Four Chromo-domain Proteins of *Schizosaccharomyces pombe* Differentially Repress Transcription at Various Chromosomal Locations

Geneviève Thon and Janne Verhein-Hansen

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

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ABSTRACT

Transcription is repressed in regions of the fission yeast genome close to centromeres, telomeres, or the silent mating-type cassettes *mat2-P* and *mat3-M*. The repression involves the chromo-domain proteins Swi6 and Clr4. We report that two other chromo-domain proteins, Chp1 and Chp2, are also important for these position effects. Chp1 showed a specificity for centromeric regions. Its essentiality for the transcriptional repression of centromeric markers correlates with its importance for chromosome stability. Chp2 appeared more pleiotropic. Its effects on centromeric silencing were less pronounced than those of Chp1, and it participated in telomeric position effects and transcriptional silencing in the mating-type region. We also found that PolII-transcribed genes were repressed when placed in one of the *Schizosaccharomyces pombe* rDNA clusters, a situation analogous to that in the budding yeast *Saccharomyces cerevisiae*. Chp2, Swi6, Clr4, and, to a lesser extent, Chp1 participated in that repression.

EUKARYOTIC chromosomes are organized in domains with distinctive properties, among which are the potential for transcription and for recombination. These two parameters can vary along chromosomes according to the DNA sequence, the proximity of specialized structures such as telomeres and centromeres, and the specific state of individual cells. Repetitive sequences and the proteins that interact with them often define regions where transcription and recombination are reduced. For example, heterochromatic areas, which are predominantly composed of repeated sequences, are poorly transcribed. In Drosophila they can silence euchromatic genes translocated nearby (reviewed by Weiler and Wakimoto 1995). In the yeast *Saccharomyces cerevisiae*, recombination and PolII transcription occur differently in the rDNA, a chromosomal region where repeats occur naturally in many eukaryotes, compared with other genomic regions (see references in Smith *et al.* 1999).

We are interested in position effects in the fission yeast *Schizosaccharomyces pombe* and in determining whether repeated sequences influence these effects. *S. pombe* has three chromosomes of respectively 5.7, 4.6, and 3.5 Mb (Fan *et al.* 1988; Hoheisel *et al.* 1993; Mizukami *et al.* 1993). Each chromosome contains a 40- to 100-kb centromeric region comprising a 4- to 7-kb central core of unique sequence flanked by large inverted repeats and repeats placed in tandem (Takahashi *et al.* 1992; Steiner and Clarke 1994 and references therein). Because of their partially symmetrical arrangement relative to the central core, centromeric sequences have been proposed to fold into a hairpin. The rDNA forms two large clusters of ~500–1000 kb at both ends of chromosome 3 (Umesono *et al.* 1983; Toda *et al.* 1984; Allshire *et al.* 1987; Hoheisel *et al.* 1993). Telomeres consist of degenerate repeats of the sequence TTACAGG. These repeats are ~300 bp long. They are adjacent to the rDNA genes at both ends of chromosome 3 and to telomere-associated sequences at the ends of chromosomes 1 and 2 (Sugawara 1989).

Position effects on fission yeast transcription have been observed near centromeres, telomeres, and in the vicinity of the *mat2-P* and *mat3-M* silent mating-type cassettes in the right arm of chromosome 2. The *mat2-P* and *mat3-M* cassettes are linked and 4.3 kb of the DNA that separates them displays striking sequence homology with a centromeric repeat (Grewal and Klar 1997). Marker genes introduced near centromeres, telomeres, or in the mating-type region are repressed, either stringently or in a variegated fashion, by mechanisms that utilize the *trans*-acting factors Clr1, Clr2, Clr3, Clr4, Swi6, and Rik1 (Lorentz *et al.* 1992; Thon and Klar 1992; Allshire *et al.* 1994, 1995; Ekwall and Ruusala 1994; Nimmo *et al.* 1994; Thon *et al.* 1994). In addition to repressing transcription, these six factors inhibit meiotic recombination in the mating-type region (Egel *et al.* 1989; Klar and Bonaduce 1991; Lorentz *et al.* 1992; Thon *et al.* 1994), indicating that position effects on transcription and recombination can involve related structures or mechanisms. Whether these silencing factors are also responsible for the reduced recombination observed in centromeric areas (Nakaseko *et al.* 1986; Chikashige *et al.* 1989), or whether they repress tran-

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

scription or recombination at other genomic locations, is not known. In addition to the position effects on transcription, position effects on recombination have been documented in *S. pombe* by a transplacement study of the *ade6-M26* hot spot for recombination which showed that the ability of the *M26* mutation to promote recombination in the *ade6* gene depends on the chromosomal context (Virgin *et al.* 1995).

Among the proteins that associate with chromatin and determine its properties are proteins containing a chromatin organization modifier (chromo) domain (for review see Cavalli and Paro 1998). This motif was first identified in the Drosophila HP1 and Pc proteins (Paro and Hogness 1991) and subsequently in >40 proteins from various organisms (Aasland and Stewart 1995; Koonin *et al.* 1995; current sequence databases). It is found, either alone or in combination with other domains, in both repressors and activators of transcription and in several proteins that localize to pericentric heterochromatin. The chromo-domain and the related chromo-shadow domain mediate the formation of protein complexes and their association with chromatin (Messmer *et al.* 1992; Powers and Eissenberg 1993; Saunders *et al.* 1993; Platero *et al.* 1995; Strutt and Paro 1997). Many potential partners have been proposed to interact with chromo-domain proteins, including components of the nuclear membrane (Ye and Worman 1996; Ye *et al.* 1997) and components of the origin recognition complex (Pak *et al.* 1997).

Two chromo-domain proteins, Swi6 and Clr4, influence position effects in *S. pombe* (Lorentz *et al.* 1992; Ekwall and Ruusala 1994; Thon *et al.* 1994; Allshire *et al.* 1995). Swi6 contains a chromo- and chromo-shadow domain (Lorentz *et al.* 1994; Aasland and Stewart 1995). Clr4 contains a chromo- and SET domain [***Su(var)3-9***, ***E****nhancer of zeste*, ***tr****ithorax*; Tschiersch *et al.* 1994; Ivanova *et al.* 1998]. When either *swi6* or *clr4* is mutated or deleted, genes normally subject to transcriptional silencing near centromeres, telomeres, or in the mating-type region are derepressed. Consistent with these phenotypes, Swi6 localizes at centromeres, telomeres, and in the mating-type region (Ekwall *et al.* 1995). Clr4 is important for the localization of Swi6 (Ekwall *et al.* 1996) and, when expressed from a plasmid, is itself seen in the rDNA (Sawin and Nurse 1996). We tested whether two other chromo-domain proteins of *S. pombe*, Chp1 (Doe *et al.* 1998) and Chp2, were involved in the same processes as Swi6 and Clr4. In addition to assaying the effects of Chp1 and Chp2 in the regions where silencing was previously observed, we investigated whether Swi6, Clr4, Chp1, or Chp2 modified the efficiency of PolII transcription in the rDNA.

MATERIALS AND METHODS

Sequence of *chp1* and *chp2*: Chp1 (GenBank accession no. Q10103) is encoded in the cosmid SPAC18G6 (Z68198) and Chp2 (CAA16917) in SPBC16C6 (AL021767).

Bacterial strains used for cloning: Cloning of plasmid DNA was performed in the *Escherichia coli* strains DH5 (Hanahan 1983) or S1754 (Thon *et al.* 1999).

Cloning of *chp1*: *chp1* is contained within a 4.3-kb *Bam*HI-*Ecl*136I fragment of genomic DNA. That fragment was purified from cosmid ICRFc60G0618 (Reference Library, ICRF; Lehrach 1990) and cloned into Bluescribe (Stratagene, La Jolla, CA) digested with *Bam*HI and *Hin*cII, to create pGT121. The presence of *chp1* in pGT121 was ascertained by restriction mapping and partial sequencing.

Partial replacement of *chp1* with *LEU2* or *ura4*$^+$: *Eco*RV cleaves pGT121 75 bp downstream of the predicted initiating ATG of the *chp1* open reading frame (ORF) and *Bgl*II cleaves pGT121 1355 bp upstream of the predicted stop codon. A total of 1450 bp of pGT121 DNA between those *Eco*RV and *Bgl*II sites were replaced with the 2.2-kb *Ehe*I-*Bam*HI fragment of pJJ250 (Jones and Prakash 1990), which contains the *S. cerevisiae LEU2* gene, to create pGT122, or with the 1.8-kb *Hin*cII-*Bam*HI fragment of pON94 (a gift from Olaf Nielsen), which contains the *S. pombe ura4*$^+$ gene, to create pGT162. pON94 was constructed by filling in the ends of the 1.8-kb *Hin*dIII fragment, which contains *ura4*$^+$ (Grimm *et al.* 1988), adding *Sph*I linkers, and ligating the product into the *Sph*I site of a modified version of pUC19 (Yannish-Perron *et al.* 1985) where the *Hin*dIII site was converted to a *Bam*HI site. The insert of pGT122 was released by digesting with *Bam*HI and *Sca*I and used to transform a diploid strain produced by the mating of PG9 and PG1141. *chp1*$^+$/*chp1*Δ*::LEU2* diploids were identified among the Leu$^+$ transformants by Southern blot of genomic DNA restricted with *Sph*I or *Sph*I and *Nsi* and hybridized with the *chp1* probe described below (Southern and Northern blot analyses). Two such diploids were subjected to tetrad dissection. The insert of pGT162 was released with *Bam*HI and *Fsp*I and used to transform SP837. Ura$^+$ transformants with the *chp1*Δ*::ura4*$^+$ allele were identified by Southern blot of genomic DNA digested with *Nsi*I or *Sph*I and *Sac*I and probed with the *chp1* probe.

Deletion of *chp1* with no substituted marker: pGT121 was digested with *Bsa*AI and *Acc*I, filled in at the ends with the Klenow fragment of *E. coli* DNA polymerase I, and religated, to create pGT187. The entire *chp1* ORF is deleted in pGT187, as well as 33 nucleotides 5′ and 77 nucleotides 3′ to the *chp1* ORF. The insert of pGT187 was released with *Bam*HI and *Sph*I and used to transform PG1544. The DNA of FOA^R transformants was digested with *Nsi*I or *Bam*HI and *Ecl*136III and hybridized with the *chp1* probe, which allowed us to identify transformants in which the *chp1*Δ*::ura4*$^+$ allele of PG1544 had been replaced with the *chp1*Δ allele.

Replacement of *chp2* with *ura4*$^+$: The 1.8-kb *Hin*dIII fragment containing the *S. pombe ura4*$^+$ gene (Grimm *et al.* 1988) was cloned in Bluescript KSII(+) (Statagene) with the *Eco*RV site in the *ura4*$^+$ gene close to the *Eco*RV site in the polylinker, to create pGT188. The oligonucleotides GTO-150 (5′ TCCCC CCGGGAGCTCAGATCGTTATACACTTTACGTATCTAGG 3′) and GTO-151 (5′ CGGGATCCGTCGACTATTAAGACTTTC CAGATATACCAAC 3′) were used to amplify 777 bp of genomic DNA on one side of the *chp2* ORF. GTO-152 (5′ AACTGC AGTCGACTTGATCTTTGGACTTAATAATTAGAATTAC G 3′) and GTO-153 (5′ TCCCCCGGGAAGCTTCTCGAGATG GCGCTTGAAGGGCTTAGTGCGC 3′) were used to amplify 776 bp of genomic DNA on the other side of the *chp2* ORF. The oligonucleotides were purchased from Operon Technologies (Alameda, CA). The PCR were performed with Native Pfu DNA polymerase (Stratagene) in 80 μl containing the buffer provided by the manufacturer, 1 μg *S. pombe* genomic DNA, 1 μm of each primer, and 0.5 mm dNTPs. The amplification cycles were as follows: 3 min 94°; 15 times [1 min 94°, 1 min 60°, 1 min 72°]; 7 min 72°. The PCR products were gel purified. The product amplified with GTO-150 and GTO-151

was digested with *Sac*I and *Bam*HI. The product amplified with GTO-152 and GTO-153 was digested with *Sal*I and *Sma*I. These two fragments were ligated in a four-way ligation with Bluescript SKII(−) (Stratagene) digested with *Sac*I and *Sma*I and with the 1.8-kb *Bam*HI-*Sal*I fragment of pGT188, which contains *ura4*$^+$. A clone containing the *ura4*$^+$ gene flanked by sequences normally flanking the *chp2* ORF was obtained from this ligation and named pGT190. The insert of pGT190 was released with *Sac*I and *Xho*I and used to transform SP837. The DNA of Ura$^+$ transformants was digested with *Hin*dIII or *Bam*HI and *Pst*I and hybridized to the *chp2* probe described below (Southern and Northern analyses). This identified a strain with the *chp2*Δ*::ura4*$^+$ allele, PG1744.

Replacement of *chp2* with *LEU2*: The 3.7-kb *Bam*HI-*Pst*I fragment and the 0.8-kb *Pst*I-*Sal*I fragment of pGT190 were ligated in a three-way ligation with the 2-kb *Bam*HI-*Sal*I of pJJ250, which contains the *S. cerevisiae LEU2* gene (Jones and Prakash 1990). A clone similar to pGT190, but containing *LEU2* instead of *ura4*$^+$, was obtained from this ligation, pGT193. The insert of pGT193 was released by digesting with *Sac*I and *Xho*I and used to transform a diploid strain produced by the mating of PG1636 and PG1637. *chp2*$^+$/*chp2*Δ*::LEU2* diploids were identified among the Leu$^+$ transformants by a Southern blot analysis similar to that described above for the *chp2*Δ*::ura4*$^+$ allele and two such diploids were subjected to tetrad dissection.

Deletion of *chp2* with no substituted marker: pGT190 was digested with *Sal*I. The 3.7-kb *Sal*I fragment, which contains Bluescript and 777 bp of DNA flanking *chp2* on its centromere-proximal side, was ligated with the 0.8-kb *Sal*I fragment, which contains 776 bp of DNA flanking *chp2* on its centromere-distal side. A clone with a precise deletion of the *chp2* ORF was obtained from this ligation, pGT191. The insert of pGT191 was released by digesting with *Sac*I and *Xho*I and used to replace the *chp2*Δ*::ura4*$^+$ allele of PG1753. The DNA of FOA^R transformants was digested with *Cla*I or *Bam*HI and *Pst*I and hybridized to the *chp2* probe. This identified a strain with the *chp2*Δ allele, PG1776.

Introduction of *ura4*$^+$ in the rDNA repeats: The 1.8-kb *Hin*dIII fragment containing *ura4*$^+$ (Grimm *et al.* 1988) was cloned into pUC8 (Viera and Messing 1982). The resulting construct was linearized with *Bsr*FI and used to transform PG1634. Ura$^+$ transformants were analyzed by Southern blots of genomic DNA restricted with *Eco*RV, *Hin*dIII, or *Bam*HI and hybridized with a *LEU2* or *ura4* probe. A transformant with pUC8 and *ura4* integrated in the pBR322 sequence present in the rDNA repeat of PG1634 was saved as PG1661.

Yeast media and culture conditions: YES (Thon and Friis 1997) was used as rich medium; MSA (Egel *et al.* 1994) supplemented with 100 mg adenine, 100 mg uracil, and 200 mg l-leucine per liter was used as sporulation medium; drop-out media (Rose *et al.* 1990), FOA medium (Thon *et al.* 1999), and YE (Moreno *et al.* 1991) were used to test auxotrophies. Plates were incubated at 33°.

Yeast transformation and strain construction: A lithium acetate protocol adapted from Heyer *et al.* (1986) and Moreno *et al.* (1991) was used for transforming *S. pombe*. Strains transformed with constructs containing *LEU2* or *ura4*$^+$ were selected on drop-out media lacking leucine or uracil. FOA medium was used to select for loss of *ura4*$^+$. The strains produced by transformation and their progeny from subsequent crosses are listed in Table 1.

Southern and Northern blot analyses: *S. pombe* DNA was prepared according to Moreno *et al.* (1991) and RNA according to Schmitt *et al.* (1990) from liquid YES cultures incubated at 30°. Blotting and hybridization conditions were as described previously (Thon *et al.* 1999). The probes used for Southern hybridization were made with a random priming kit (Promega, Madison, WI), 3000 Ci/mmol [α-^{32}P]dCTP from Amersham (Piscataway, NJ), and the following templates: the *Bam*HI-*Sph*I fragment of pGT121 for *chp1*; a purified PCR product amplified from genomic DNA with GTO-150 and GTO-153 for *chp2*; the 1.8-kb *Hin*dIII fragment for *ura4* (Grimm *et al.* 1988); and the 2-kb *Bam*HI-*Sal*I of pJJ250 for *LEU2* (Jones and Prakash 1990). Antisense *ura4* riboprobes were made for Northern hybridization with a Riboprobe II core system (Promega), 3000 Ci/mmol [α-^{32}P]UTP (Amersham), T7 RNA polymerase (Promega), and pON140 (a gift from Olaf Nielsen) linearized with *Bam*HI as template. pON-140 is a clone of the *ura4*$^+$ gene in pGEM4 (Promega).

Stability of Ura$^-$ phenotype: FOA^R colonies were purified by two rounds of streaking on FOA-containing plates and patched on YES plates. Following growth under nonselective conditions on YES for ~15 generations, cells were suspended in water and used for spot tests on selective media or for DNA preparation.

Chromosome stability: Mitotic loss of the minichromosome Ch16m23*::ura4*$^+$-Tel[72] was assayed as that of the minichromosome Ch16 (Allshire *et al.* 1995): cells containing the *ade6-M210* allele on chromosome 3, the complementing allele *ade6-M216* on Ch16m23*::ura4*$^+$-Tel[72], and mutations of interest were propagated in medium lacking adenine (AA-ade) at 33°, plated on medium containing a small amount of adenine (MSA supplemented with 100 mg uracil, 200 mg l-leucine, and 15 mg adenine per liter), and incubated at 33°. The loss rate of Ch16m23*::ura4*$^+$-Tel[72] was determined as the number of colonies with a red sector equal to or greater than half the colony divided by the sum of white and sectored colonies.

RESULTS

Deletion of two *ch*romo-domain *p*rotein genes, *chp1* and *chp2*, in *S. pombe*: A gene capable of encoding a 109-kD protein with an N-terminal chromo-domain is present in the left arm of *S. pombe* chromosome 1. That gene was the subject of a previous study and named *chp1* (**ch**romo-domain protein in *S. p*ombe; Doe *et al.* 1998). Another gene, which we will refer to as *chp2*, is located in the right arm of chromosome 2 and encodes a 43-kD protein displaying extensive sequence similarity with the *S. pombe* Swi6 protein (Figure 1). Like Swi6 and a family of proteins related to Drosophila HP1, Chp2 contains both a chromo- and chromo-shadow domain. The *chp1* and *chp2* ORFs were both discovered in the *S. pombe* genome sequencing project (Sanger Center).

We created several deletion alleles of *chp1* and *chp2*: part of the *chp1* or the entire *chp2* ORF was replaced with the *S. cerevisiae LEU2* gene, a functional homolog of the *S. pombe leu1*$^+$ gene, or with the *S. pombe ura4*$^+$ gene. Deletion of the entire ORF with no substituted markers was also obtained. The deletions were originally introduced in the chromosome of diploid cells. The *chp1* and *chp2* deletions both proved viable in the haploid progeny of the transformed diploids. This had been observed independently for *chp1* (Doe *et al.* 1998). *chp1*-deleted cells formed colonies significantly smaller than the wild type, whereas *chp2*-deleted cells formed colonies as large as the wild type.

Deleting *chp1* or *chp2* alleviates centromeric position effects: *S. pombe* centromeres are organized in large inverted repeats (Takahashi *et al.* 1992; Steiner and

TABLE 1

Strains and their genotypes

Strain	Genotype	Source[a]
FY336	*h*$^+$ *cnt1*/TM1(*Nco*I)::*ura4*$^+$ *leu1-32 ura4-DS/E ade6-210*	Allshire *et al.* (1994)
FY340	*h*$^+$ *TM1(Nco*I)-*ura4*$^+$ Random int. leu1-32 ura4-DS/E ade6-210	Allshire *et al.* (1994)
FY489	*h*$^-$ *leu1-32 ura4-DS/E ade6-210*	Allshire *et al.* (1995)
FY501	*h*$^+$ *imr*L(*Nco*I)::*ura4*$^+$ oriII leu1-32 ura4-DS/E ade6-210	Allshire *et al.* (1995)
FY520	*h*$^+$ Ch16 m23::*ura4*$^+$-TEL[72] leu1-32 ura4-DS/E ade6-210 (ch16 *ade6-216*)	Nimmo *et al.* (1995)
FY521	*h*$^-$ Ch16 m23::*ura4*$^+$ leu1-32 ura4-DS/E ade6-210 (Ch16 *ade6-216*)	Allshire *et al.* (1995)
FY611	*h*$^+$ Ch16 m23::*ura4*$^+$-TEL[72] leu1-32 ura4-DS/E ade6-210 (Ch16 *ade6-216*) *swi6-115*	Allshire *et al.* (1995)
FY648	*h*$^+$ *otr1*R(*Sph*I)::*ura4*$^+$ *leu1-32 ura4-DS/E ade6-210*	Allshire *et al.* (1995)
FY939	*h*$^+$ *otr1*L(dh/*Bgl*II)::*ura4*$^+$ oriII leu1-32 ura4-DS/E ade6-210	Allshire *et al.* (1995)
FY967	*h*$^+$ *otr1*L(dh/*Hin*dIII)::*ura4*$^+$ oriII leu1-32 ura4-DS/E ade6-210	Allshire *et al.* (1995)
FY986	*h*$^+$ *otr1*L(dg1a/*Hin*dIII)::*ura4*$^+$ oriII leu1-32 ura4-DS/E ade6-210	Allshire *et al.* (1995)
PG9	*mat3-M(Eco*RV)::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M216*	Thon and Klar (1992)
PG445	*mat1-P*Δ*17::LEU2 leu1-32 ura4-D18 ade6-M216*	Thon *et al.* (1998)
PG596	*mat3-M(Eco*RV)::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M216 swi6-115*	Thon *et al.* (1994)
PG912	*mat3-M(Eco*RV)::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M210 clr4-681*	Thon *et al.* (1994)
PG1032	*mat1-Msmt-0 mat2-P(Xba*I)::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M216 clr4-681*	This study
PG1141	*h*^{90} *leu1-32 ura4-D18, ade6-210*	Thon *et al.* (1999)
PG1402	*mat1-P*Δ*17::LEU2* Δ*(482)mat3-M leu1-32 ura4-D18 ade6-M216*	This study
PG1419	*mat1-P*Δ*17::LEU2* Δ*(482)mat3-M leu1-32 ura4-D18 ade6-M216 clr4-681*	This study
PG1454	*mat3-M(Eco*RV)::*ura4*$^+$ *leu1-32 ura4-D18 chp1*Δ::*LEU2*	This study
PG1544	*h*^{90} *leu1-32 ura4-D18 ade6-216 chp1*Δ::*ura4*$^+$	This study
PG1572	*mat1-Msmt-0 leu1-32 ura4-D18 Ylp2.4 chp1*Δ::*ura4*$^+$	This study
PG1594	*mat1-P*Δ*17::LEU2 leu1-32 ura4-D18 ade6-M216 clr4-681*	This study
PG1600	*h*$^+$ Ch16 m23::*ura4*$^+$-TEL[72] leu1-32 ura4-DS/E or *-D18 ade6-210* (Ch16 *ade6-216*) *chp1*Δ::*LEU2*	This study
PG1605	*mat1-Msmt-0 mat2-P(Xba*I)::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M216 chp1*Δ::*LEU2*	This study
PG1634	*h*$^+$ *ura4-DS/E leu1/YIp2.4 swi6-115*	This study
PG1636	*h*^{90} *leu1-32 ura4-DS/E ade6-210*	This study
PG1637	*h*^{90} *leu1-32 ura4-DS/E ade6-216*	This study
PG1639	*mat1-Msmt-0* Δ*(Bgl*II-*Bss*HII)*mat2-P(Xba*I)::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M216 chp1*Δ::*LEU2*	This study
PG1640	*mat1-Msmt-0* Δ*(Bgl*II-*Bss*HII)*mat2-P(Xba*I)::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M216 swi6-115*	This study
PG1661	*h*$^+$ *ura4-DS/E leu1/YIp2.4 pUCura4*$^+$*-7 swi6-115*	This study
PG1691	*h*$^+$ *imr*L(*Nco*I)::*ura4*$^+$ oriII leu1-32 ura4-DS/E ade6-210 chp1Δ::LEU2	This study
PG1699	*h*$^+$ *otr1*L(dh/*Bgl*II)::*ura4*$^+$ oriII leu1-32 ura4-DS/E ade6-210 chp1Δ::LEU2	This study
PG1703	*h*$^+$ *otr1*L(dh/*Hin*dIII)::*ura4*$^+$ oriII leu1-32 ura4-DS/E ade6-210 chp1Δ::LEU2	This study
PG1709	*h*$^+$ *otr1*L(dg1a/*Hin*dIII)::*ura4*$^+$ oriII leu1-32 ura4-DS/E ade6-210 chp1Δ::LEU2	This study
PG1717	*h*$^+$ *otr1*R(*Sph*I)::*ura4*$^+$ *leu1-32 ura4-DS/E ade6-210 chp1*Δ::*LEU2*	This study
PG1721	*h*$^+$ *cnt1*/TM1(*Nco*I)::*ura4*$^+$ *leu1-32 ura4-DS/E ade6-210 chp1*Δ::*LEU2*	This study
PG1731	*h*$^+$ Ch16 m23::*ura4*$^+$-TEL[72] leu1-32 ura4-DS/E ade6-210 (Ch16 *ade6-216*) *clr4-681*	This study
PG1732	*h*$^+$ Ch16 m23::*ura4*$^+$-TEL[72] leu1-32 ura4-DS/E ade6-210 (Ch16 *ade6-216*) *chp1*Δ::*LEU2*	This study
PG1734	*h*^{90} *ura4-DS/E leu1/YIp2.4 pUCura4*$^+$*-7*	This study
PG1736	*h*^{90} *ura4-DS/E leu1/YIp2.4 pUCura4*$^+$*-7 clr4-681*	This study

(*Continued*)

TABLE 1

(Continued)

Strain	Genotype	Source[a]
PG1744	*h*^{90} *ura4-D18 leu1-32 ade6-216 chp2*Δ*::ura4*	This study
PG1753	*h*^{90} *ura4-DS/E leu1-32 ade6-210 chp2*Δ*::ura4*	This study
PG1763	*h*^{90} *cnt1*/TM1(*Nco*I)*::ura4*$^{+}$ *leu1-32 ura4-DS/E ade6-210 chp2*Δ*::LEU2*	This study
PG1764	*h*$^{+}$ *otr1*R(*Sph*I)*::ura4*$^{+}$ *leu1-32 ura4-DS/E ade6-210 chp2*Δ*::LEU2*	This study
PG1765	*h*$^{+}$ *imr1*L(*Nco*I)*::ura4*$^{+}$ oriII leu1-32 ura4-DS/E ade6-210 chp2Δ::LEU2	This study
PG1767	*h*$^{+}$ *otr1*L(dh/*Bgl*II)*::ura4*$^{+}$ oriII leu1-32 ura4-DS/E ade6-210 chp2Δ::LEU2	This study
PG1769	*h*$^{+}$ *otr1*L(dh/*Hin*dIII)*::ura4*$^{+}$ oriII leu1-32 ura4-DS/E ade6-210 chp2Δ::LEU2	This study
PG1771	*h*$^{+}$ *otr1*L(dg1a/*Hin*dIII)*::ura4*$^{+}$ oriII leu1-32 ura4-DS/E ade6-210 chp2Δ::LEU2	This study
PG1773	*h*^{90} Ch16 m23::*ura4*$^{+}$-TEL[72] leu1-32 ura4-DS/E ade6-210 (Ch16 *ade6-216*) *chp2*Δ*::LEU2*	This study
PG1774	*h*$^{+}$ Ch16 m23::*ura4*$^{+}$-TEL[72] leu1-32 ura4-DS/E ade6-210 (Ch16 *ade6-216*) *chp2*Δ*::LEU2*	This study
PG1776	*h*^{90} *ura4-DS/E leu1-32 ade6-210 chp2*Δ	This study
PG1778	*mat1-P*Δ*17::LEU2* Δ*(482)mat3-M leu1-32 ura4-D18 ade6-216 chp2*Δ*::ura4*$^{+}$	This study
PG1779	*mat1-P*Δ*17::LEU2* Δ*(482)mat3-M leu1-32 ura4-D18 ade6-216 chp1*Δ*::ura4*$^{+}$	This study
PG1781	*mat1-P*Δ*17::LEU2* Δ*(482)mat3-M leu1-32 ura4-D18 ade6-216 swi6*Δ*::ura4*$^{+}$	This study
PG1783	*mat3-M(Eco*RV*)::ura4*$^{+}$ *leu1-32 ura4-DS/E ade6-210 chp2*Δ*::LEU2*	This study
PG1787	*mat1-Msmt-0* Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4*$^{+}$ *leu1-32 ura4-D18 ade6-M216 chp2*Δ*::LEU2*	This study
PG1790	*mat1-Msmt-0 mat2-P(Xba*I*)::ura4*$^{+}$ *leu1-32 ura4-D18 ade6-M216 chp2*Δ*::LEU2*	This study
PG1792	*h*$^{+}$ *ura4-DS/E leu1/YIp2.4 pUCura4*$^{+}$*-7 chp1*Δ	This study
PG1794	*h*^{90} *ura4-DS/E leu1/YIp2.4 pUCura4*$^{+}$*-7 chp2*Δ	This study
PG1796	*mat1-P*Δ*17::LEU2 leu1-32 ura4-D18 ade6-216 chp1*Δ*::ura4*$^{+}$	This study
PG1797	*mat1-P*Δ*17::LEU2 leu1-32 ura4-D18 ade6-210 swi6*Δ*::ura4*$^{+}$	This study
PG1799	*mat1-P*Δ*17::LEU2 leu1-32 ura4-D18 ade6-216 chp2*Δ*::ura4*$^{+}$	This study
PG1970	*h*^{90} Ch16 m23::*ura4*$^{+}$-TEL[72] *leu1-32 ura4-DS/E ade6-210* (Ch16 *ade6-216*) *swi6-115 chp1*Δ*::LEU2*	This study
PG1972	*h*^{90} Ch16 m23::*ura4*$^{+}$-TEL[72] *leu1-32 ura4-DS/E ade6-210* (Ch16 *ade6-216*) *clr4-681 chp1*Δ*::LEU2*	This study
PG1974	*h*$^{+}$ Ch16 m23::*ura4*$^{+}$-TEL[72] *leu1-32 ura4-DS/E ade6-210* (Ch16 *ade6-216*) *swi6-115 chp2*Δ*::LEU2*	This study
PG1976	*h*^{90} Ch16 m23::*ura4*$^{+}$-TEL[72] *leu1-32 ura4-DS/E ade6-210* (Ch16 *ade6-216*) *clr4-681 chp2*Δ*::LEU2*	This study
PG1978	*h*$^{+}$ Ch16 m23::*ura4*$^{+}$-TEL[72] *leu1-32 ura4-D18 ade6-210* (Ch16 *ade6-216*) *chp1*Δ*::LEU2 chp2*Δ*::LEU2*	This study
PG1984	*h*$^{+}$ Ch16 m23::*ura4*$^{+}$-TEL[72] *leu1-32 ura4-DS/E ade6-210* (Ch16 *ade6-216*) *swi6-115 clr4-681*	This study
SP837	*h*^{90} *leu1-32 ura4-D18 ade6-216*	Thon *et al.* (1994)
SP1124	*mat1-Msmt-0 mat2-P(Xba*I*)::ura4*$^{+}$ *ura4-D18 ade6-M216*	Thon *et al.* (1994)
SP1126	*mat1-Msmt-0 mat2-P(Xba*I*)::ura4*$^{+}$ *ura4-D18 ade6-M210 swi6-115*	Thon *et al.* (1994)
SP1151	*mat1-Msmt-0* Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4*$^{+}$ *ura4-D18 ade6-M216*	Thon *et al.* (1994)
SP1165	*mat1-Msmt-0* Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4*$^{+}$ *leu1-32 ura4-D18 ade6-M216 clr4-681*	Thon *et al.* (1994)
YIp2.4-1	*h*$^{-}$ *leu1/YIp2.4*	Toda *et al.* (1984)

[a] The complete genealogy of strains constructed in this study is available upon request. Mutated and engineered alleles used for the strain constructions were described in the following studies: *mat1-P*Δ*17::LEU2*: Arcangioli and Klar (1991); *mat1-Msmt-0*: Engelke *et al.* (1987) and Styrkarsdottir *et al.* (1993); *mat3-M(Eco*RV*)::ura4*$^{+}$: Thon and Klar (1992); *mat3-P(Eco*RV*)::ura4*$^{+}$: Thon and Klar (1993); *mat2-P(Xba*I*)::ura4*$^{+}$ and Δ*(Bgl*II-*Bss*HII*)mat2-P(Xba*I*)::ura4*$^{+}$: Thon *et al.* (1994); Δ*(482)mat3-M*: Thon *et al.* (1999); *ura4-D18*: Grimm *et al.* (1988); *ade6-M210* and *ade6-M216*: Gutz (1963); *swi6-115*: Gutz and Schmidt (1985); *swi6*Δ*::ura4*$^{+}$: Lorentz *et al.* (1994); *clr4-681*: Thon *et al.* (1994).

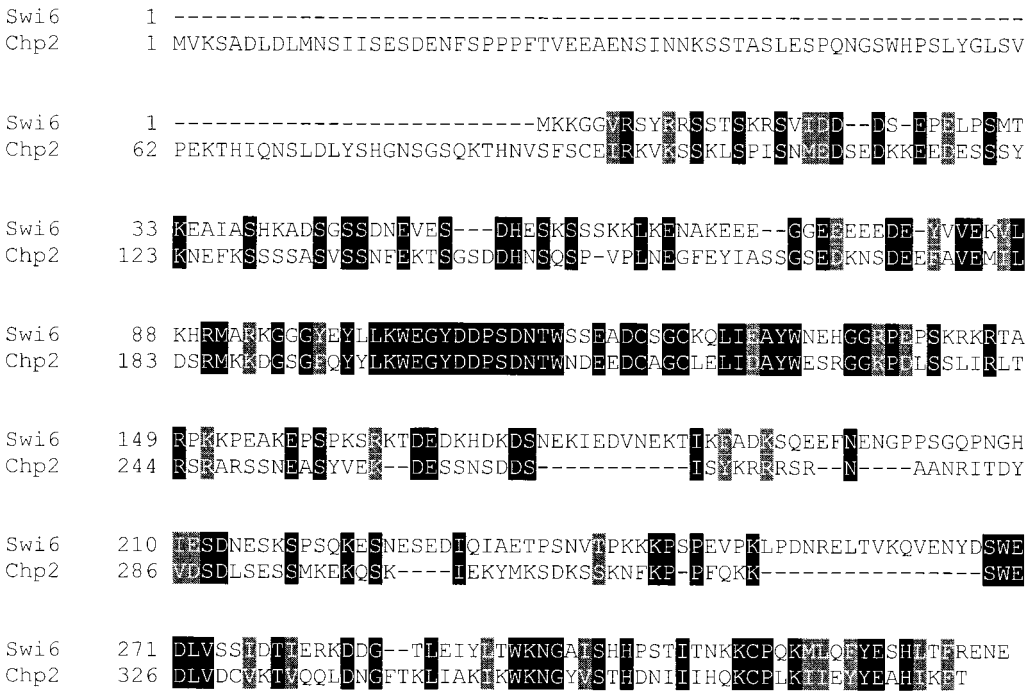


Figure 1.—Sequence comparison of Chp2 and Swi6. The predicted protein sequences of Chp2 and Swi6 were aligned using the CLUSTAL W program (Thompson *et al.* 1994).

Clarke 1994 and references therein). The *ura4*$^+$ gene was placed at several locations within the centromere of chromosome 1 by Allshire *et al.* (1994, 1995). Insertion at most locations results in a level of expression inferior to that of the *ura4*$^+$ gene at its wild-type location and in a variegated phenotype where clonally derived fractions of the cell population express *ura4*$^+$, whereas the rest of the cells keep it repressed. Swi6 and Clr4 are required for this centromeric repression (Allshire *et al.* 1995).

We tested the effect of Chp1 and Chp2 on the expression of *ura4*$^+$ at six centromeric integration sites (Figure 2). In the first experiment, we compared the ability of wild-type, *chp1-*, or *chp2*-deleted cells containing a centromeric *ura4*$^+$ gene to form colonies on medium lacking uracil or medium containing FOA, a toxigenic substrate of the Ura4 protein (Grimm *et al.* 1988; Figure 2A). In the second experiment, we examined the amount of *ura4*$^+$ transcript present in cells of the same strains by Northern blot analysis (Figure 2B). Transcripts originating from the *ura4DS/E* allele (Allshire *et al.* 1994), a truncated *ura4* gene occupying its natural chromosomal location, were used as an internal control.

In wild-type cells, the tightest repression of *ura4*$^+$ was observed in the outer centromeric repeats (otr1L) within the dg1 region. Insertion within the central area (cnt) or the inner most repeats (imr1L) allowed a higher expression of *ura4*$^+$. These phenotypes agree with those reported by Allshire *et al.* (1994, 1995). Some strains (FY939, FY967, FY501, FY336) grew more poorly on FOA medium in our experiments than originally observed (compare Figure 2A with Figure 1C and Figure 2B in Allshire *et al.* 1995). We believe that this is due to a difference in the FOA-containing media used. The amount of *ura4*$^+$ transcript relative to the control transcript from *ura4-DS/E* was similar in both studies. As noted before (Allshire *et al.* 1995), an amount of *ura4*$^+$ transcript inferior to that found in the wild type could support effective growth on medium lacking uracil.

Deletion of *chp1* completely derepressed five centromeric loci and caused a small derepression at *cnt1*, the most central centromeric insertion site (Figure 2, A and B). Deletion of *chp2* partially derepressed *ura4*$^+$ at the six centromeric sites tested. It increased growth on medium lacking uracil and reduced growth on medium containing FOA (Figure 2A). It also increased the amount of *ura4*$^+$ transcript detected in Northern blot relative to the amount arising from the same sites in wild-type cells (Figure 2B). However, the derepression was strikingly small compared with that caused by deletion of *chp1*. This was true for all sites examined except possibly cnt1.

The FOA resistance of strains containing a *ura4*$^+$ centromeric marker was generally reduced by deletion of *chp1* or *chp2*, but we noted that approximately one cell in a thousand was able to generate a full-sized colony on FOA-containing medium; the frequency of such cells varied from culture to culture. *chp*$^+$ strains gave rise to a similar kind of papillation (Figure 2A). We investigated the nature of the FOA resistance by propagating cells from well-growing FOA^R colonies under nonselective conditions and by measuring their ability to form colonies in the absence of uracil. We also performed Southern blots to test for possible alterations of the *ura4* gene. Two independent FOA^R colonies from each strain

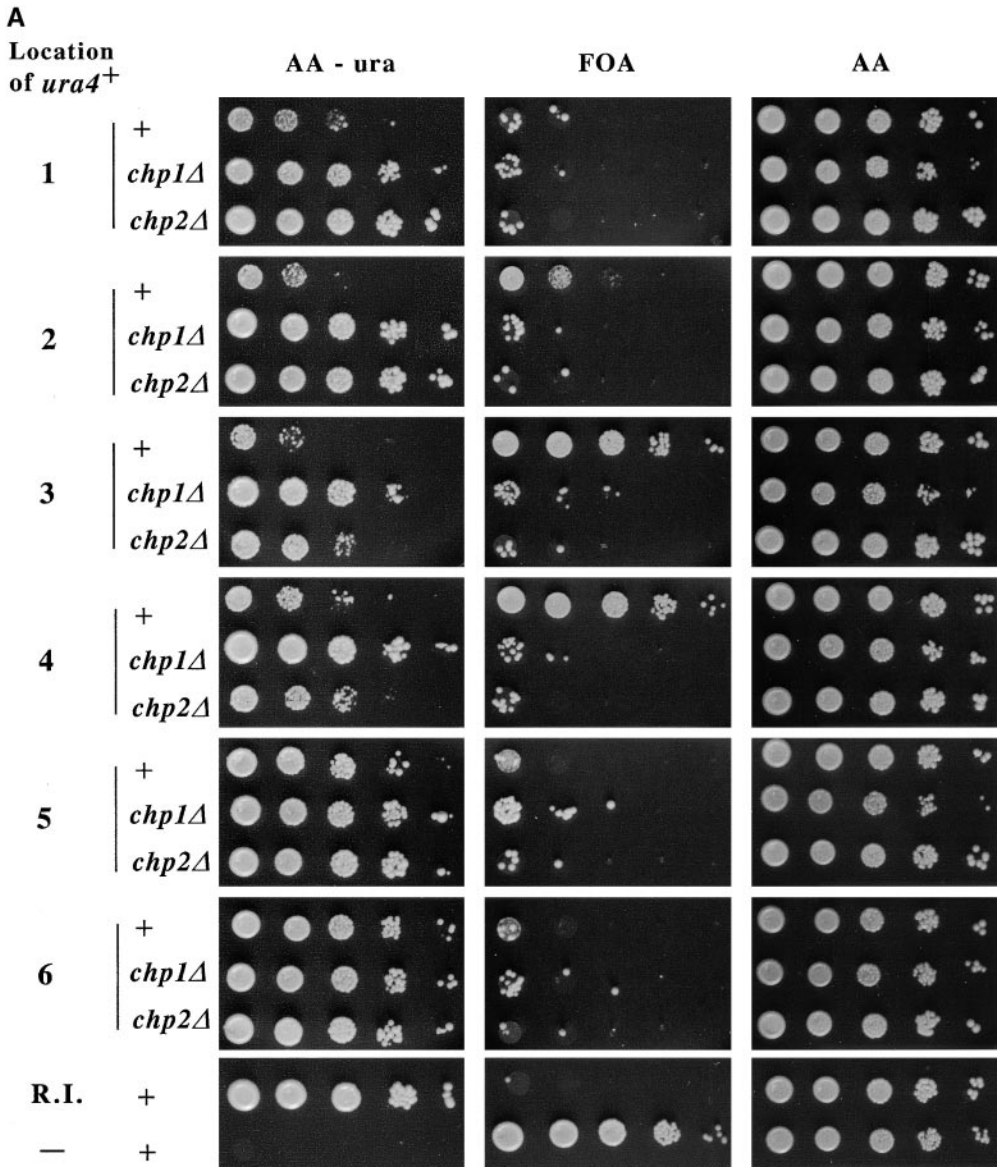


Figure 2.—Effect of Chp1 and Chp2 on gene expression near centromere 1. (A) Expression of the *ura4*$^+$ gene assayed by plating efficiency on selective media. Serial 10-fold dilutions of cell suspensions were spotted on the indicated media. The cells contained centromeric insertions of *ura4*$^+$ that are depicted in B (1–6), a random integration of *ura4*$^+$ (R.I.), or no full-length *ura4* gene (−). The strains were: 1: +, FY939; *chp1*Δ, PG1699; *chp2*Δ, PG1767; 2: +, FY967; *chp1*Δ, PG1703; *chp2*Δ, PG1769; 3: +, FY986; *chp1*Δ, PG1709; *chp2*Δ, PG1771; 4: +, FY648; *chp1*Δ, PG1717; *chp2*Δ, PG1764; 5: +, FY501; *chp1*Δ, PG1691; *chp2*Δ, PG1765; 6: +, FY336; *chp1*Δ, PG1721; *chp2*Δ, PG1763; R.I., FY340; −, FY489. (B) Northern blot analysis of the *ura4*$^+$ transcript. RNA was prepared from the strains displayed in A and the amount of *ura4*$^+$ transcript was estimated by hybridization to a *ura4* riboprobe.

displayed in Figure 2, 1–6, were tested in this manner. We found that the FOA resistance was due to a loss of the *ura4*$^+$ gene in all strains examined except for FY986 and FY648. In FY986 and FY648, the FOA-resistant state returned efficiently to Ura$^+$ and the *ura4*$^+$ gene appeared unchanged in Southern blots. This epigenetically controlled resistance is likely to have masked deletion events such as those occurring in the other strains tested.

Deletion of *chp2*, but not of *chp1*, alleviates telomeric position effects: Ch16 is a 530-kb *S. pombe* minichromosome derived from chromosome 3 by radiation-induced breakage (Matsumoto *et al.* 1987). It contains the *ade6-M216* allele, which allows one to monitor its presence in cells bearing the *ade6-M210* allele due to the intragenic complementation between the *ade6-M216* and *ade6-M210* alleles (Gutz 1963). A truncated version of Ch16, Ch16m23::*ura4*$^+$-Tel[72], where the *ura4*$^+$ gene is ~1 kb from the telomere, was constructed in two steps (Niwa *et al.* 1989; Nimmo *et al.* 1994). *ura4*$^+$ is expressed in a variegated manner in Ch16m23::*ura4*$^+$-Tel[72] (Nimmo *et al.* 1994). Clr4 and, to a lesser extent, Swi6 contribute to the repression of *ura4*$^+$ at that location (Allshire *et al.* 1995). We crossed the Ch16m23::*ura4*$^+$-Tel[72] minichromosome into, respectively, *chp1*- and *chp2*-deleted strains. Crosses with *swi6-115* and *clr4-681* were performed at the same time for comparison. We assayed the expression of *ura4*$^+$ in three mutant and three wild-type strains issued from each cross by spot tests on selective media. Strains with identical genotypes proved to have very similar plating efficiencies on the various selective plates (data not shown). Spot tests were repeated with one strain of each genotype and the amount of *ura4*$^+$ transcript was estimated by Northern blot. As can be seen in Figure 3, deleting *chp1* had no influence on telomeric position effect, but deleting *chp2*

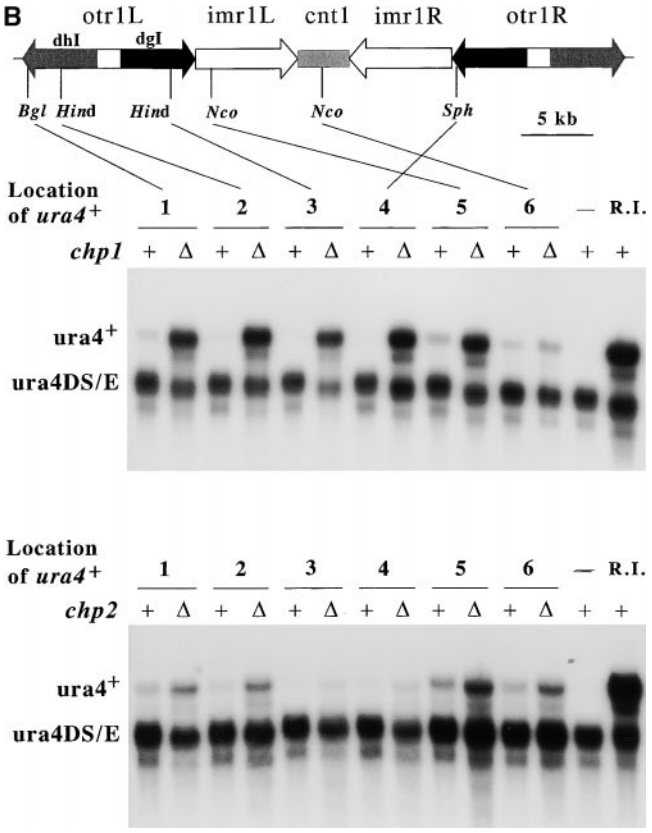


Figure 2.—(*Continued*).

caused an increased expression of *ura4*$^+$. The increased expression caused by the *chp2* deletion was similar to that caused by a mutation in *clr4*.

Role of *S. pombe* chromo-domain proteins in chromosome segregation: We measured the rate of loss of the minichromosome Ch16m23::*ura4*$^+$-Tel[72] in wild-type, *chp1*Δ::*LEU2*, *chp2*Δ::*LEU2*, *swi6-115*, and *clr4-681* cells. The results are presented in Table 2. *swi6-115* and *chp1*Δ::*LEU2* increased loss of Ch16m23::*ura4*$^+$-Tel[72], in accordance with previous reports (Allshire *et al.* 1995; Doe *et al.* 1998). In contrast, deletion of *chp2* or the *clr4-681* mutation had a modest and possibly not significant effect on the rate of loss compared with the wild type. We constructed strains containing pairwise combinations of the *chp1*Δ::*LEU2*, *chp2*Δ::*LEU2*, *swi6-115*, and *clr4-681* alleles. We found that all double mutants were viable and that the rate of loss of Ch16m23::*ura4*$^+$-Tel[72] was greater in the *chp1*Δ::*LEU2 swi6-115* and *chp1*Δ::*LEU2 clr4-681* double mutants than it was in any of the single mutants (Table 2). For all other combinations tested, the rate of loss was similar to that caused by the stronger mutation in the pair. The *chp2* deletion did not increase chromosome loss in any of the backgrounds tested. The minichromosome loss assay we used relies on measuring changes from a white Ade$^+$ phenotype displayed by cells containing the minichromosome to a red Ade$^-$ phenotype displayed after chromosome loss (materials and methods; Allshire *et al.* 1995). In all mutant strains tested, we noticed the presence of colonies that were mostly red with a narrow white sector (Table 2). This phenotype is indicative of multiple consecutive missegregation events and it is expected to occur very infrequently according to our calculated rates of chromosome loss. The existence of such a phenotype suggests that the mutations tested destabilize chromosomes in a heritable manner in a fraction of the cell population. This property could also account for the relatively large variations in loss rates observed in independent cultures of the same strain (Table 2). Alternatively, red colonies with a narrow white sector could be produced if Ade$^+$ cells temporarily stopped dividing after a missegregation event.

Deletion of *chp2*, but not of *chp1*, derepresses transcription in the mating-type region: The mating-type region of *S. pombe* comprises three linked cassettes, *mat1*, which is transcribed, and *mat2-P* and *mat3-M*, which are silenced (Egel and Gutz 1981; Beach 1983). Silencing extends to the regions flanking *mat2-P* and *mat3-M* and to the 10.9-kb region that separates the two cassettes (Thon and Klar 1992; Thon *et al.* 1994; Grewal and Klar 1997). It affects the *ura4*$^+$ gene when placed at an *Xba*I site located ~400 bp centromere-distal to the *mat2-P* cassette [*mat2-P(XbaI)::ura4*$^+$ allele; Thon *et al.* 1994; Figure 4A] or at an *Eco*RV site located ~150 bp centromere-distal to the *mat3-M* cassette [*mat3-M(EcoRV)::ura4*$^+$ allele; Thon and Klar 1992; Figure 4B]. The products of *clr1*, *clr2*, *clr3*, *clr4*, *clr6*, *swi6*, *rik1*, *esp1*, *esp2*, and *esp3* are essential for full repression. These factors seem to act in partially redundant pathways, such that double mutations are required to completely abolish silencing within the mating-type region. Double mutations with strong synergistic effects can either reside in two *trans*-acting factors not belonging to the same class or consist in a combination of *cis*- and *trans*-acting mutations (Thon *et al.* 1994, 1999; Thon and Friis 1997; Grewal *et al.* 1998). We examined whether Chp1 or Chp2 contributed to the repression of *ura4*$^+$ placed near *mat2-P* or *mat3-M*, or to the repression of the mating-type genes at *mat2-P* or *mat3-M*. The tests were performed in strains with or without deletion of *cis*-acting silencing elements.

First, we assayed *ura4*$^+$ expression from the mating-type region in cells lacking *chp1* or *chp2* by spot tests on selective media (Figure 4). Wild-type, *swi6-115*, and *clr4-681* cells were spotted on the same plates for comparison. Cells lacking Chp1 grew as well on FOA and as poorly on medium lacking uracil as cells containing a functional protein, indicating that Chp1 had no effect on transcription of the *ura4*$^+$ gene placed in the mating-type region. In contrast, lack of Chp2 allowed increased growth in the absence of uracil, indicating that Chp2 was required for the repression of *ura4*$^+$ near *mat2-P* and *mat3-M*. In addition to forming colonies in the absence of uracil, cells deleted for *chp2* and containing

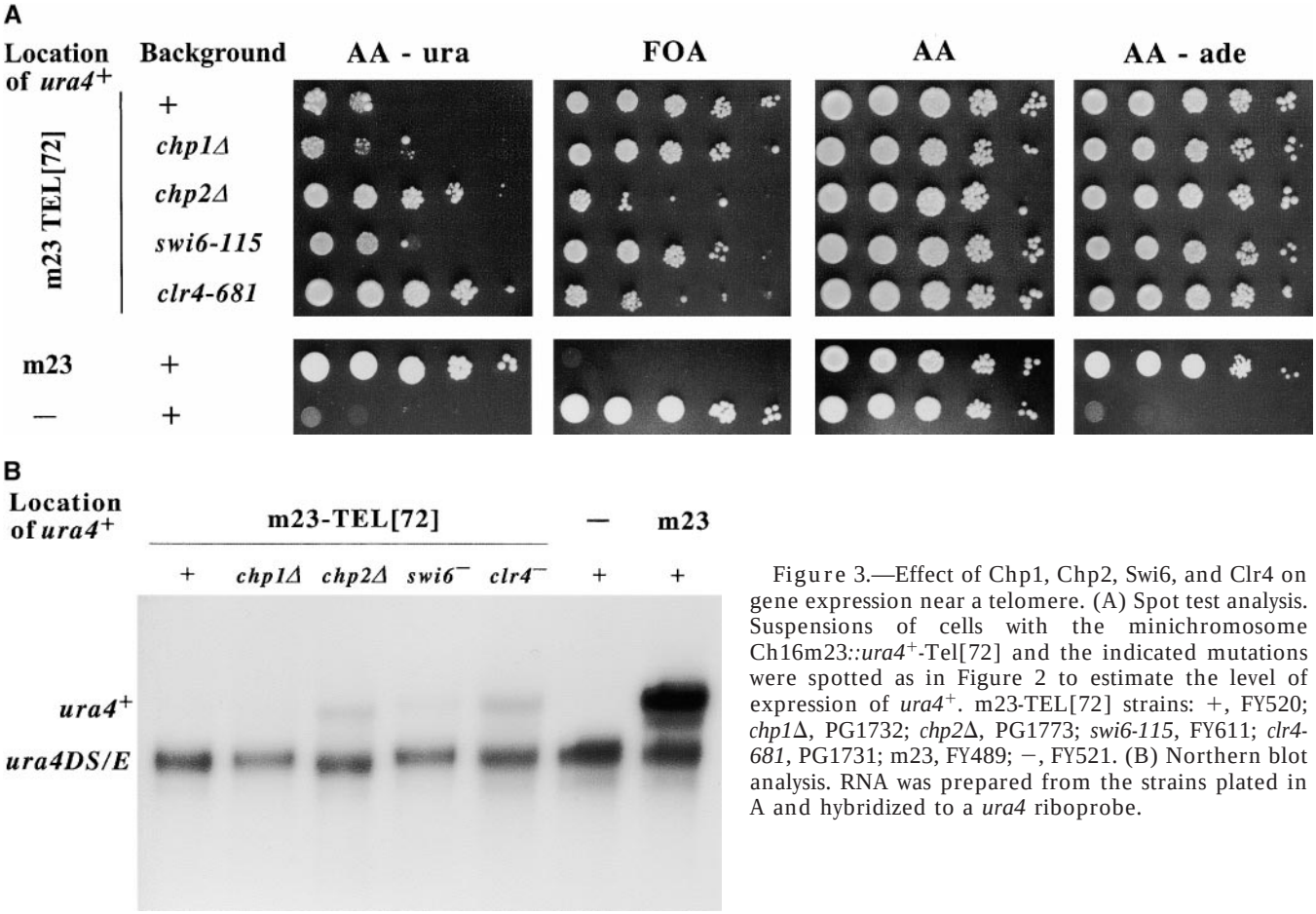


Figure 3.—Effect of Chp1, Chp2, Swi6, and Clr4 on gene expression near a telomere. (A) Spot test analysis. Suspensions of cells with the minichromosome Ch16m23::*ura4*$^+$-Tel[72] and the indicated mutations were spotted as in Figure 2 to estimate the level of expression of *ura4*$^+$. m23-TEL[72] strains: +, FY520; *chp1*Δ, PG1732; *chp2*Δ, PG1773; *swi6-115*, FY611; *clr4-681*, PG1731; m23, FY489; −, FY521. (B) Northern blot analysis. RNA was prepared from the strains plated in A and hybridized to a *ura4* riboprobe.

ura4$^+$ near *mat2-P* could also form colonies on FOA-containing medium, indicating that deletion of *chp2* did not derepress *ura4*$^+$ near *mat2-P* as efficiently as mutations in *swi6* or *clr4* (Figure 4A, top). A stronger derepression of *ura4*$^+$ was obtained by simultaneously deleting *chp2* and a *mat2-P cis*-acting element (Figure 4A, bottom). The derepression seen at *mat3-M* following deletion of *chp2* appeared equivalent to that in *swi6* and *clr4* mutants (Figure 4B).

Wild-type *S. pombe* cells efficiently switch the content of their *mat1* cassette between *P* and *M* by copying the silent information present at *mat2-P* and *mat3-M*. Following these switches, cells can mate and form zygotic asci within isolated colonies. Strains unable to switch their

TABLE 2

Effect of Chp1, Chp2, Swi6, and Clr4 on the stability of a minichromosome[a]

Strain	Background	Half-sectored colonies	Mostly red colonies	Colonies examined	Loss rate per cell division (%)	Mutant/ WT ratio
FY520	WT	1,0,0,3	0,0,0,0	1098,1081,1022,2642	0.07	1
PG1600	*chp1*Δ::*LEU2*	6,4,7,9,12	2,4,9,6,9	1105,867,1085,421,1791	1.29	18
PG1774	*chp2*Δ::*LEU2*	1,1,1,1,6,3	0,0,2,0,0,1	896,984,1097,977,3519	0.21	3
FY611	*swi6-115*	4,3,12,4,40	3,2,0,0,13	956,1016,568,663,1804	1.52	22
PG1731	*clr4-681*	3,0,0,2,0	0,0,0,1,0	1230,973,998,854,651	0.13	2
PG1978	*chp1*Δ::*LEU2 chp2*Δ::*LEU2*	4,6,2	1,4,0	536,723,536	0.95	14
PG1970	*chp1*Δ::*LEU2 swi6-115*	25,5,24	11,4,31	896,269,618	5.60	80
PG1972	*chp1*Δ::*LEU2 clr4-681*	21,14,8	7,21,23	749,707,542	4.75	68
PG1974	*chp2*Δ::*LEU2 swi6-115*	15,4,2,11	1,3,0,0	774,456,231,1150	1.38	20
PG1976	*chp2*Δ::*LEU2 clr4-681*	2,0,2,3	0,0,0,0	1116,486,884,905	0.21	3
PG1984	*swi6-115 clr4-681*	6,10,9	7,3,4	690,969,785	1.60	23

[a] Three to six independent cultures were assayed as described in materials and methods. WT, wild type.

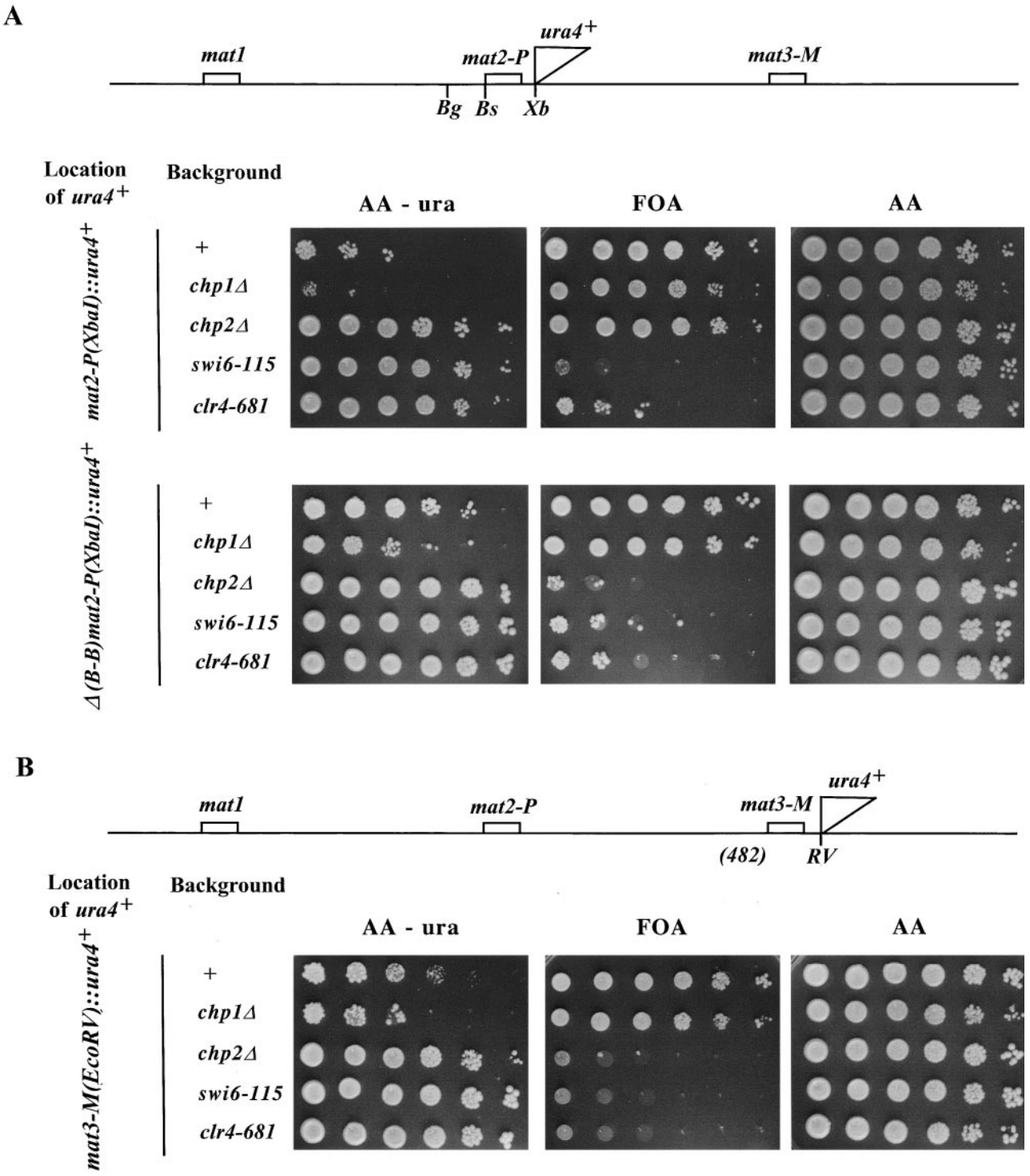


Figure 4.—Effects of Chp1, Chp2, Swi6, and Clr4 on the expression of *ura4*$^+$ from the mating-type region. (A) Effects near *mat2-P*. Cells with *ura4*$^+$ at the *Xba*I site centromere-distal to *mat2-P* (*Xb*) were spotted on the indicated media as in Figure 2. (Top) Strains have intact *mat2-P* silencing elements. (Bottom) Strains have a *mat2-P* centromere-proximal deletion of ~1.5 kb between a *Bgl*II (*Bg*) and a *Bss*HII (*Bs*) site. *mat2-P(XbaI)::ura4* strains: +, SP1124; *chp1*Δ, PG1605; *chp2*Δ, PG1790; *swi6-115*, SP1126; *clr4-681*, PG1032; Δ*(B-B)mat2-P(XbaI)::ura4* strains: +, SP1151; *chp1*Δ, PG1639; *chp2*Δ, PG1787; *swi6-115*, SP1640; *clr4-681*, SP1165. (B) Effects near *mat3-M*. Cells with the *ura4*$^+$ gene at the *Eco*RV site centromere-distal to *mat3-M* were spotted as in Figure 2. +, PG9; *chp1*Δ, PG1454; *chp2*Δ, PG1783; *swi6-115*, PG596; *clr4-681*, PG912.

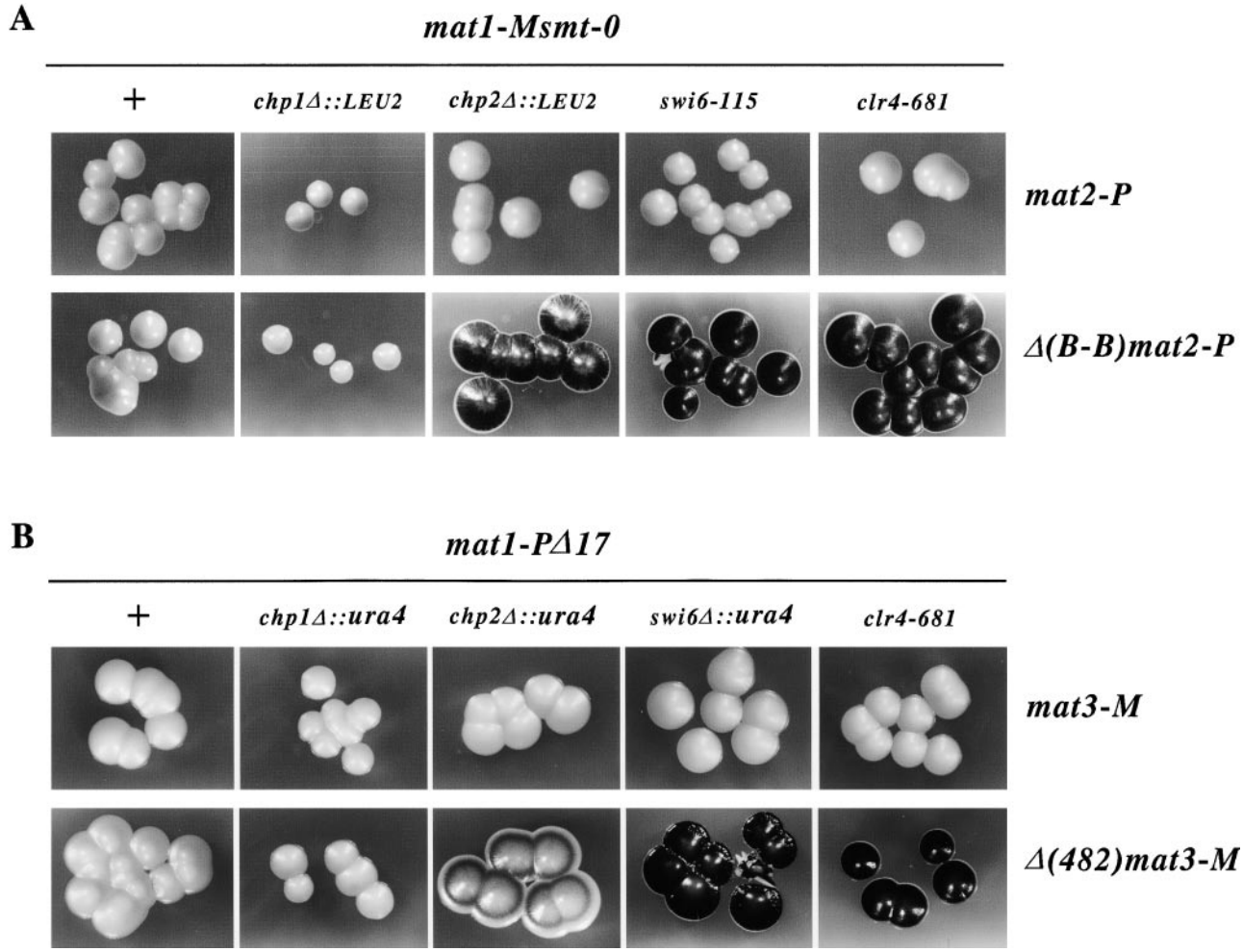


Figure 5.—Effects of Chp1, Chp2, Swi6, and Clr4 on the expression of *mat2-P* and *mat3-M*. (A) Effects on *mat2-P*. Sporulated colonies of stable *mat1-M* strains grown on MSA plates supplemented with leucine, uracil, and adenine were exposed to iodine vapors and photographed. Strains in the top row have intact *mat2-P cis*-acting elements. Strains in the bottom row have the 1.5-kb deletion depicted in Figure 4A. *mat2-P* strains: +, SP1124; *chp1*Δ, PG1605; *chp2*Δ, PG1790; *swi6-115*, SP1126; *clr4-681*, PG1032; Δ*(B-B)mat2-P* strains: +, SP1151; *chp1*Δ, PG1639; *chp2*Δ, PG1787; *swi6-115*; PG1640; *clr4-681*, SP1165. (B) Effects on *mat3-M*. Sporulated colonies of stable *mat1-P* strains were photographed as in A. Strains in the top row have intact *mat3-M cis*-acting elements. Strains in the bottom row have the 482-bp deletion depicted in Figure 4B. *mat3-M* strains: +, PG445; *chp1*Δ, PG1796; *chp2*Δ, PG1799; *swi6*Δ, PG1797; *clr4-681*, PG1594; Δ*(482)mat3-M* strains: +, PG1402; *chp1*Δ, PG1779; *chp2*Δ, PG1778; *swi6*Δ, PG1781; *clr4-681*, PG1419.

mat1 allele usually form colonies that do not contain spores. Under particular circumstances, such as when both mating types are coexpressed in single cells, haploid cells can sporulate without mating in a process referred to as haploid meiosis. Therefore, sporulation in unswitchable *mat1-M* cells can be used to monitor *mat2-P* expression and sporulation in unswitchable *mat1-P* cells can be used to monitor *mat3-M* expression. We examined various strains for the presence of haploid sporulation by microscopic examination (data not shown) and by exposing colonies to iodine vapors, which selectively stains spores black (Bresch *et al.* 1968; Figure 5). According to this assay, deletion of neither *chp1* nor *chp2* significantly derepressed the silent cassettes. Deletion of *chp2*, however, had a synergistic effect with deletion of either a *mat2-P* or a *mat3-M* centromere-proximal silencing element. A qualitatively similar effect was observed in *swi6* or *clr4* mutant strains (Thon *et al.* 1994, 1999; Figure 5), but not following deletion of *chp1* (Figure 5). Hence, Chp2, but not Chp1, appears to play a role similar to the other chromo-domain proteins Swi6 and Clr4 in silencing transcription of the mating-type region.

We constructed six *mat1-Msmt-0* strains containing all pairwise combinations of the *chp1*Δ*::LEU2*, *chp2*Δ*::LEU2*, *swi6-115*, and *clr4-681* alleles. These strains were used to monitor the levels of expression of *mat2-P*, as revealed by haploid meiosis after growth on limiting nitrogen. The strains were examined by iodine staining and microscopic examination. None of the combinations led to an easily observable derepression of *mat2-P* (data not shown). This is consistent with our previous conclusion

that Chp1 does not affect transcriptional silencing in the mating-type region and indicates that Chp2, Swi6, and Clr4 act in a single pathway.

Effects on mating-type switching: Switching to the opposite mating type is a regulated and extremely efficient process in homothallic strains with a *mat1 mat2-P mat3-M* mating-type region, also designated h^{90}. Swi6 and Clr4 are required for that process (Egel *et al.* 1984; Gutz and Schmidt 1985; Thon and Klar 1993; G. Thon and A. J. S. Klar, unpublished results; Ivanova *et al.* 1998). More specifically, Swi6 and Clr4 seem to be important for the choice of the silent cassette that converts *mat1* since mutations in *swi6* or *clr4* not only reduce switching in h^{90} cells, but increase switching in cells with an engineered *mat1 mat2-M mat3-P* mating-type region (h^{09} cells; Thon and Klar 1993). Iodine staining and microscopic examination of the type of asci produced within a colony allow for estimating the efficiency of mating-type switching. High switching rates lead to homogenous mixtures of P and M cells within colonies and hence to a high efficiency of zygotic ascus formation and to a dark iodine staining phenotype. On the other hand, low switching rates lead to a reduced frequency of zygotic asci and to speckled iodine staining phenotypes. Because of the resemblance between Chp2 and Swi6, we investigated whether deleting the *chp2* gene affected mating-type switching in h^{90} or h^{09} cells, in two different backgrounds known to affect switching, *swi6-mod*$^+$ and *swi6-mod*$^-$ (Thon and Klar 1993). We found that the iodine staining and sporulation phenotypes of wild-type and *chp2*-deleted strains were similar (data not shown), an indication that mating-type switching can occur efficiently in the absence of Chp2. Mating-type switching also occurs normally in the absence of Chp1 as judged by the iodine staining phenotype of *chp1*-deleted h^{90} colonies (Doe *et al.* 1998; data not shown).

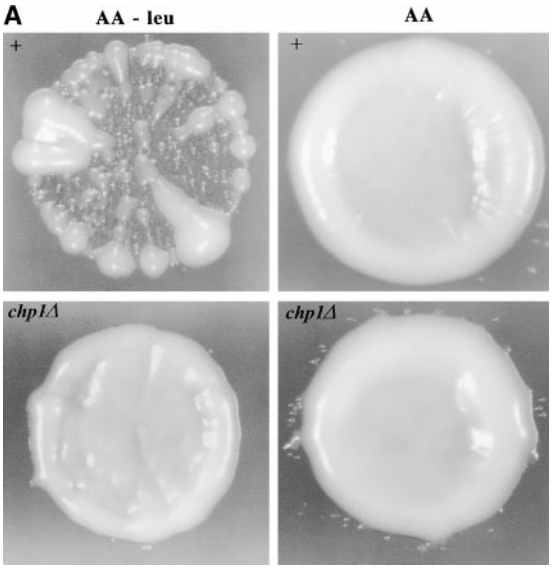
Effect of *swi6*, *clr4*, *chp1*, and *chp2* on the transcription of PolII genes in the rDNA repeats: Genes for the 25S, 18S, and 5.8S ribosomal RNAs are present in 70–100 copies in the genome of *S. pombe* (Hoheisel *et al.* 1993). Most of these genes are organized in two large clusters located at both ends of chromosome 3 and consisting in tandem repeats of a 10.4-kb unit (Schaak *et al.* 1982; Toda *et al.* 1984; Hoheisel *et al.* 1993). The *S. cerevisiae* *LEU2* gene and plasmid DNA were integrated in the rDNA by Toda *et al.* (1984) for the purpose of mapping the rRNA genes. We observed that the *LEU2* marker was expressed rather poorly in a strain with such an insertion, YIp2.4-1. When replicated from complete medium to medium lacking leucine, colonies with the *YIp2.4* insertion did not grow evenly (Figure 6A). Growth was limited to sectors of the colonies, giving rise to wheel-like patterns. This phenotype was suppressed by deleting *chp1*, *chp2*, *swi6*, or by a mutation in *clr4* (Figure 6A; data not shown).

We introduced the *S. pombe ura4*$^+$ gene near the *LEU2* gene of *YIp2.4* (Figure 6B). The integration was obtained in a *swi6-115* background and tested by Southern blots (data not shown). As expected, the integrated *ura4*$^+$ gene displayed tight linkage to *LEU2* in tetrad dissections (0 recombinants/45 viable spores examined). We compared its expression with the expression of *ura4*$^+$ integrated at a random site (Allshire *et al.* 1994; Figure 6, C and D) and with the expression of the truncated *ura4-DS/E* allele.

In a background with no known silencing defect, expression of *ura4*$^+$ from the rDNA supported only limited growth on medium lacking uracil (Figure 6C). Many cells could form colonies on FOA-containing plates (Figure 6C). We tested whether the FOA resistance was due to genetic alteration of the *ura4*$^+$ gene or to an epigenetic phenomenon by isolating FOA-resistant colonies from 24 independent cultures and determining whether they could give rise to Ura$^+$ colonies after growth on nonselective medium. Out of 24 independent FOA-resistant cultures, 17 displayed a phenotype undistinguishable from their parental Ura$^+$ strain upon replating on selective media (Figure 6C, row 1; data not shown).

We found that the four chromo-domain proteins Chp1, Chp2, Swi6, and Clr4 participated in the repression of *LEU2* and *ura4*$^+$ integrated in the rDNA (Figure 6). Altering the genes encoding these proteins by deletion or mutation improved growth on media lacking leucine or uracil (Figure 6C) and led to an accumulation of *ura4*$^+$ transcripts (Figure 6D). Chp2, Swi6, and Clr4 had the most obvious effects and Chp1 had a smaller but consistently reproducible effect. The *ura4*$^+$ transcript originating from the rDNA was of the same size as that

Figure 6.—Position effects in the rDNA repeats. (A) Expression of the *S. cerevisiae LEU2* gene in *chp1*$^+$ and *chp1*$^-$ cells assayed on replica. Colonies of *chp1*$^+$ (top; YIp2.4-1) or *chp1*-deleted (bottom; PG1572) cells with an integration of *LEU2* in the rDNA were obtained on complete AA medium and replicated onto medium lacking (AA-leu) or containing (AA) leucine as indicated. (B) Integration of *ura4*$^+$ in the rDNA. The *S. pombe ura4*$^+$ gene contained in the pUC8 vector was allowed to recombine by homology with the *YIp2-4* allele (Toda *et al.* 1984). The locations of *Bam*HI (Bam), *Bsr*FI (Bs), and *Hin*dIII (Hin) restriction sites are indicated. (C) Expression of *LEU2* and *ura4*$^+$ assayed by plating efficiency. Cells containing a *LEU2* and *ura4*$^+$ gene in the rDNA and the indicated mutations were spotted on selective plates as in Figure 2. 1, PG1734; 2, PG1792; 3, PG1794; 4, PG1661; 5, PG1736. (D) Northern blot analysis. RNA was prepared from the strains displayed in C and probed with an antisense *ura4* probe: 1–5, as in C; 6, FY489; 7, FY340. (E) Southern blot analysis. DNA was prepared from the cultures used in D, digested with *Hin*dIII, blotted, and probed as in D.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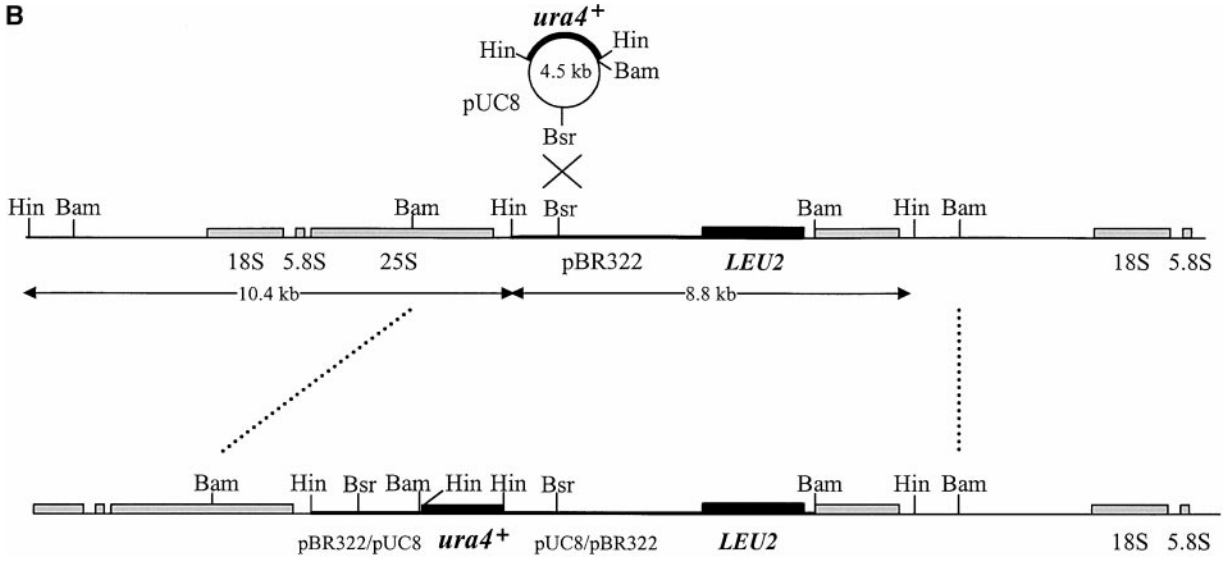
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Hin
Hin
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10.4 kb
8.8 kb
Bam
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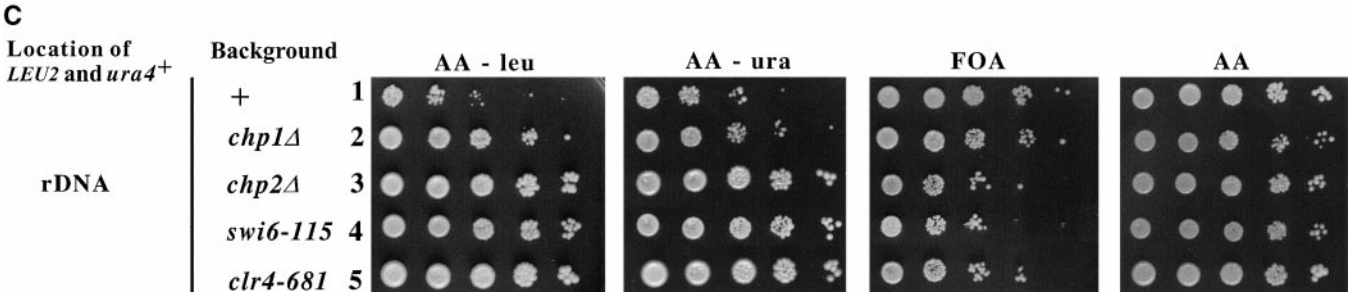
C
Location of LEU2 and ura4+
Background
rDNA
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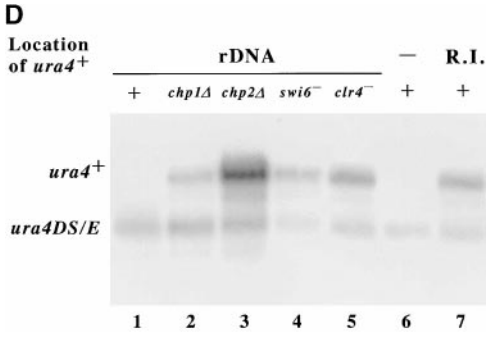
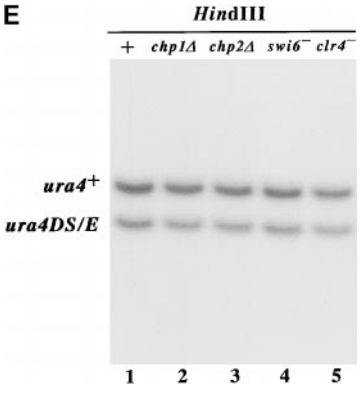
chp2Δ 3
swi6-115 4
clr4-681 5
AA - leu
AA - ura
FOA
AA


D
Location of ura4+
rDNA
—
R.I.
+ chp1Δ chp2Δ swi6− clr4−
+
+
ura4+
ura4DS/E
1 2 3 4 5 6 7


E
HindIII
+ chp1Δ chp2Δ swi6− clr4−
ura4+
ura4DS/E
1 2 3 4 5

from the wild-type *ura4*$^+$ gene, indicating that initiation and termination of transcription occurred normally. We determined by Southern blot analysis that the increased expression of *ura4*$^+$ was not caused by amplification of the *ura4*$^+$ gene (Figure 6E). We tested the stability of the Ura$^-$ phenotype in *chp1*Δ, *chp2*Δ, *swi6-115*, or *clr4-681* colonies formed on FOA. The 24 independent FOA-resistant derivatives tested for each *chp2*Δ, *swi6-115*, and *clr4-681* had a frequency of reversion to Ura$^+$ lower than 1 in 10^6 cells and 9 tested by Southern blot proved to have lost the *ura4*$^+$ gene. Among the 24 independent FOA-resistant isolates of *chp1*-deleted cells, 13 had a frequency of reversion to Ura4$^+$ lower than 1 in 10^6 and 11 could return to Ura$^+$ very efficiently. Hence, the FOA resistance in *chp1*-deleted cells could result either from mutations in or loss of the *ura4*$^+$ gene (in this case, loss was not tested by Southern blot), or from reversible silencing of the *ura4*$^+$ gene. This phenotype is similar to the phenotype of *chp*$^+$ cells and different from the phenotype of *chp2*Δ, *swi6-115*, or *clr4-681* cells, where reversible silencing of *ura4*$^+$ could not be observed. We conclude from this experiment that the epigenetic repression of *ura4*$^+$ in the rDNA is dependent to a small extent on Chp1 and more significantly on Chp2, Swi6, and Clr4.

DISCUSSION

We have examined the contribution of four chromo-domain proteins, Chp1, Chp2, Swi6, and Clr4, to position effects on transcription in the genome of fission yeast. We have determined that PolII-transcribed genes can be repressed when placed in the rDNA cluster located in the right arm of chromosome 3, and that the repression of transcription is dependent on the presence of these four chromo-domain proteins. We have also shown that Chp1 is crucial to centromeric silencing, but not to transcriptional silencing in the mating-type region or near telomeres. Conversely, the major effects of Chp2, in addition to those observed in the rDNA, were in the mating-type region and near telomeres. We will compare these phenotypes with those reported for mutations in *swi6* and *clr4* and discuss them in the light of the reported subcellular localization of these proteins.

Chp2 *vs.* Swi6: *chp2* and *swi6* encode the most closely related of the known *S. pombe* chromo-domain proteins. Both ORFs are of about the same size and both contain a chromo- and chromo-shadow domain, a structure similar to the HP1 protein of flies and mammals. We found that *chp2* and *swi6* had overlapping but distinguishable functions. Altering either gene alleviated centromeric and telomeric silencing (Allshire *et al.* 1995; this study). It also allowed transcription in the mating-type region and the derepression observed near *mat2-P* or *mat3-M* was in both cases increased by the simultaneous deletion of *cis*-acting elements located near the cassettes. In contrast to their effects on transcription, *swi6* and *chp2* affected mating-type switching differently. Swi6 is important for the choice of the silent cassette used to convert specific *mat1* alleles, and lack of Swi6 causes characteristic switching defects where switching is reduced in strains with a wild-type mating-type region but increased in cells with swapped silent cassettes (Egel *et al.* 1984; Gutz and Schmidt 1985; Thon and Klar 1993). *chp2*-deleted cells were able to form colonies with a sporulation phenotype similar to the wild type, indicating that, unlike Swi6, Chp2 is not absolutely required for efficient mating-type switching. One explanation for these different effects of Swi6 and Chp2 might reside in the degree to which each protein contributes to the structure of the mating-type region. Lack of Chp2 might not have as deleterious an effect as lack of Swi6. Indeed, the derepression of transcription caused by deleting *chp2* is not as marked as that caused by deleting *swi6* (Figures 4 and 5). Hence, the organization remaining in *chp2*-deleted cells might be sufficient to support efficient mating-type switching, albeit not sufficient for complete transcriptional silencing. Another possibility is that Swi6 interacts with a protein required for switching whereas Chp2 does not. Other differences between *swi6*- and *chp2*-deleted cells could be seen in the mitotic and meiotic viability of mutant strains. Cultures of *swi6* mutant cells contain higher proportions of dead cells than do wild-type cultures and produce an increased number of three-spored asci. *chp2*-deleted strains were as healthy as the wild type in these respects (data not shown). Consistent with these phenotypes, mitotic loss of a minichromosome was not increased by lack of Chp2 whereas it is in the absence of Swi6 (Table 2).

Effects on chromosome segregation—Epistasis analysis: Chromo-domain proteins are found in association with the pericentric heterochromatin of many eukaryotes. In addition to repressing transcription, they possibly participate in the formation of structures important for chromosome function, in particular the kinetochore. Consistent with such a function, the *S. pombe* chromo-domain proteins Swi6, Clr4, and Chp1 are required for efficient chromosome segregation (Allshire *et al.* 1995; Ekwall *et al.* 1996; Bernard *et al.* 1998; Doe *et al.* 1998). Swi6 is present at centromeres (Ekwall *et al.* 1995) and two proteins are known to be required for its localization, Rik1 and Clr4 (Ekwall *et al.* 1996). The mutant allele of *clr4* used in this study, *clr4-681*, did not increase the rate of chromosome loss to the same extent as the previously tested allele *clr4-S5* (Table 2; Allshire *et al.* 1995; Ekwall *et al.* 1996). The *clr4-681* allele contains a point mutation causing an amino-acid change in the Clr4 SET domain (G^{486}D mutation; Ivanova *et al.* 1998). It will be interesting to determine whether that allele or other *clr4* mutations affecting the SET domain of the protein influence the localization of Swi6 to the same extent as the *clr4-S5* allele. The *clr4-681* or *swi6-115* allele had a synergistic effect with a

deletion of *chp1* (Table 2). Hence, Swi6 and Clr4 seem to act in a pathway different from that in which Chp1 participates. The observed synergy also reveals that the *clr4-681* allele does reduce the efficiency of chromosome segregation although this effect could not be readily seen in a wild-type background. Consistent with our epistasis analysis, Chp1 localizes to one or a few discrete spots in the nucleus, which might coincide with centromeres, in a manner that does not depend on Rik1 or Clr4 (Doe *et al.* 1998), whereas the centromeric localization of Swi6 depends on Rik1 and Clr4. As expected, *swi6-115* was epistatic to *clr4-681* (Table 2). Deleting *chp2* increased chromosome loss only threefold compared with the wild type, a relatively modest effect (Table 2). Further experiments will be required to assess its role in chromosome segregation.

Chp1 target recognition: In contrast to the pleiotropic effects of Chp2, Chp1 displayed a specificity for centromeric regions. A DNA segment homologous to a centromeric repeat is found in the mating-type region between *mat2-P* and *mat3-M* (Grewal and Klar 1997). In that area, the sequence similarity with centromere 2 reaches 96% over 4.3 kb of DNA. Deletions in the mating-type region that include the 4.3-kb repeat lead to a partial derepression of transcription, where "on" and "off" states of expression are clonally inherited, and to defects in mating-type switching, which covariegate with the defects in transcription (Grewal and Klar 1996; Thon and Friis 1997). The *ura4*$^+$ gene is silenced when introduced within the repeat in the mating-type region (Grewal and Klar 1997) or within the homologous repeat near centromere 1 (Allshire *et al.* 1995). Whereas the Swi6 and Clr4 proteins are required for the repression at both locations (Allshire *et al.* 1995; Grewal and Klar 1997), we showed here that Chp1 was required for silencing centromeric insertions of *ura4*$^+$, but not for silencing insertions of *ura4*$^+$ in the mating-type region, nor the mating-type genes. The lack of effect of a *chp1* deletion on gene expression in the mating-type region was not simply explained by the redundancy of the silencing mechanism operating in that region, which occasionally masks silencing defects, since no cumulative effect was observed following combination of the *chp1* deletion with other silencing deficiencies (Figure 5; data not shown). The simplest interpretation of our results is that Chp1 does not act in the mating-type region, although we cannot rule out that it has a very localized action at locations that we have not tested. The restriction of its repressive effects to centromeres suggests that it is tethered there by a protein/DNA complex other than that associated with the 4.3-kb sequence. Chp1 could then exert an effect on the 4.3-kb repeat at a distance, possibly via other proteins such as Swi6 and Clr4, or it might spread along the DNA by oligomerization.

Loss of the *ura4*$^+$ gene from centromeric regions: Meiotic recombination is inefficient in *S. pombe* chromosomal regions close to centromeres (Nakaseko *et al.* 1986; Chikashige *et al.* 1989). This inhibition possibly reflects the existence of an intramolecular secondary structure that would compete with pairing between homologs, or could also be due to steric hindrance by the kinetochore. We discovered that a significant fraction of cells containing a centromeric *ura4*$^+$ insertion could lose the *ura4*$^+$ gene during mitotic growth. One likely mechanism leading to the loss of *ura4*$^+$ when placed in a centromeric repeat is an intramolecular gene conversion involving the untouched repeat placed symmetrically to that containing *ura4*$^+$. Such interactions have been proposed to be responsible for the very high degree of sequence conservation between centromeric repeats and for the presence of identical nucleotide changes in central repeats occupying symmetrical positions relative to centromere 1 in some strains (Takahashi *et al.* 1992). *ura4*$^+$ could also be lost from an insertion site located within the central core of chromosome 1. The central core has a unique sequence and is therefore not an expected target for mitotic gene conversion. We have not determined whether the central core was altered in cells having lost *ura4*$^+$ from the *cnt1* integration site.

Transcriptional silencing in the rDNA: Transcriptional silencing has in some cases a clear function of its own. For example, silencing of the *mat2-P* and *mat3-M* mating-type cassettes is crucial to the yeast sexual cycle and to yeast survival in the absence of a nitrogen source. In other cases, transcriptional silencing might be a consequence of other phenomena, or of structural or localization requirements. The role of transcriptional silencing in the rDNA is the subject of speculation.

Ty1 elements and prototrophic markers introduced in the rDNA of *S. cerevisiae* are poorly transcribed (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997). Their transcription is increased by disruption of the silencing gene *SIR2*, of the ubiquitin-conjugating enzyme gene *UBC2*, by reduced levels of histone H2A and H2B, as well as by mutations in a large number of genes including genes involved in DNA replication or regulation of chromatin structure (Bryk *et al.* 1997; Smith and Boeke 1997; Smith *et al.* 1999). Repression of PolII transcription in the rDNA repeats might be a by-product of PolI regulation (Smith and Boeke 1997), of the rDNA control of mitotic and meiotic recombination (Gottlieb and Esposito 1989 and references therein), and/or of life span regulation (Kennedy *et al.* 1997). It might reflect functions of the nucleolus distinct from the production of rRNA and linked to its constituting a discrete nuclear subcompartment. Such a function was proposed recently for the RENT complex, a nucleolar protein complex associated with Sir2, which sequesters Cdc14 in the nucleolus during most of the cell cycle and releases it at telophase (Shou *et al.* 1999; Straight *et al.* 1999).

The transcriptional repression we observed in the

rDNA of *S. pombe* is reminiscent of that in *S. cerevisiae*. At least one *S. pombe* ORF encodes a protein related to *S. cerevisiae* Sir2 (CAB38511 in cosmid SPBC16D10) and it will be interesting to investigate its participation in rDNA silencing. The four chromo-domain proteins whose roles were assayed here have no homologs in *S. cerevisiae*. *S. cerevisiae* has only two chromo-domain proteins: a member of the CHD family (Woodage *et al.* 1997; AC P32657) whose *S. pombe* closest relatives are Hrp1 (Jin *et al.* 1998) and an uncharacterized ORF (AC O14139) and Esa1 (Hilfiker *et al.* 1996; Clarke *et al.* 1999). We have found that *S. pombe* proteins other than the chromo-domain proteins described here are important for the transcriptional repression in the rDNA, including the histone deacetylase Clr3 (data not shown). As for *S. cerevisiae*, one can speculate that the repression of transcription is part of growth rate regulation, that *S. pombe* PolI has template preferences different from those of PolII, or that the nucleolus constitutes a compartment with special properties, as suggested by its ultrastructure (Leger-Sylvestre *et al.* 1997). A regulatory function by the dynamic sequestering of proteins involved in mitosis can also be envisioned for the *S. pombe* nucleolus. Clr4, a protein important for chromosome segregation and rDNA silencing, is localized to the nucleolus in interphase when expressed from a plasmid as a green fluorescent protein (GFP)-fusion protein. During mitosis, a fraction of the Clr4 pool relocalizes to the mitotic spindle (S26 in Sawin and Nurse 1996).

Direct or indirect effects—Localization: rDNA insertions of the *LEU2* and *ura4*$^+$ gene were transcribed more efficiently in *swi6-115* than in wild-type cells. The *swi6-115* mutation substitutes an arginine for the tryptophan at position 269, a residue conserved between chromo-shadow domains (Allshire 1996). The *swi6-115* mutation also results in undetectable levels of the Swi6 protein (Ekwall *et al.* 1996) and therefore probably constitutes a loss of function. Hence, our observation indicates that the wild-type Swi6 protein represses PolII transcription in the rDNA. The interpretation of that repression is not straightforward because Swi6 is not localized within the nucleolus of wild-type cells (Ekwall *et al.* 1995, 1996). One possible explanation is that Swi6 recognizes and silences PolII-transcribed genes when placed in the context of the rDNA. In this model, localization of Swi6 to the nucleolus would not precede introduction of the marker gene and would therefore not have been observed in past experiments. Alternatively, the influence of the *swi6-115* mutation on transcription in the rDNA could be indirect. In the absence of Swi6 a factor normally present in the rDNA and able to repress PolII transcription might not be synthesized, or might be titrated away to sites normally occupied by Swi6, such as the centromeres. Titration effects could account for other observed phenotypes. As mentioned above, a GFP-Clr4 fusion protein localizes to the nucleolus (Sawin and Nurse 1996) and in *clr4*$^-$ cells, Swi6 does not associate with centromeres, but is found in the nucleolus (Ekwall *et al.* 1996). One could then conceive that nucleolar sites normally occupied by Clr4 can be recognized by Swi6, which in the absence of Clr4 is titrated away from the centromeres to the nucleolus. In wild-type cells, Clr4 would repress transcripion of PolII genes in the rDNA. In *clr4* mutant cells, Swi6 would mislocalize to sites normally occupied by Clr4, but would fail to exert the same repression as Clr4. In *swi6* mutant cells, Clr4 would be titrated to sites normally occupied by Swi6 and would fail to repress transcription in the rDNA. Other indirect effects due to alterations in *clr4* are also possible since *clr4* controls the transcription of several genes, both positively and negatively (Ivanova *et al.* 1998). In another type of model, one could imagine that changes in centromere or telomere structure sterically affect the nucleolus, possibly facilitating entrance of PolII. Additional localization studies and overexpression experiments ought to help distinguish between some of these models.

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