in the dominant mutant is revealed as black staining colonies whereas the recessive mutant shows no staining.

tions were separately combined with the *mat1* deletion and tested for conjugation phenotypes. In this genetic background *swi6-S115* cells were M-maters while *clr1-1* or *clr1-6* cells were bi-maters. Both of these mutants and the *rik1* (EGEL, WILLER and NIELSON 1989) mutant relieve the extremely strong meiotic recombination block between the two loci (see discussion). Therefore we tested if the *rik1* mutation also could derepress the two silent loci. A *mat1* deleted *rik1-304* strain conjugated efficiently as an *M* cell (Figure 3).

Twelve of the 16 newly isolated mutants in the *ade6-M210* background were successfully fused to or crossed with *swi6*, *clr1* and *rik1* mutant strains in the *ade6-M216* background, respectively, and ade$^+$ diploids were selected. These diploids were subjected to quantitative conjugation tests with *P* and *M* cells, respectively. We found three members of the *swi6* complementation group, but no new members of the *clr1* or *rik1* complementation groups.

We focused the following analysis on the bi-mating mutants because of the relative ease of making crosses as compared to making protoplast fusions. The two bi-maters, *E22* and *E36*, were crossed with each other and *clr1*, *swi6* and *rik1* mutants. Ade$^+$ diploids were selected and tested for conjugation. The *E22* haploid strain and the *E22/E22* homozygous diploid strain efficiently mated with *P* and *M* cells with frequencies of 4–20%, whereas in *E22/clr1*, *E22/swi6*, *E22/rik1* and *E22/E36* diploid strains the mating fraction was reduced to less than 0.4% for P-mating and less than 4% for M-mating (Table 2 and Figure 3). The E36 haploid strain mated at 0.8–4% in both directions, while *E36/clr1*, *E36/swi6* and *E36/rik1* diploid strains displayed less than 0.4% of P-mating and less than 0.8% of M-mating. Therefore, all five mutations could fully or partially complement each other and belong to different complementation groups. We name the two new groups *clr2* and *clr3* (for cryptic loci regulators, see THON and KLAR 1992). One mutation from each of these groups *clr2-E22* and *clr3-E36* were fused to or crossed with ten of the other new mutants and the resulting diploids were tested for conjugation (data not shown). Two mutants could not complement *E22* and therefore belong to the *clr2* group. Interestingly, one of these two, *E45*, also belongs to the *swi6* complementation group (see discussion). One mutation did not complement *E36* and belongs to the *clr3* group. Five mutations could not be assigned to any of these groups indicating that they belong to additional complementation groups. One of these (*S5*) was further characterized. The S5 mutant strain showed M-mating level of 4.0%. M-mating was reduced to 0.8% in the *S5/clr1* strain and to less than 0.4% in *S5/swi6*, *S5/rik1*, *S5/clr2* and *S5/clr3* strains. Therefore *S5* belongs to a sixth complementation group called *clr4* (Table 2 and Figure 3). The remaining four mutations have not yet been grouped with respect to each other.

A gray iodine staining phenotype of *clr* mutants: Cells of h^{90} strains frequently switch their mating-type by transposition of *P* and *M* genes from the silent mating-type loci into *mat1* resulting in colonies with a homogenous mixture of *M* and *P* cells. Upon nitrogen starvation the cells will conjugate and form spores that contain amylose. Therefore h^{90} colonies show a homogenously black iodine staining phenotype while colonies of mutants that decrease the mating-type switching rate exhibit a mottled staining (GUTZ and SCHMIDT 1985) To investigate if the *clr2-E22*, *clr3-E36*, *clr4-S5* and *E45* mutations could affect switching they were transferred to an homothallic h^{90} genetic background. The mutants were crossed with the h^{90}, *ade6-M216* strain Eg363, and ade$^+$ diploids were selected and haploidized. Leu$^+$ colonies were selected and stained with iodine vapors. Since *leu1* is linked (12 cM) to the *mat1Δ::ura4*$^+$ allele it follows that most of the Leu$^+$ colonies should be of h^{90} configuration after this procedure. The *E45* mutant gave segregation of black (wild type) and mottled colonies with roughly equal frequencies. These mottled colonies were restreaked and shown to reproduce only mottled colonies. According to GUTZ and SCHMIDT (1985) switching mutants can be classified as either class I or II. Class I yield only mottled colonies like *E45*, while class II yield mottled and iodine negative colonies. The mutations *clr2-E22*, *clr3-E36* and *clr4-S5* gave segregation of black and grey staining colonies. The grey staining colonies contained cells with immature spores and reduced levels of zygotic four spored asci (see THON and KLAR 1992). Both *clr2* and *clr3* displayed two types of grey staining colonies: those with

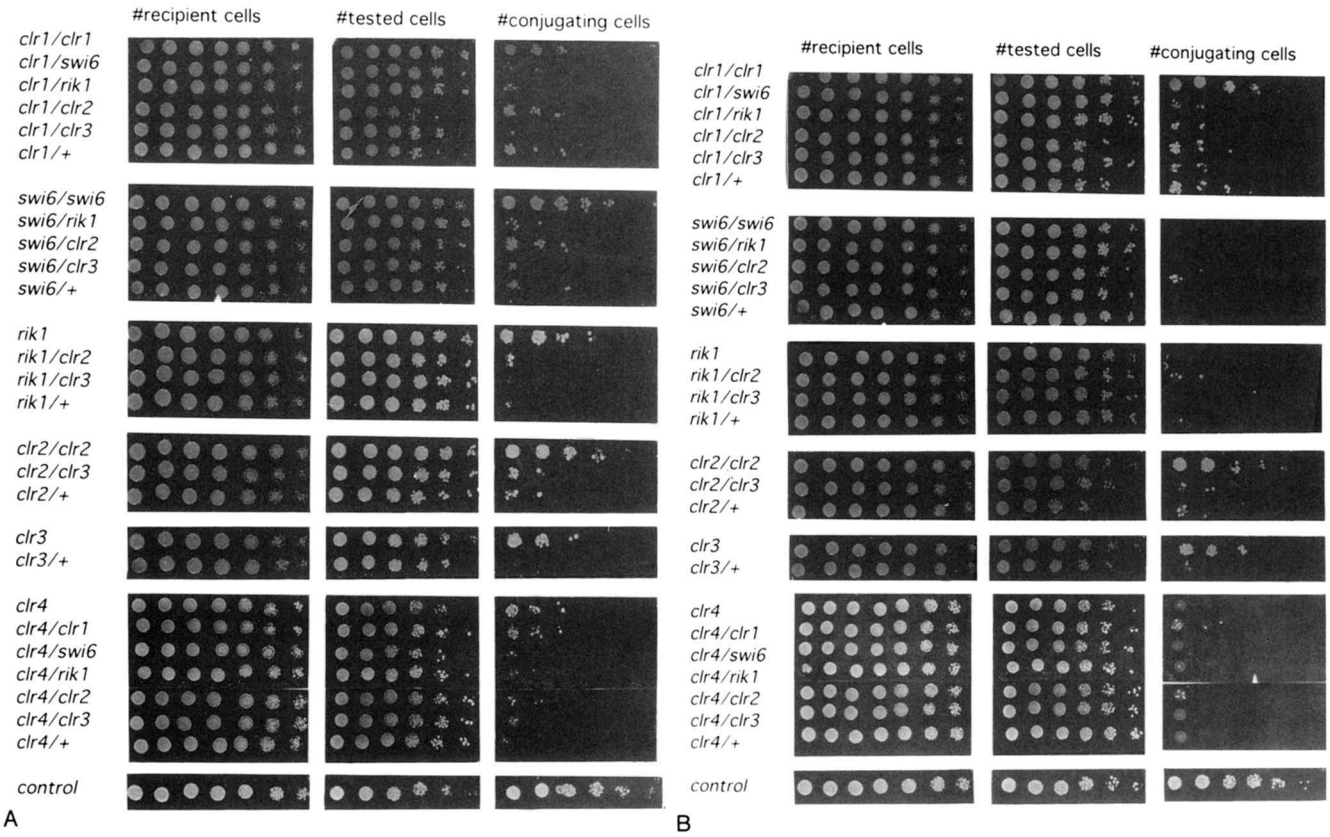


FIGURE 3.—Six complementation groups of mutations involved in repression of the *mat2* and *mat3* loci. The figure shows quantitative conjugation tests (see MATERIALS AND METHODS) of diploid strains containing all pairwise combinations of *clr1*, *swi6*, *rik1*, *clr2*, *clr3* and *clr4* mutations. The three panels represent minimal medium plates supplemented either with uracil, adenine (scores the number of recipient cells), leucine, lysine, adenine (scores the total number of tested cells) or adenine (scores the number of conjugating cells). (A) M-mating test using the *P* strain KE06 as a recipient and a h^- strain as positive control. (B) P-mating test using the *M* strain KE05 as a recipient and a h^+ strain as positive control. The plates were photographed after 3 days of incubation at 30°. See Table 2 for the conjugation levels scored in this type of test.

TABLE 2

Six complementation groups of mutations that derepress the silent mating type loci

	clr1-1 *clr1-6*	*swi6-S115*	*rik1-304*	*clr2-E22*	*clr3-E36*	*clr4-S5*	Wild type
M-mating							
clr1-1 or *clr1-6*	2–10	0.2–0.8	0.2	0.8–4	0.2–0.8	0.8	0.2–0.8
swi6-S115		4–20	0.1–0.4	0.2–0.8	0.1–0.4	<0.1	0.1–0.4
rik1-304			2–10[a]	0.3	0.2	0.2	<0.1
clr2-E22				4–20	0.2–0.8	0.4	0.2–0.8
clr3-E36					0.8–4[a]	0.4	<0.2
clr4-S5						4.0[a]	<0.1
P-mating							
clr1-1 or *clr1-6*	4–20	0.1–0.4	0.1–0.4	0.1–0.4	0.1–0.4	0.1–0.4	0.1–0.4
swi6-S115		<0.1	<0.1	0.1–0.4	0.2	<0.1	<0.1
rik1-304			<0.1[a]	0.1	0.1	<0.1	<0.1
clr2-E22				4–20	0.1–0.4	0.1	0.1–0.4
clr3-E36					0.8–4[a]	0.1	<0.1
cir4-S5						<0.1[a]	<0.1

Percentage of mating cells in a diploid strain having the mutations as indicated.
[a] A haploid mutant strain was tested instead of a homozygous diploid.

light staining and those with significantly darker staining (Figure 4). Upon restreaking a light staining colony it could produce darker staining colonies and a dark staining colony could produce lighter staining colonies, indicating that these two states are reversible. Mottled and grey mutants were crossed with a wild-type h^{90} strain, Ade^+ diploids were selected and tetrads were dissected. Gray and black or speckled

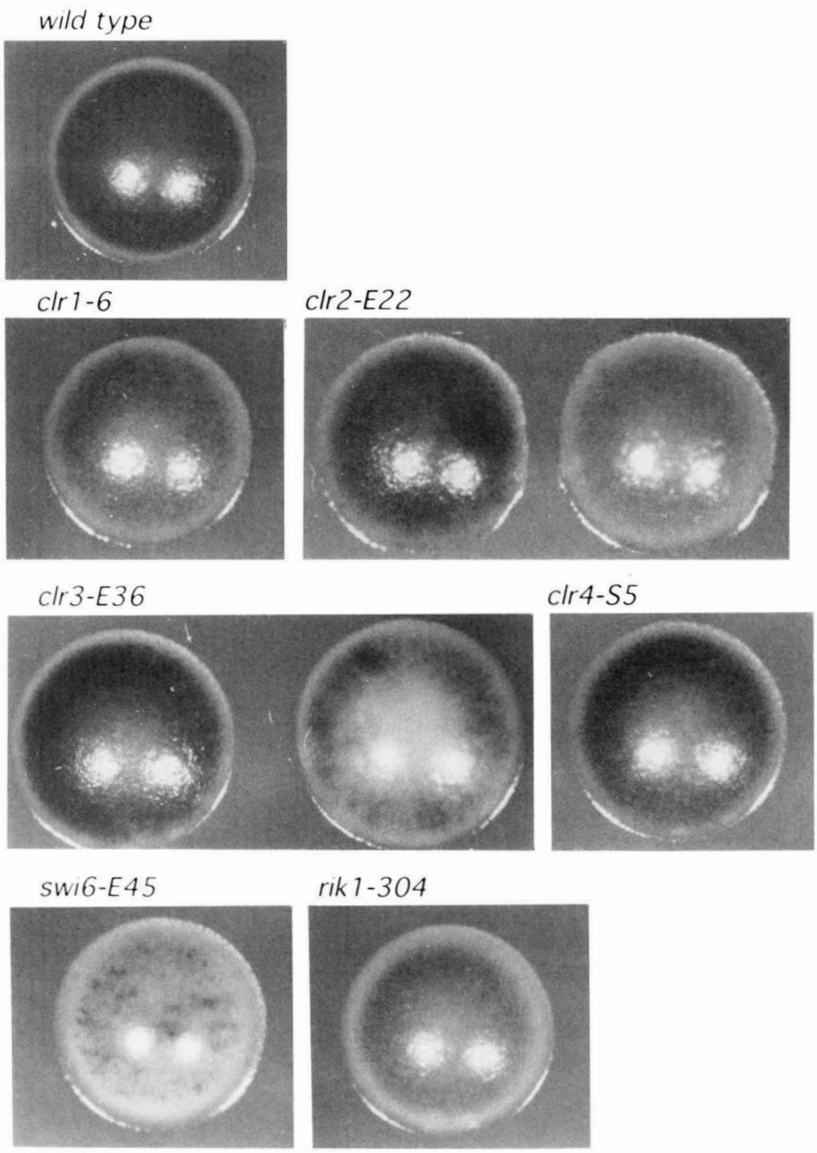


FIGURE 4.—Reduced sporulation phenotypes of homothallic *clr1*, *clr2*, *clr3*, *clr4*, *rik1* and *swi6* mutants. The strains Eg363 (h^{90}), PG339 (h^{90}, *clr1-6*), KE78 (h^{90}, *clr2-E22*), KE81 (h^{90}, *clr3-E36*), KE108 (h^{90}, *clr4-S5*), Eg388 (h^{90}, *rik1-304*), and KE76 (h^{90}, *swi6-E45*) were grown on malt extract plates for four days at 25° before the colonies were subjected to iodine staining. The two different (light and dark staining) phenotypes of *clr2* and *clr3* mutants are shown. The two bright spots in each colony are reflections of the light source.

and black iodine staining colonies segregated 2:2, showing that the phenotypes were caused by single mutations.

Allelism of the mutations: As judged from the quantitative conjugation tests we have found at least three new complementation groups (see above). Preliminary observations indicate that *clr2-E22* and *clr3-E36* map on chromosome *II*, because of non-linkage to *lys1* and *ade6*$^+$ markers after haploidization (data not shown). *swi6* is assigned to chromosome *I* (A. LORENTZ and H. SCHMIDT, personal communication) and *rik1* is mapped to chromosome *III* (EGEL, WILLER and NIELSEN 1989). *clr1* is located on chromosome *II* (THON and KLAR 1992). To investigate if mutations in *clr2*, *clr3* and *clr4* complementation groups define new genes, homothallic *swi6-S115*, *clr1*, *rik1*, *clr2-E22*, *E45*, *clr3-E36* and *clr4-S5* mutants were crossed pairwise with each other. Ade$^+$ diploids were selected, sporulated and subjected to tetrad dissection and/or random spore analysis (Table 3). *clr2-E22*, *clr3-E36* and *clr4-S5* did produce black staining h^{90} recombinants with frequencies expected for unlinked genes (about 25%) with all of the tested mutations, thus defining three new *clr* genes, *clr2*, *clr3* and *clr4*. In addition *E45* was crossed with some of the switching mutants of class I (*swi1*, *2*, *3* and *6*) and random spores from the diploids were plated. h^{90}, wild-type recombinants were found at expected frequencies in crosses with *swi1*, *2* and *3* (data not shown). In the *swi6-S115* cross no wild-type recombinants were detected. Also only tetrads with four speckled iodine staining colonies (parental ditype asci) were found in tetrad dissection of the *swi6-S115/E45* strain. Thus, *E45* appears to be allelic with *swi6-S115*, and therefore is designated *swi6-E45*.

Transcription of the silent loci: To verify that the conjugation phenotypes of the mutants were indeed due to transcription of the silent mating type loci total

TABLE 3

Meiotic recombination analysis demonstrating that *clr2-E22*, *clr3-E36* and *clr4-S5* define novel genes, and that *E45* is allelic to *swi6-S115*

Cross	Tetrad analysis[a]	Random spore analysis[b]
clr2-E22/swi6-S115	7/8	30.1
clr2-E22/clr1-6	9/11	26.3
clr2-E22/rik1-304	4/6	28.6
clr2-E22/E45	5/6	28.7
clr2-E22/clr2-E22	NT	0
clr2-E22/clr3-E36	NT	28.7
clr3-E36/swi6-S115	NT	23.0
clr3-E36/clr1-6	NT	28.7
clr3-E36/rik1-304	NT	25.6
clr4-S5/E45	NT	27.7
clr4-S5/rik1-304	NT	20.7
clr4-S5/clr1-6	NT	28.3
clr4-S5/clr2-E22	NT	29.4
clr4-S5/clr3-E36	NT	19.0
E45/clr1-6	5/6	20.6
E45/rik1-304	3/4	26.5
E45/swi6-S115	0/5	0
swi6-S115/swi6-S115	0/4	0

[a] Nonparental ditype and tetratype asci divided by the total number of asci. A ratio between 1/2 and 5/6, dependent on their relative distance to the centromeres, is the theoretical expectancy for nonlinked mutations. Not tested = NT.

[b] Percentage of wild-type recombinants in a sample of 300–500 random spores.

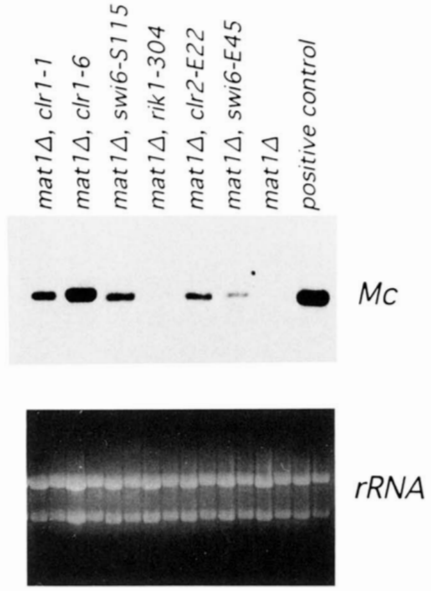


FIGURE 5.—Northern analysis showing derepression of *mat3-Mc* gene in *clr1*, *swi6*, *rik1* and *clr2* mutants. RNA was extracted from cells having genotypes as indicated in the figure. Strains are KE61 (*mat1Δ::ura4⁺*, *clr1-1*), KE54 (*mat1Δ::ura4⁺*, *clr1-6*), KE55 (*mat1Δ::ura4⁺*, *swi6-S115*), KE73 (*mat1Δ::ura4⁺*, *rik1-304*), KE89 (*mat1Δ::ura4⁺*, *clr2-E22*), KE91 (*mat1Δ::ura4⁺*, *swi6-E45*), KE46 (*mat1Δ::ura4⁺*), and Eg325 (h^{90}, wild type). Northern blotting and hybridization with *Mc* probe showed expression in nitrogen starved *clr1*, *swi6*, *rik1*, *clr2* cells and in the positive control h^{90} cells. Expression in the *rik1* strain was very weak and only detected after longer exposure. Bottom: To control the relative RNA amounts in the preparations, they were run into an agarose gel and stained with ethidium bromide. Note that RNA extracted both from cultures with (left) and without nitrogen (right) is shown for each strain.

RNA was extracted from cells grown with nitrogen and from cells starved for nitrogen. Northern blots were tested with *Mc* and *Pc* probes, respectively (Figure 5). *Mc* gene transcripts were detected in all mutant strains but not in the negative control, KE46. The *rik1* strain gave a relatively weak band, but was unambiguously detected after long exposure while no band was visible in the negative control. *Mc* transcripts were in all cases induced by nitrogen starvation (data not shown). In contrast *Pc* gene transcript was only detected in the positive control after 2 days of exposure. After one week of exposure some diffuse bands could be seen in *clr1* and *clr2* mutants. To confirm the presence of *Pc* mRNA we made cDNA from the total RNA preparations using a *Pc* gene specific primer. Upon PCR amplification of this cDNA all mutants tested (*clr1-1*, *rik1*, *swi6-E45* and *clr2-E22*) gave strong bands in the 10-fold dilutions and weaker bands in the 100-fold dilutions, whereas the negative control gave only a weak band in the 10-fold dilution. The positive control, however gave a band also in the 1000-fold dilution (Figure 6). This indicates that *mat2* locus is partially derepressed in these mutants. Surprisingly, PCR detected *Pc* transcription did not correlate with the P-mating behaviour of *swi6* and *rik1* mutants. We thought that a possible explanation for this is that the conjugation tests were performed at 25° while the RNA used for PCR and Northern analysis was extracted after growth at 30°. It is known for another position effect in *S. pombe* that the repression of genes inserted in centromeres is temperature sensitive (R. ALLSHIRE, personal communication). However this could not be confirmed for the *mat2-P* locus in *swi6* and *rik1* mutants because they still could not conjugate as *P* cells at 30°.

Additional phenotypes of the *clr* mutants: According to KELLY *et al.* (1988) simultaneous expression of all four mating-type genes (*Pc*, *Pm*, *Mc* and *Mm*) are needed for meiosis. Expression of *Pm* and *Mm* are not needed for conjugation. Therefore, to examine the extent of *mat2* and *mat3* derepression, the mutants from all six complementation groups lacking the active *mat1* cassette were examined under the microscope. Elongated cells could only be seen in *clr1–1*, *clr2-E22* and *clr3-E36* mutants after 1 or 2 days incubation on ME plates. After four days up to 50% of the cells were elongated (Figure 7). This morphology was strictly dependent on nitrogen starvation.

To see if the *clr2* mutant could derepress a nonrelated gene and also to see if this effect could extend distal to *mat3*, we used a strain that is deleted for the *ura4* gene at its normal location, but has the *mat3-*

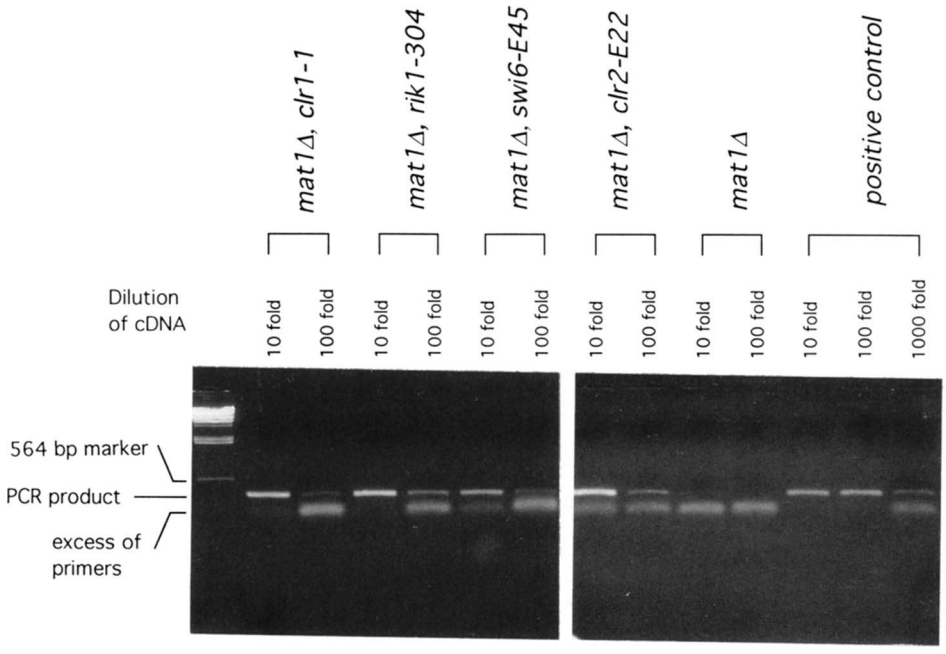


FIGURE 6.—PCR analysis showing derepression of *mat2-Pc* gene in *clr1*, *rik1*, *swi6* and *clr2* mutants. cDNA was made from RNA preparations from nitrogen starved cells with mutations as indicated in the figure. See the legend of Figure 5 for the names of strains. cDNA from the h^{90} strain was used as positive control and cDNA from the *mat1* deleted strain as negative control. Different dilutions of cDNA was subjected to PCR amplification (see MATERIALS AND METHODS) and the PCR product of 311 bp was separated from the excess of primers in a 1.1% agarose gel.

M::ura4$^+$ insertion allele constructed by THON and KLAR (1992). This strain (PG9) was crossed with the *clr2-E22* strain KE78, tetrads were dissected and tested for growth with and without uracil (Figure 8). The *ura4* gene is repressed in wild-type cells when located in the vicinity of the *mat3* locus, leading to a poor growth on plates lacking uracil. Both in the positive control, *clr1–6*, and in *clr2-E22* the *ura4* gene was derepressed resulting in faster growth on minimal medium. Iodine staining of the same dissection replicated on malt extract showed that in all cases the faster growing colonies also displayed the grey staining phenotype described above, confirming they were of *clr1*, *mat3-M::ura4*$^+$ or *clr2*, *mat3-M::ura4*$^+$ genotype.

DISCUSSION

The aim of this work was to identify genes encoding *trans*-acting factors involved in positional regulation of mating type loci in *S. pombe*. We employed a selection procedure in which a strain made sterile by deletion of the active mating type locus was mutagenized and cells able to conjugate were selected as prototrophic diploids. This approach resulted in the identification of three new genes called *clr2*, *clr3* and *clr4*. Mutations in *clr2* and *clr3* genes cause derepression of both silent mating type loci, whereas mutation in *clr4* only derepresses the silent *mat3* locus. In addition, the previously identified *rik1* gene (EGEL, WILLER and NIELSEN 1989) was shown to be required for repression of *mat3*.

Earlier attempts to identify *trans*-acting factor genes have resulted in the identification of four genes. The *mrf1* and *mrf2* genes (EKWALL, OLSSON and RUUSALA 1992) were found as recessive mutations that alleviate repression of an antibiotic resistance gene inserted in the plasmid-borne silent *P* locus. However, mutations in the *mrf* genes only derepressed the plasmid-borne mating-type locus but not the corresponding chromosomal locus. The reason for this discrepancy is not understood. We have verified that the *mrf* mutants are not allelic to *clr1*, *swi6* or *rik1*. Furthermore, we have tested the *mrf clr1*, *mrf swi6* and *mrf rik1* double mutants for effects on derepression of the *kanR* gene inserted into the plasmid-borne *mat2* locus and on chromosomal loci by examining the sporulation phenotype in the h^{90} configuration. In both cases no additive effects could be observed (data not shown). The *clr1* gene was defined by mutations that increase iodine staining of colonies of h^{09} mating type configuration (THON and KLAR 1992). In this configuration the location of silent *M* and *P* genes is the reverse to that in h^{90} (*mat2-M* instead of *mat2-P* and *mat3-P* instead of *mat3-M*) and these colonies are stained faintly by iodine vapors due to poor sporulation. The darker staining in *clr1* mutants was shown to be caused by haploid meiosis. THON and KLAR further showed that the *clr1* mutation derepresses both silent loci and increases expression of the *ura4* gene inserted near the silent *M* locus. The *swi6* gene was originally defined by a mutation that gives a mottled iodine staining of colonies, due to reduced mating type switching (EGEL, BEACH and KLAR 1984). This mutation was subsequently shown to cause haploid meiosis in h^{+N}, h^{-U} and h^{90} configurations, indicating derepression of the silent mating-type loci (LORENTZ, HEIM and SCHMIDT 1992).

Among 12 mutants tested in this study we found three members of the *swi6* complementation group. We did not find any new members of the *clr1* or *rik1*

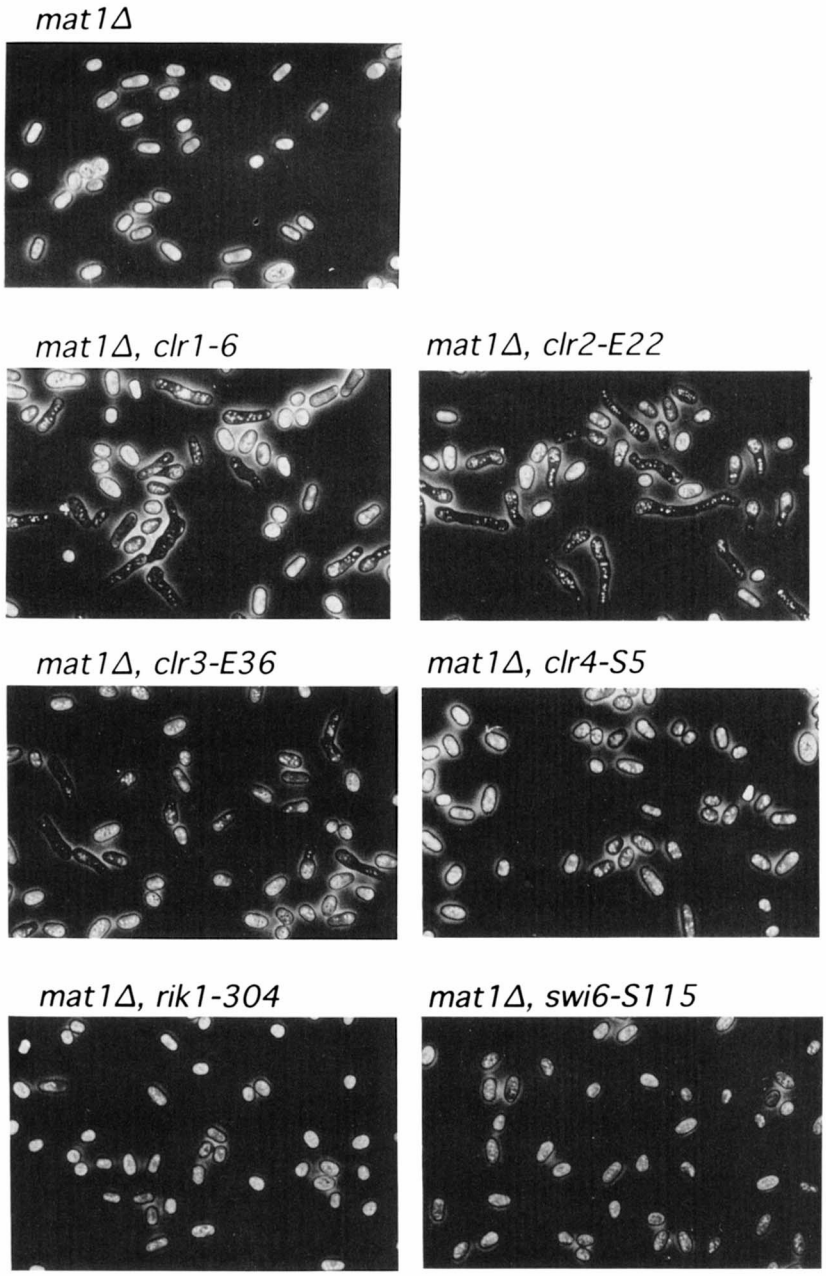


FIGURE 7.—Elongated cells in the *mat1* deleted bi-mating mutants. The strains KE50 (*mat1Δ::ura4*$^+$), KE54 (*mat1Δ::ura4*$^+$, *clr1-6*), KE88 (*mat1Δ::ura4*$^+$, *clr2-E22*), KE90 (*mat1Δ::ura4*$^+$, *clr3-E36*), KE102 (*mat1Δ::ura4*$^+$, *clr4-S5*), KE55 (*mat1Δ::ura4*$^+$, *swi6-S115*) and KE73 (*mat1Δ::ura4*$^+$, *rik1-304*) were grown on malt extract plates. The photographs were taken after 4 days of incubation at 25°.

complementation groups. Instead our mutants define at least three new complementation groups, *clr2*, *clr3* and *clr4*. Because *clr1* and *rik1* mutations were not found we believe that this search was not extensive enough. The reason for not finding additional *clr1* alleles is probably that they belong to the dominant class of mutations that was not analyzed in this study. Indeed *clr1* mutations are dominant with respect to sporulation in combination with both tester strains, and preliminary experiments identify a sporulation-dominant, bi-mating mutant that do not complement *clr1* (data not shown). In contrast *rik1* is much more recessive with respect to sporulation, so in this case the reason for not finding additional alleles remains unclear. Therefore, with this in mind it will be interesting to analyze more members of the dominant group of P-mating mutants. The two orders of magnitude difference observed for P-mating as compared to M-mating mutants could be due early sporulation in the selected diploids. This can be prevented by using a meiosis deficient recipient strain (*mat1-Mm*$^-$). In the case of M-mating mutants additional *trans*-acting factor genes can probably be identified by analyzing a larger collection of mutations, as well as the four unassigned mutations found in this study.

The morphology of haploid *mat1* deleted *clr1*, *clr2* and *clr3* mutants under nitrogen starvation argues that these three factors give a similar type of derepression. The same morphology has been observed in heterothallic *M* cells artificially expressing the M-factor receptor gene, *map3*, normally expressed only in *P* cells (TANAKA *et al.* 1993). According to TANAKA *et al.* (1993) expression of *Pc* gene is likely to be a prerequisite for *map3* expression. Therefore the elon-

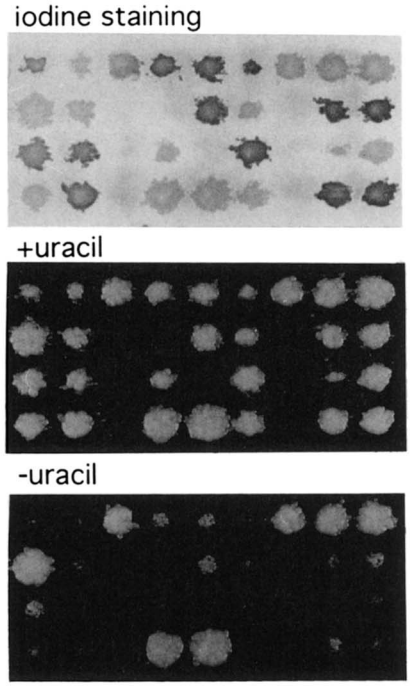


FIGURE 8.—Derepression of the *ura4*$^+$ gene inserted close to the *mat3* locus in the *clr2* mutant. An Ade$^+$ diploid strain from the cross KE78 (*h*^{90}, *clr2-E22*, *ura4*, *ade6-M210*) × PG9 (*h*^{90}, *mat3-M::ura4*$^+$, *leu1-32*, *ura4-D18*, *ade6-M216*) was subjected to tetrad analysis. The separated spores were incubated on a rich medium plate for 4 days at 30° and then replica plated to one malt extract plate and two minimal medium plates, one with and one without uracil. The malt extract plate was iodine stained and photographed after two days at 25° showing a 2:2 segregation of gray (*clr2*^{minus}) and black (*clr2*$^+$) colonies in each tetrad. The minimal medium plates were photographed after 2 days of growth at 30°. All colonies grew well on minimal medium with uracil but without uracil only *mat3-M::ura4*$^+$, *clr2-E22* segregants gave large colonies whereas *mat3-M::ura4*$^+$, *clr2*$^+$ segregants produced significantly smaller colonies.

gated cells found under nitrogen starvation in *clr* mutants could be a result of simultaneous expression of *map3* due to derepression of *mat2-Pc* and of M-factor, due to derepression of *mat3-M* locus resulting in an autostimulation of sexual activity. Indeed both *Pc* and *Mc* expression were detected in nitrogen starved *clr* mutants (Figures 5 and 6). THON and KLAR (1992) showed that *clr1* caused haploid meiosis in an unswitchable *P* (*mat1-PΔ17*, *mat2-P*, *mat3-M*) strain and to a lesser extent in an unswitchable *M* (*mat1-Msmt-0*, *mat2-P*, *mat3-M*) strain. Since all four genes *Pc*, *Pm*, *Mc* and *Mm* are required for meiosis (KELLY *et al.* 1988), this argues that one of the *P* genes is limiting in the *clr1* mutant. *mat2-Pc* and *Pm* transcripts were not detected in *clr1* while *mat3-Mc* was readily detected by Northern analysis (THON and KLAR 1992). Taken together with the present data this strongly suggests that under nitrogen starvation *Pm* gene transcript is limiting in all three *clr* mutants. This leads to sexually induced, but meiosis deficient cells in a *mat1* deleted background.

Mutations in *clr1*, *rik1* and *swi6* genes all have pleiotropic phenotypes (THON and KLAR 1992; EGEL, WILLER and NIELSEN 1989; LORENTZ, HEIM and SCHMIDT 1992). Altogether, four different effects can be distinguished; unequal spore size and reduced spore viability (*rik1*), derepression of silent mating type loci for gene expression (all three genes), reduced mating type switching, *i.e.*, mitotic gene conversion using silent loci as donors (*swi6*) and increased meiotic recombination in the *K*-region between the silent loci (all three genes). According to a model proposed by KLAR and BONADUCE (1991) the *swi6* gene product plays a vital role in folding the mating-type region to make the donor loci accessible to *mat1*, and this structure is thought to inhibit meiotic recombination between the donor loci. The sporulation defects of *rik1* are thought to be caused by poor meiotic segregation of chromosomes (EGEL, WILLER and NIELSEN 1989). *S. pombe* centromeres have an unusual chromatin structure (TAKAHASHI *et al.* 1992), lack meiotic recombination (NAKASEKO *et al.* 1986) and insertion of genes in the middle of the centromeres causes position effect variegation (R. ALLSHIRE, personal communication). These observations suggest that *S. pombe* mating-type region and centromeres may share heterochromatin-like properties. It is possible that the *clr1*, *swi6*, *rik1*, *clr2*, *clr3* and *clr4* gene products act as modifiers of these effects, and therefore it will be very interesting to test if these mutants can effect centromeric functions. One of the newly isolated mutations, *swi6-E45*, displayed an unusual complementation behavior. It is fully recessive against wild type, but cannot efficiently complement *clr2-E22* unlike the allelic *swi6-S115* which fully complements this mutation. This situation is similar to that in mating-type silencing in budding yeast where certain recessive *SIR3* and *SIR4* alleles fail to complement recessive mutations in *SIR1* and *SIR2* (RINE and HERSKOWITZ 1987). According to the authors this failure to complement could be due to that the noncomplementing gene products form a complex. We are currently following up this observation.

In the analysis of transcription from the silent loci a striking asymmetry was observed; The *mat3-M* locus was in all mutants significantly more derepressed than the *mat2-P* locus (see Figures 5 and 6). *Pc* transcripts could only be detected after PCR amplification while *Mc* was readily detected on Northern blots. One explanation for this could be that we have a built in bias in our selection procedure, *i.e.*, symmetrically derepressing mutants will not be detected. This could perhaps be due to haploid meiosis caused by the simultaneous expression of all four mating-type genes. However, the fact that the *swi6*, *rik1* and *clr1* mutants were isolated by different procedures also show the same asymmetry argues against the first hypothesis. A second more interesting hypothesis is that the asym-

metry is a consequence of the spreading of heterochromatin in a manner discussed by LOCKE, KOTARSKI and TARTOF (1988). Let us imagine that there is a center for spreading in the vicinity of *mat2*, perhaps already included in the defined *cis*-acting elements (EKWALL, NIELSEN and RUUSALA 1991), and that the spreading is dependent on the products of *clr1*, *swi6*, *rik1*, *clr2*, *clr3* and *clr4* genes. In the case of a mutation in one of these genes the spreading is narrower and disappears first from areas more remote from the spreading center. This could explain why *mat3* is more sensitive to the mutations than *mat2*. The recent finding that the *swi6* protein sequence contains chromodomains (A. LORENTZ and H. SCHMIDT, personal communication) similar to those of proteins known to be associated with heterochromatin in Drosophila (SHAFFER, WALLRATH and ELGIN 1993) supports this type of interpretation.

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