Histone Deacetylase Homologs Regulate Epigenetic Inheritance of Transcriptional Silencing and Chromosome Segregation in Fission Yeast

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ABSTRACT

Position-effect control at the silent *mat2-mat3* interval and at centromeres and telomeres in fission yeast is suggested to be mediated through the assembly of heterochromatin-like structures. Therefore, *trans*-acting genes that affect silencing may encode either chromatin proteins, factors that modify them, or factors that affect chromatin assembly. Here, we report the identification of an essential gene, *clr6* (*c*ryptic *l*oci *r*egulator), which encodes a putative histone deacetylase that when mutated affects epigenetically maintained repression at the *mat2-mat3* region and at centromeres and reduces the fidelity of chromosome segregation. Furthermore, we show that the Clr3 protein, when mutated, alleviates recombination block at *mat* region as well as silencing at donor loci and at centromeres and telomeres, also shares strong homology to known histone deacetylases. Genetic analyses indicate that silencing might be regulated by at least two overlapping histone deacetylase activities. We also found that transient inhibition of histone deacetylase activity by trichostatin A results in the increased missegregation of chromosomes in subsequent generations and, remarkably, alters the imprint at the *mat* locus, causing the heritable conversion of the repressed epigenetic state to the expressed state. This work supports the model that the level of histone deacetylation has a role in the assembly of repressive heterochromatin and provides insight into the mechanism of epigenetic inheritance.

PROPAGATION of stable states of gene expression is thought to be critical for the development and maintenance of differentiated cell types. Examples in higher eukaryotes include *X*-chromosome inactivation in female mammals (reviewed by Riggs and Porter 1996), parental imprinting (reviewed by Barlow 1995; Ainscough and Surani 1996), and stable expression of homeotic genes in Drosophila (Paro 1993). The underlying mechanisms by which differentiated cells establish and maintain stable patterns of gene expression are not clear. Studies of position-effect variegation (PEV), a phenomenon in which heterochromatin variably but stably silences nearby genes, have provided a model for understanding the propagation of committed states of gene expression. It was recently suggested that polycomb-mediated stable inactivation of homeotic genes and PEV in Drosophila are related phenomena (Orlando and Paro 1995), supporting the idea that position effects are mediated through long-range control exerted by repressive chromatin structures.

Silenced genomic regions in the distantly related yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, such as silent donor mating-type loci (reviewed by Klar 1989), telomeres (Gottschling *et al.* 1990; Nimmo *et al.* 1994), and centromeres (reviewed by Allshire 1996), share many parallels with heterochromatic regions in higher eukaryotes. In fission yeast, the *mat2* and *mat3* loci, which serve as donors for *mat1* alleles during mating-type interconversion, are silenced even though they contain genetic information identical to that of the expressed *mat1-P* or *mat1-M* alleles, respectively (reviewed by Klar *et al.* 1998). Furthermore, it was recently observed that an ~11-kb recombinationally "cold" region between the donor loci, referred to as the *K* region, is also subject to position effects; *i.e.*, expression of the *ura4*$^+$ marker gene inserted in this region is repressed severely (Thon *et al.* 1994; Grewal and Klar 1997). Mutations in six *trans*-acting genes [*clr1*, *clr2*, *clr3*, *clr4* (*c*ryptic *l*oci *r*egulator), *swi6*, and *rik1*] partially derepress the donor loci and alleviate the repression of *ura4*$^+$ at different locations within the *mat2-mat3* interval (Egel *et al.* 1989; Lorentz *et al.* 1992; Thon and Klar 1992; Ekwall and Ruusala 1994; Thon *et al.* 1994; Grewal and Klar 1996, 1997), suggesting that these gene functions affect this region in a global fashion. These genes are also shown to prohibit meiotic and mitotic recombination in the entire *mat2-mat3* region (Egel *et al.* 1989; Klar and Bonaduce 1991; Lorentz *et al.* 1992; Thon and Klar 1992; Thon *et al.* 1994).

All six *trans*-acting genes, originally identified for their role in silencing at the mating-type region, were subsequently shown to affect position-effect control of expression of a marker gene integrated at centromere 1 (*cen1*; Allshire *et al.* 1995), indicating their involvement in

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the functional organization of centromeres. A mechanistic link between centromeric and mating-type region silencing is further suggested by our recent findings that approximately one-third of the *K* region is homologous to the centromeric repeat sequences (Grewal and Klar 1997). Furthermore, analogous to the existence of an epigenetic component in centromere function (Steiner and Clarke 1994; Ekwall *et al.* 1997), silencing, mating-type interconversion, and recombinational suppression at the mating-type region are also regulated by an epigenetic mechanism (Grewal and Klar 1996). Many studies have suggested that centromeres and the mating-type locus are assembled into a higher-order chromatin structure refractory to transcription and to recombination machinery, yet recombination required for *mat1* switching is allowed. Similarly, the *clr1-clr4*, *swi6*, and *rik1* gene products are believed to facilitate the assembly of a heterochromatin-like structure, which, once assembled, is capable of self-templating (Grewal and Klar 1996). Consistent with the chromatin model, Swi6 (Lorentz *et al.* 1994) and Clr4 (Ivanova *et al.* 1998) proteins share homology with the chromodomain motif found in heterochromatin-associated proteins in higher eukaryotes (reviewed by Singh 1994), and Swi6 colocalizes with centromeres, telomeres, and the mating-type region (Ekwall *et al.* 1995). The exact mechanism of the assembly of heterochromatin-like structures is not known. However, genetic and biochemical evidence from other systems suggests that the acetylation status of conserved lysine residues in the amino-terminal tails of histones H3 and H4 plays a role in chromatin assembly and transcription regulation (see Braunstein *et al.* 1993; Brownell and Allis 1996; Wolffe 1996; Hartzog and Winston 1997; Pazin and Kadonaga 1997).

Independent of any perceived molecular model of repression, we have used a genetic approach to identify *trans*-acting genes essential for silencing. A new locus, *clr6*, was identified that encodes a putative histone deacetylase. We have investigated the effects of *clr6-1* mutation on silencing at the mating-type region and at centromeres and telomeres. We also examined the effects of general inhibition of histone deacetylation on epigenetic inheritance and demonstrated that transient inhibition by the drug trichostatin A (TSA) alters the imprint at the *mat2-mat3* region. We also describe the molecular characterization of the previously identified *clr3* gene and discuss the implications of our findings for current models of structural organization at the mating-type region and at centromeres in *S. pombe*, as well as for the mechanism of epigenetic inheritance in general.

MATERIALS AND METHODS

Strains and culture conditions: Most of the *S. pombe* strains used in this study were constructed in our laboratory (Table 1). Strains FY336, FY498, FY520, and FY648 have been described by Allshire *et al.* (1995). The construction of HM248 and PRZ119 was described by Niwa *et al.* (1986) and Reynolds *et al.* (1990), respectively. The *KΔ::ura4*$^+$ allele was constructed as part of an earlier study (Grewal and Klar 1996). We used standard genetic crosses to construct all other strains.

Standard conditions were used for growth, sporulation, random-spore analysis, tetrad analysis, gap repair, transformations, and construction of diploids from haploid cells (Moreno *et al.* 1991). YEA and PMA (supplemented with appropriate amino acids) were used as rich and sporulation media, respectively. Synthetic medium lacking an appropriate nutrient such as uracil (AA-URA) was used in most of the experiments. FOA medium contains 5-fluoroorotic acid (0.8 g/liter) in synthetic complete medium. Ura$^+$ and Ura$^-$ derivatives of *KΔ::ura4*$^+$ strains were obtained by selecting for their growth on AA-URA or FOA medium, respectively. Quantitation of *ura4*$^+$ expression was carried out by growing cells on synthetic Edinburgh minimal medium containing glutamate, adenine, leucine, and uracil, each at 75 mg/liter (N/S), URA$^-$ (N/S lacking uracil), and FOA (N/S supplemented with FOA). The temperature and ultraviolet radiation (UV) sensitivities were determined as described in Figure 1.

Iodine-staining assay: We used the iodine-staining assay to estimate the efficiency of mating-type switching and the level of derepression of donor loci. Individual colonies grown on sporulation medium for 3 days were exposed to iodine vapors. Synthesis of a starch-like compound by sporulating cells results in black staining of colonies after exposure to iodine vapors (Bresch *et al.* 1968). In switching-competent cells, the intensity of iodine staining indicates the efficiency of mating-type switching; in a nonswitching background, it indicates the level of "haploid meiosis," a phenotype resulting from concurrent expression of both *P* and *M* information in a haploid cell (Thon and Klar 1992).

Isolation of the *clr6-1* mutation strain: SP1167 containing the nonswitchable *mat1-Msmto* (Engelke *et al.* 1987) allele and the mutant *clr1-5* allele displays only partial derepression of the *mat2-P* locus (Thon *et al.* 1994). Therefore, when grown on sporulation (PMA$^+$) medium, SP1167 colonies showed weak haploid meiosis and, hence, stained very lightly with iodine vapors. To identify new mutants, SP1167 was mutagenized with ethylmethanesulfonate to 50% viability by following the procedure of Moreno *et al.* (1991). The mutagenized cells were plated onto PMA$^+$ medium and allowed to grow at 25° for 6 days. Several mutants showing dark iodine staining and high levels of haploid meiosis were isolated. One mutant, *clr6-1*, conferred a growth defect at 37° and was investigated further.

Cloning and sequencing: To clone the *clr6* gene and the previously identified *clr3* (Thon *et al.* 1994) gene, we transformed SP1359 and SP1195, respectively, with the *S. pombe* partial *Sau*3A genomic library cloned in the pWH5 vector (Wright *et al.* 1986). The vector contains the *S. cerevisiae LEU2* gene, which upon transformation, can complement the *leu1-32* defect in *S. pombe.* The screening of Leu$^+$ transformants identified plasmids containing 8.0-kb (pAK86) and 5.0-kb (pClr6) inserts that were able to complement the recessive *clr3-735* and *clr6-1* alleles, respectively. Upon retransformation, both plasmids complemented mutant phenotypes of the respective strains. To confirm that cloned DNA contained wild-type copies of *clr3* and *clr6* rather than extragenic suppressors, the plasmids were integrated by homologous recombination and analyzed for linkage. The plasmid-borne *LEU2* marker showed tight linkage to the respective loci because only the parental ditype segregation pattern was found in all the tetrads we analyzed (69 tetrads for *clr3* and 44 tetrads for *clr6* locus).

The nucleotide sequence of the subcloned genomic fragments was determined by using the Prism Ready Reaction Dye

TABLE 1

***S. pombe* strains**

Strain	*ura4*$^+$ insertion	*mat1*	Auxotrophic markers/Ch16	*clr* loci
SP412		*mat1-M mat2,3Δ*	*leu1-32*	
SP837		h^{90}	*leu1-32 ura4-D18 ade6-216*	
SP1001		*mat1-Msmt-o*	*his2 ade6-216*	
SP1005		*mat1-PΔ17::LEU2*	*leu1-32 ura4 ade6-216*	
SP1167	*mat2-Pint::ura4*$^+$	*mat1-Msmt-o*	*ura4-D18 ade6-216*	*clr1-5*
SP1173		*mat1-Msmt-o*	*leu1-32 ura4-D18 his2 ade6-216*	
SP1195	*mat2-Pint::ura4*$^+$	*mat1-Msmt-o*	*ura4-D18 ade6-216*	*clr1-5 clr6-1*
SP1240	*mat2-Pint::ura4*$^+$	*mat1-Msmt-o*	*leu1-32 ura4-D18 ade6-216*	*clr6-1*
SP1242	*mat2-Pint::ura4*	h^+	*leu1-32 ura4-D18 ade6-210*	*clr6-1*
SP1249	*mat2-Pint::ura4*$^+$	*mat1-PΔ17::LEU2*	*leu1-32 ura4-D18 ade6-216*	*clr1-5 clr6-1*
SP1251	*mat2-Pint::ura4*$^+$	*mat1-Msmt-o*	*leu1-32 ura4-D18 ade6-210*	*clr1-5 clr6-1*
SP1286	*mat2-Pint::ura4*$^+$	*mat1-Msmt-o*	*ura4-D18 ade6-210*	*clr3-735 clr6-1*
SP1302	*mat3-Mint::ura4*$^+$	h^{90}	*leu1-32 ura4-D18 ade6-210*	
SP1306	*mat2-Pint::ura4*$^+$	*mat1-Msmt-o*	*leu1-32 ura4 ade6-210*	*clr1-5 clr6-1::LEU2::clr6*$^+$
SP1359	*mat3-Mint::ura4*$^+$	h^{90}	*leu1-32 ura4-D18 ade6-210*	*clr3-735*
SP1464	*KΔ::ura4*$^+$	h^{90}	*ura4-D18 ade6-210*	*clr6-1*
SPG27	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4 ade6-210 his2*	
SPG32	*KΔ::ura4*$^+$	*mat1-Msmt-o*	*leu1-32 ura4 ade6-210*	
SPG51	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4 ade6-216*	
SPG60	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4 his2 ade6-216*	*clr1-5*
SPG62	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4 his2 ade6-210*	*clr3-735*
SPG133	*mat2-Pint::ura4*$^+$	*mat1-PΔ17::LEU2*	*ura4 ade6-216*	*clr6-1*
SPG134		*mat1-PΔ17::LEU2*	*ura4 ade6-210* (Ch16 *ade6-M216*)	*clr1-5*
SPG135	*mat2-Pint::ura4*$^+$	*mat1-PΔ17::LEU2*	*leu1-32 ura4 ade6-210* (Ch16 *ade6-216*)	*clr6-1*
SPG136	*mat2-Pint::ura4*$^+$	*mat1-Msmt-o*	*ura4 ade6-210* (Ch16 *ade6-216*)	*clr1-5 clr6-1*
SPG137	*KΔ::ura4*$^+$	h^{90}	*ura4 his2 ade6-210*	*clr1-5 clr6-1*
SPG138	*cnt1*/TM1(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-216*	*clr6-1*
SPG139	*cnt1*/TM1(*Nco*I)::*ura4*$^+$	h^{90}	*leu1-32 ura4 his2 ade6-216*	*clrl-5*
SPG140	*cnt1*/TM1(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-216*	*clr1-5 clr6-1*
SPG141	*imr1*R(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-210*	*clr6-1*
SPG142	*imr1*R(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-216*	*clr1-5*
SPG143	*imr1*R(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-210*	*clr1-5 clr6-1*
SPG144	*otr1*R(*Sph*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-216*	*clr6-1*
SPG145	*otr1*R(*Sph*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-210*	*clr1-5*
SPG146	*otr1*R(*Sph*I)::*ura4*$^+$	h^{90}	*leu1-32 ura4 his2 ade6-210*	*clr1-5 clr6-1*
SPG147	Ch16m23::*ura4*$^+$-TEL	h^+	*leu1-32 ura4 ade6-210* (Ch16 *ade6-216*)	*clr6-1*
SPG148	Ch16m23::*ura4*$^+$-TEL	h^+	*leu1-32 ura4 ade6-210* (Ch16 *ade6-216*)	*clr1-5*
SPG149	Ch16m23::*ura4*$^+$-TEL	h^{90}	*leu1-32 ura4 his2 ade6-210* (Ch16 *ade6-216*)	*clr1-5 clr6-1*
SPG155	*mat2-Pint::ura4*$^+$	*mat1-PΔ17::LEU2*	*leu1-32 ura4 ade6-210* (Ch16 *ade6-216*)	*clr3-735*
SPG156		*mat1-M mat2,3Δ*	*leu1-32 ura4 ade6-210* (Ch16 *ade6-216*)	*clr3-735 clr6-1*
SPG157	*KΔ::ura4*$^+$	h^{90}	*ura4 his2 ade6-210*	*clr3-735 clr6-1*
SPG158	*imr1*R(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-210*	*clr3-735*
SPG159	*imr1*R(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-216*	*clr3-735 clr6-1*
SPG160	*otr1*R(*Sph*I)::*ura4*$^+$	h^+	*ura4 ade6-216*	*clr3-735*
SPG161	*otr1*R(*Sph*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-210*	*clr3-735 clr6-1*
SPG162	*cnt1*/TM1(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4 ade6-216*	*clr3-735*
SPG163	*cnt*/TM1(*Nco*I)::*ura4*$^+$	*mat1-M mat2,3Δ*	*leu1-32 ura4 ade6-210*	*clr3-735 clr6-1*
PG383		*mat1-PΔ17::LEU2*	*leu1-32 ura4-D18 ade6-210*	*clr1-5*
FY336	*cnt1*/TM1(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4-DS/E ade6-210*	
FY498	*imr1*R(*Nco*I)::*ura4*$^+$	h^+	*leu1-32 ura4-DS/E ade6-210*	
FY520	Ch16m23::*ura4*$^+$-TEL	h^+	*leu1-32 ura4-DS/E ade6-210* (Ch16 *ade6-216*)	
FY648	*otr1*R(*Sph*I)::*ura4*$^+$	h^+	*leu1-32 ura4-DS/E ade6-210*	
HM248		h^-	*his2 ade6-210* (Ch16 *ade6-216*)	
PRZ119		h^+	*leu1-32 ura4-D18 lys1-131 ade6-210*	*rhp6-1Δ*

Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Samples were applied to the Applied Biosystems model 373A sequencing system. Data were analyzed by using GCG software (University of Wisconsin). To determine the location of introns, cDNA was amplified from the *S. pombe* cDNA library and sequenced. The nucleotide sequences of the *clr6* and *clr3* genes have been deposited in the GenBank database (accession numbers AFO64206 and AFO64207, respectively).

Construction of the *clr6* disruption allele: A 0.9-kb fragment containing part of the *clr6* gene was subcloned into pBS/KS (Stratagene, La Jolla, CA). The *ura4+* gene in a 1.8-kb *Hin*dIII fragment was then inserted into the *Hin*dIII site to construct pBS/*clr6::ura4*$^+$. A 2.7-kb *Xho*I-*Pst*I insert containing the disrupted *clr6* open reading frame (ORF) was used to transform the *clr6*$^+$/*clr6*$^+$ *ura4*/*ura4* diploid strain to uracil prototrophy. Transformants with the correct integration were identified by Southern analysis.

DNA and RNA analyses: DNA from *S. pombe* cultures grown overnight was prepared as described by Moreno *et al.* (1991), and RNA was prepared as described by Schmitt *et al.* (1990). Southern and Northern hybridizations were carried out as described by Sambrook *et al.* (1989). The probes used in this study were as follows: a 1.8-kb *Hin*dIII fragment containing *ura4*$^+$, a 0.9-kb fragment containing part of the *clr6* gene, a 1.0-kb *Bcl*I-*Taq*I DNA fragment carrying part of the *M* cassette, and a *Nde*I-*Pst*I fragment containing *cdc2*.

Fluctuation test: The fluctuation test was carried out as described by Luria and Delbruck (1943). The Ura$^-$ and Ura$^+$ derivatives of SPG27 and SP1464 were diluted to a concentration of about six cells per milliliter in YEA nonselective liquid medium. Aliquots (100-μl) of cultures were dispensed into microtiter plates and allowed to grow at 30° for 24 hr before plating onto YEA plates. After 3 days at 30°, the colonies were replicated onto PMA$^+$ sporulation and AA-URA media. Iodine staining and *KΔ::ura4*$^+$-expression phenotypes were scored after 3 days, and the rate of appearance of the variant (μ) was calculated by using the formula $\mu = -[\ln(B/A)]/N$, where N is the average number of colony-forming units per culture, B is the number of cultures without variation, and A is the total number of cultures in the experiment.

Chromosome segregation assay: The chromosome loss experiment was carried out as described by Allshire *et al.* (1995). Cells from Ade$^+$ colonies were plated onto adenine-limited medium (YE) and incubated at 30° for 4 days. If chromosome loss occurs in the first division of a cell plated on YE, half of the resultant colony carrying Ch16 will be white, whereas the other half without Ch16 will be red. The number of half-sectored red/white colonies was determined, and the rate of chromosome loss per cell division was calculated by dividing the number of half-sectored colonies by the total number of white colonies plus half-sectored colonies. For TSA treatment, 2 μl of TSA [10 μg/ml dissolved in dimethylsulfoxide (DMSO)] was spotted onto filter paper discs (1-cm diameter) laid over a lawn of cells (HM248) spread on a plate. The plates were incubated at 33° for different time intervals, and cells growing underneath the filters were plated onto YE medium.

RESULTS

Isolation and characterization of the *clr6-1* mutation: Our past studies have indicated that silencing of the donor mating-type loci is regulated by redundant mechanisms and that deletion of a 1.5-kb *cis*-acting *mat2-P*-proximal region potentiates the derepressing effect of mutations in all the known *trans*-acting (*clrl-clr4* and *swi6*) genes (Thon *et al.* 1994). Therefore, it is likely that there exist additional *trans*-acting factors that are involved in silencing. To identify the hypothesized additional factors, we mutagenized a nonswitchable *mat1-Msmto* strain carrying the mutant *clr1-5* allele (SP1167) and sought mutants exhibiting strong derepression of the *mat2-P* locus. We obtained several *trans*-acting mutations that increased haploid meiosis, a phenotype that occurs in haploid cells expressing both *P* and *M* information (Thon and Klar 1992) and causes increased iodine staining of colonies formed by nonswitching strains. Here we present the analysis of one of these mutations, *clr6-1*, which, according to our linkage analysis, was distinct from previously identified *trans*-acting mutations and caused increased haploid meiosis (Figure 1A). Analysis of the *clr6-1* mutant cells revealed that the mutation caused a temperature-sensitive (Ts$^-$) growth defect at 37° (Figure 1B). Interestingly, the *clr6-1* mutant cells did not show the Ts$^-$ growth defect in the first five to six cell divisions after shifting to the restrictive temperature of 37°. In addition to the Ts$^-$ phenotype, we also found that the mutant cells displayed increased UV sensitivity (Figure 1C).

The observed increase in haploid meiosis could be caused by a defect in the *pat1* or *mei2* genes, which control meiosis and whose mutants confer this phenotype, even in the absence of donor loci (reviewed by Egel 1994). Alternatively, the increase in haploid meiosis could result from derepression of donor loci. We have differentiated between these possibilities. First, genetic analysis showed that the haploid meiosis phenotype was dependent on the *mat2-P* and *mat3-M* donor loci (data not shown). Second, Northern analysis (Figure 2) revealed an increase in the *mat3* transcript level in the *clr1-5 clr6-1* double mutant as compared with *clr1-5* alone, indicating that the *clr6* gene regulates silencing of *mat3*. Furthermore, the *clr6-1* mutation, originally isolated for its effect on *mat2-P*, also derepresses *mat3-M* in a *clr1* background. To test whether the *clr6-1* mutation had a cumulative effect with *clr1-5* or was epistatic to *clr1* function, we constructed a *clr1*$^+$ *clr6-1* strain. As expected for a mutation that affects only part of the redundant or overlapping silencing mechanisms, the *clr6-1* allele by itself had no visible effect on the silencing of *mat2* and *mat3* (Figures 1A and 2). We also analyzed the cumulative effect of the *clr6-1* allele with mutations in *clr2*, *clr3*, *clr4*, and *swi6*, which are suggested to work in a single pathway in conjunction with *clr1*, as pairwise combinations do not show cumulative effect (Thon *et al.* 1994). Surprisingly, the *clr6-1* allele caused increased haploid meiosis only in the *clr3* mutant background (SP1286; data not shown). Furthermore, the *clr6-1* mutation did not show a cumulative effect when combined with *mat2-P*-proximal deletion. We explain these findings by suggesting that Clr6 acts in an overlapping manner with Clr1/Clr3 and that

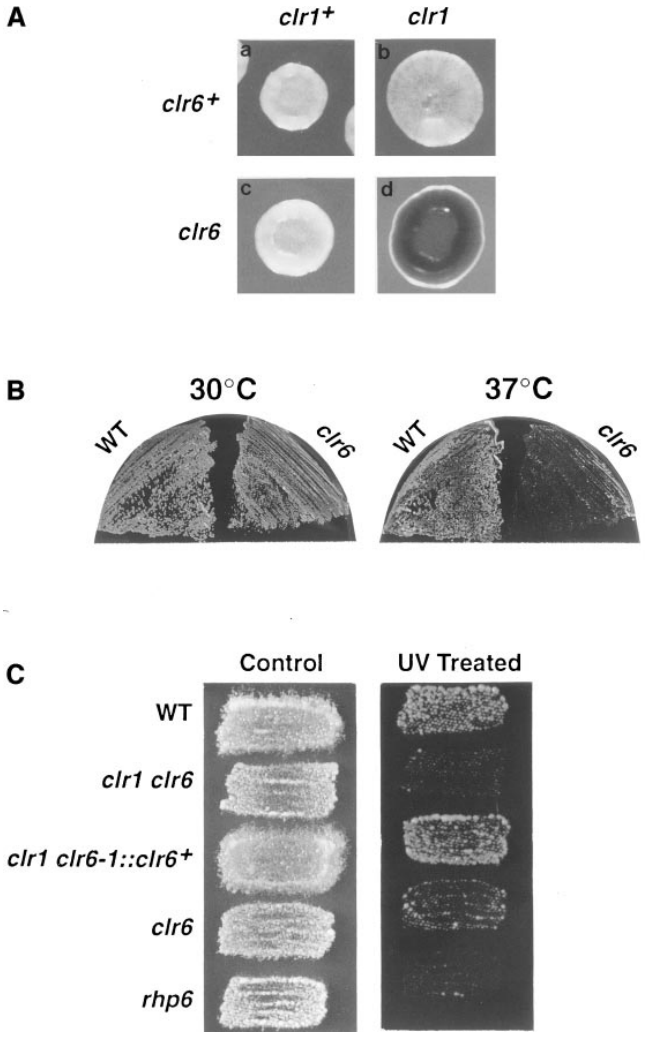


Figure 1.—The *clr6-1* mutant phenotypes. (A) Cumulative effect of *clr6-1* and *clr1-5* mutations on *mat2-P* derepression. The colonies of nonswitchable *M* cells (*mat1-Msmto*) were sporulated and exposed to iodine vapors before photography. Because *mat1-Msmto* strains are prohibited from switching, their intensity of staining indicates the level of haploid meiosis within the colonies, qualitatively reflecting the level of *mat2-P* derepression. In cells of this genotype, derepression of *mat3-M* cannot be determined by the staining assay. The strains used and their relevant genotypes were as follows: SP1173, WT; SP1167, *clr1-5*; SP1240, *clr6-1*; and SP1195, *clr1-5 clr6-1*. (B) Temperature sensitivity of *clr6-1*. The strains previously grown under permissive growth conditions (30°) were replicated onto YEA plates and incubated overnight at 30° or 37°. *clr6-1* displayed a gradual Ts^- growth defect. The cells grown overnight were further replicated onto YEA and incubated at the respective temperatures for 2 days before photography. The strains used were SPG27, WT and SP1464, *clr6-1*. (C) UV sensitivity. Patches of the indicated strains were replicated onto YEA plates in duplicate, and one of the plates was UV irradiated (200 $\mu J/cm^2$ for 2 min). Plates were incubated in the dark at 30° for 4 days before photography. As a control, cultures grown without UV treatment are shown. The strains used were as follows: SP1302, WT; SP1251, *clr1-5 clr6-1*; SP1306, *clr1-5 clr6-1::LEU2::clr6*$^+$ (a strain that is isogenic with SP1251 but carries *clr6*$^+$ integrated next to the *clr6-1* allele); SP1242, *clr6-1*; and PRZ119, *rhp6-1*Δ (a known UV-sensitive control strain).

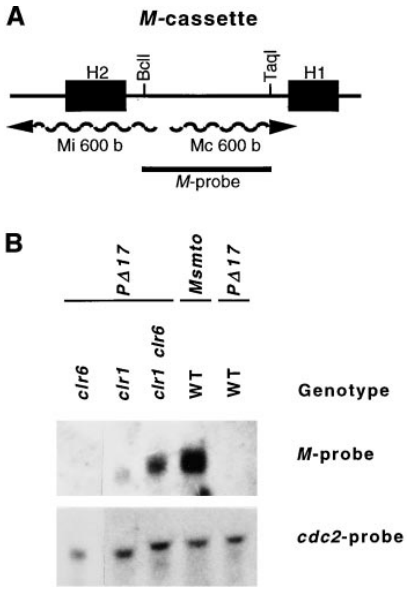
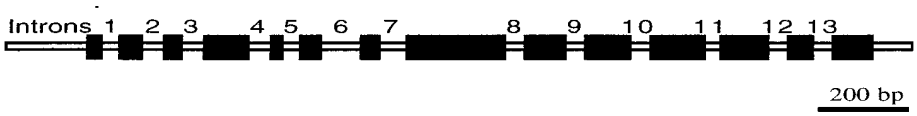


Figure 2.—Northern analysis showing derepression of *mat3-M* in a mutant background. (A) Diagrammatic representation of the *M*-cassette transcripts. The direction of transcription and size of *Mc* (constitutive) and *Mi* (inducible) transcripts are indicated. *M*-probe indicates the DNA fragment used as a probe in the Northern blot experiment shown in B. (B) Northern blot analysis of RNA from nitrogen-starved, nonswitching cells containing *mat2-P* and *mat3-M*. To directly monitor derepression of *mat3-M*, nonswitchable *mat1-P*Δ*17* (Arcongioli and Klar 1991) allele-containing strains were used. The WT (wild-type) *mat1-Msmto* (SP1001), a positive control for identifying *M* transcripts originating from the expressed *mat1* locus, and *P*Δ*17* (SP1005) strains were used as controls of the *clr*$^+$ genotype. The other strains used were as follows: SPG133, *clr6-1*; PG383, *clr1-5*; and SP1249, *clr1-5 clr6-1*. The *cdc2* mRNA was included as an internal loading control.

both these functions feed into a silencing pathway involving *clr2*, *clr4*, and *swi6* products.

Cloning and sequencing of *clr6* reveals a similarity to histone deacetylases: Because the *clr6-1* mutant is temperature sensitive, it is likely that the lethality is caused by pleiotropic effects on diverse cellular functions. An important clue to *clr6* function was provided by its nucleotide sequence. For this purpose, we isolated a clone from a partial *Sau*3A genomic library of *S. pombe* by complementing Ts^- growth and the haploid meiosis defects in the *mat1-Msmto clr1-5 clr6-1* (SP1195) mutant strain. A 5.0-kb insert complemented both defects. Sequencing of the genomic *clr6* and its cDNA clones identified an ORF that is interrupted by 13 introns. (Figure 3A). The ORF is capable of encoding a protein of 405 amino acids (Figure 3B). Comparison of the predicted Clr6 amino acid sequence with the GenBank database revealed that Clr6 shares strong homology to members of a recently described family of histone deacetylases, such as human HDAC1 (formerly HD1; Taunton *et al.* 1996; 61% identity and 78% similarity) and *S. cerevisiae* Rpd3 (Vidal and Gaber 1991; 63% identity and 77%

A



B

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      Clr6 .............MGFGKKKVSYFYDEDVGNYHYGPQHPMKPHRVRMVHNLVVNYNLYEKLNVITPVRATRNDMTRCHTDEYIEFLWRVTPDTMEKFQPH 87
      Rpd3 MVYEATPFDPITVKPSDKRRVAYFYDADVGNYAYGAGHPMKPHRIRMAHSLIMNYGLYKKMEIYRAKPATKQEMCQFHTDEYIDFLSRVTPDNLEMFKRE
     HDAC1 ..........MAQTQGTRRKVCYYYDGDVGNYYYGQGHPMKPHRIRMTHNLLLNYGLYRKMEIYRPHKANAEEMTKYHSDDYIKFLRSIRPDNMSEYSKQ
 Consensus                        V Y YD DVGNY YG  HPMKPHR RM H L  NY LY K        A    M   H D YI FL    PD

      Clr6 QLKFNVGDDCPVFDGLYEFCSISAGGSIGAAQELNSGNAEIAINWAGGLHHAKKREASGFCYVNDIALAALELLKYHQRVLYIDIDVHHGDGVEEFFYTT 187
      Rpd3 SVKFNVGDDCPVFDGLYEYCSISGGGSMEGAARLNRGKCDVAVNYAGGLHHAKKSEASGFCYLNDIVLGIIELLRYHPRVLYIDIDVHHGDGVEEAFYTT
     HDAC1 MQRFNVGEDCPVFDGLFEFCQLSTGGSVASAVKLNKQQTDIAVNWAGGLHHAKKSEASGFCYVNDIVLAILELLKYHQRVLYIDIDIHHGDGVEEAFYTT
 Consensus    FNVG DCPVFDGL E C  S GGS   A  LN      A N AGGLHHAKK EASGFCY NDI L   ELL YH RVLYIDID HHGDGVEE FYTT
                                                                                    *
      Clr6 DRVMTCSFHKFGEYFPGTGHIKDTGIGTGKNYAVNVPLRDGIDDESYESVFKPVISHIMQWFRPEAVILQCGTDSLAGDRLGCFNLSMKGHSMCVDFVKS 287
      Rpd3 DRVMTCSFHKYGEFFPGTGELRDIGVGAGKNYAVNVPLRDGIDDATYRSVFEPVIKKIMEWYQPSAVVLQCGGDSLSGDRLGCFNLSMEGHANCVNYVKS
     HDAC1 DRVMTVSFHKYGEYFPGTGDLRDIGAGKGKYYAVNYPLRDGIDDESYEAIFKPVMSKVMEMFQPSAVVLQCGSDSLSGDRLGCFNLTIKGHAKCVEFVKS
 Consensus DRVMT SFHK GE FPGTG   D G G GK YAVN PLRDGIDD  Y   F PV    M    P AV LQCG DSL GDRLGCFNL   GH  CV  VKS

      Clr6 FNLPMICVGGGGYTVRNVARVWTYETGLLAGEELDENLPYNDYLQYYGPDYKLNVLSNNMENHNTRQYLDSITSEIIENLRNLSFAPSVQMHKTPGDF.T 386
      Rpd3 FGIPMMVVGGGGYTMRNVARTWCFETGLLNNVVLDKDLPYNEYYEYYGPDYKLSVRPSNMFNVNTPEYLDKVMTNIFANLENTKYAPSVQLNHTPRDAED
     HDAC1 FNLPMLMLGGGGYTIRNVARCWTYETAVALDTEIPNELPYNDYFEYFGPDFKLHISPSNMTNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIPEDAIP
 Consensus F  PM   GGGGYT RNVAR W  ET          LPYN Y  Y GPD KL     NM N NT  YL         NL     AP VQ      D

      Clr6 FENAEKQNIAKEEIMDERV 405
      Rpd3 LGDVEEDSAEAKDTKGGSQYARDLHVEHDNEFY
     HDAC1 EESGDEDEDDPD..KRISICSSDKRIACEEEFSDSEEEGEGGRKNSSNFKKAKRVKTEDEKEKDPEEKKEVTEEEKTKEEKPEAKGVKEEVKLA
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Figure 3.—The *clr6* gene. (A) The *clr6* ORF. Thirteen introns interrupting the ORF and the filled boxes indicating exons are shown. (B) Comparison of the predicted Clr6, *S. serevisiae* Rpd3, and human HDAC1 amino acid sequences. The sequences were aligned using the PILEUP program. The consensus sequence indicating identical residues in all three sequences is shown at the bottom. The asterisk indicates the residue that is changed to aspartic acid [D] in the *clr6-1* mutant allele.

similarity; Figure 3B). Clr6 also shares similarity to the predicted amino acid sequences of two recently sequenced ORFs from *S. pombe* (Sanger Genome Sequencing Center), which we have designated homologs of *clr6* (*hoc1* and *hoc2* mapping to cosmids c3G9 and SP41410, respectively). The sequence alignments revealed that Clr6 shares greater homology to Hoc1 (50% identity and 73% similarity) than to Hoc2 (26% identity and 51% similarity).

To map the mutation in the *clr6-1* mutant, we used PCR to amplify the genomic *clr6* DNA containing *clr6* from wild-type and mutant cells. The sequencing of the PCR products identified a single G-to-A transition in codon 269, converting GGC (Gly) to GAC (Asp). Interestingly, the mutation maps to a region of Clr6 that is conserved in Rpd3 from *S. cerevisiae* and human HDAC1. The genomic location of *clr6* was determined as described by Hoheisel *et al.* (1993). The results revealed that *clr6* maps near the telomere of the short arm of chromosome *II* between markers *mei3* and *rad3*. A disruption allele of *clr6* was made by DNA-mediated transformation of a diploid strain (see materials and methods). Upon dissection of the heterozygous diploid, haploid segregants carrying the *clr6* null allele were found to be essentially inviable (data not shown). However, microscopic examination revealed that these haploid cells were able to divide for three to four generations before growth arrest, a phenotype similar to that of the *clr6-1* Ts^- strain when it was shifted to the restrictive temperature (see above). Apparently, the *clr6-1* allele must cause a partial loss of function because the deletion is lethal.

Mutation in *clr6* affects propagation of the epigenetic states regulating silencing, recombinational suppression, and directionality of switching: It has been shown that in a strain with part of the *K* region replaced by the *ura4*$^+$ marker gene (*KΔ::ura4*$^+$ allele), *ura4*$^+$ expression, recombination between flanking markers, and the efficiency of mating-type interconversion are covariegated and regulated by an epigenetic mechanism (Grewal and Klar 1996, 1997). More importantly, Ura^- efficient switching and Ura^+ inefficient switching variegated states (referred to as Ura^- and Ura^+ states, respectively) are inherited in *cis* and proposed to be the result of specific chromatin organization, with Ura^+ cells being defective in assembly of the structure (Grewal and Klar 1996). These results have been subsequently confirmed (Thon and Friis 1997) and support our suggestion that deletion of part of the *K* region compromises the establishment of a heterochromatin-like repressive structure (Grewal and Klar 1996).

Here, we determined whether a mutation in *clr6*, a histone deacetylase homolog, affects propagation of the epigenetic states. By genetic crosses, the *clr6-1* mutation was combined with derepressed (Ura^+) and repressed (Ura^-) "epialleles" of *KΔ::ura4*$^+$. First, donor loci re-

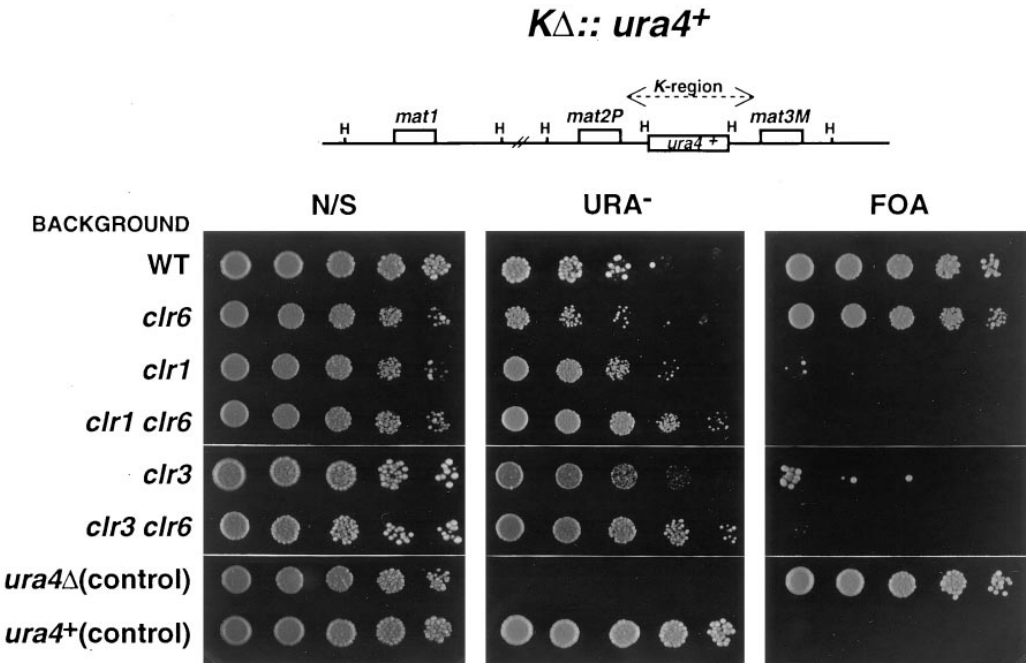

Figure 4.—The *clr6-1* mutation affects the propagation of the transcriptional states in *KΔ::ura4*$^+$ cells. The line drawing shows the arrangements of the mating-type region in *KΔ::ura4*$^+$ strains. H indicates the *Hin*dIII site. The Ura$^-$ state was combined with different mutant alleles by genetic crosses, and *ura4*$^+$ expression was assayed by dilution analysis. The cells were suspended in water, and fivefold serial dilutions were spotted onto the indicated media. The plates were incubated at 30° for 3–4 days before photography. Strains carrying either a deletion of the *ura4* gene (SP837) or the functional *ura4*$^+$ gene at an indigenous chromosomal location (SP412) were used as controls. The strains used were as follows: SPG27, WT; SP1464, *clr6-1*; SPG60, *clr1-5*; SPG137, *clr1-5 clr6-1*; SPG62, *clr3-735*; and SPG157, *clr3-735 clr6-1*. All strains containing *KΔ::ura4*$^+$ carried the mutation of the indigenous *ura4*$^+$ gene. WT indicates the *clr*$^+$ genotype.

mained silent in cells carrying either the Ura$^+$ or Ura$^-$ epistate. Second, comparison of the rate of Ura$^-$ to Ura$^+$ transition in the *clr6-1* with the rate in the *clr6*$^+$ cells by dilution assay revealed that the mutation by itself did not significantly affect the mitotic stability of the Ura$^-$ state (Figure 4). We also used the fluctuation test to quantitatively measure the effect of the *clr6* mutation on the stability of epigenetic states (see materials and methods). Confirming the results of serial dilution (Figure 4), the *clr6* mutation had only a subtle effect on the Ura$^-$ to Ura$^+$ transition, as compared with its wild-type counterpart (Table 2). However, we observed that the *clr6-1* allele caused a significant (~20-fold) increase in Ura$^+$ to Ura$^-$ conversion. This result shows that the mutation paradoxically increases the efficiency of the establishment of a transcriptionally repressed state of *KΔ::ura4*$^+$.

The silencing function of *clr6* seems to overlap with that of *clr1* and *clr3*; therefore, we tested the effect of the *clr1 clr6* and *clr3 clr6* double mutations on the propagation of epigenetic states. As a control, *KΔ::ura4*$^+$ strains carrying a mutation in *clr1* or *clr3*, which were previously shown to completely suppress variegation (Grewal and Klar 1996), were also included. Dilution analysis (Figure 4) demonstrated that like the *clr1* or *clr3* mutant strains, double mutants did not grow on FOA plates, indicating alleviation of *ura4*$^+$ repression. More significantly, Ura$^+$ cells with the double mutation were unable to revert to the Ura$^-$ state. Overall, these data suggest that mutation in *clr6* leads to increased conversion of the transcribed state into the repressed state, presumably through assembly of a higher-order chromatin structure, and that increased repression in the *clr6* mutant is dependent on the *clr1* and *clr3* gene products.

TABLE 2

Effect of the *clr6* mutation on stability of the Ura$^-$ and Ura$^+$ states

	Transition rate (per cell division)	
Genotype	Ura$^-$ to Ura$^+$	Ura$^+$ to Ura$^-$
Wild type	5.6×10^{-4}	8.4×10^{-4}
clr6-1	9.4×10^{-4}	1.6×10^{-2}

TSA treatment adversely affects propagation of the epigenetic imprint at the *mat2-mat3* region: Parallel to the *clr6-1* analysis, we also investigated the effect of inhibition of histone deacetylation on propagation of the Ura$^-$ state by treating cells with TSA, a specific inhibitor of histone deacetylases (Yoshida *et al.* 1995). A brief treatment by TSA for ~10 generations converted the majority of cells that originally displayed the Ura$^-$ state to the Ura$^+$ state. Remarkably, subsequent plating revealed that the newly acquired Ura$^+$ state was stable for many (>30) generations after the drug treatment (Figure 5). Measurement of the mitotic stability of the TSA-induced state indicated that these Ura$^+$ cells reverted to the Ura$^-$ state at a frequency comparable to that of standard Ura$^+$ cells (data not shown). Furthermore, we observed that ~30% of the cells exposed to

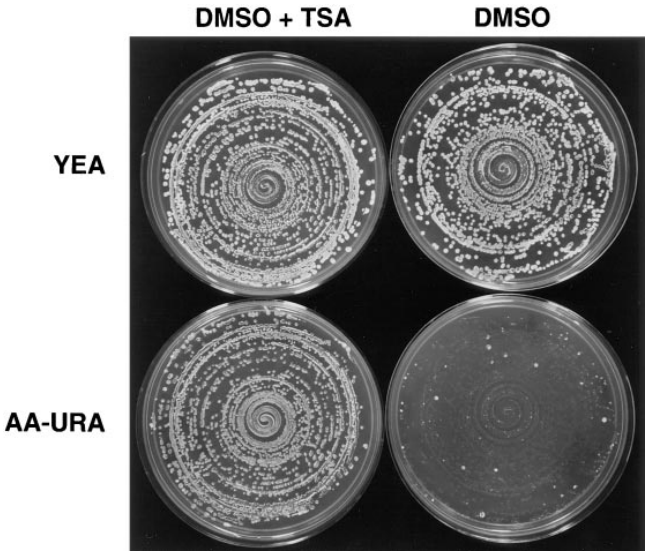

Figure 5.—TSA treatment converts the Ura$^-$ state to Ura$^+$. Ura$^-$ cells of the *KΔ::ura4*$^+$ strain (SPG27) were patched onto YEA medium and exposed to filter paper discs carrying 1 μl of 10 μg/ml TSA dissolved in DMSO. As a control, cells were likewise exposed to the DMSO solvent. The plates were incubated at 33° overnight before a loopful of cells taken from the edge of the filters was streaked onto fresh YEA plates. After growth for 3 days, *KΔ::ura4*$^+$ expression was scored by replicating colonies onto the indicated medium. Plates were photographed after overnight growth at 33°

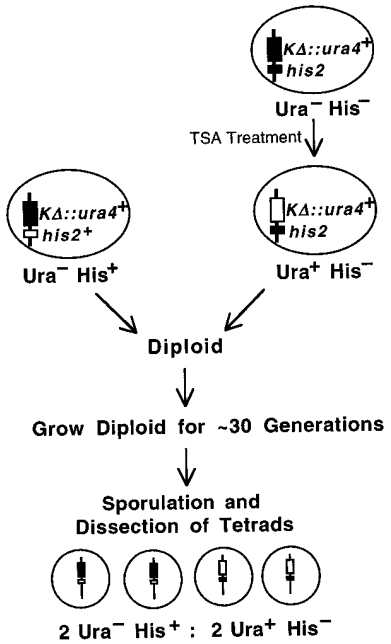

Figure 6.—The TSA-induced Ura$^+$ sate is tightly linked to the *mat* locus. Cells showing the Ura$^+$ state, obtained by TSA treatment of the *KΔ::ura4*$^+$ *his2* Ura$^-$ (SPG27) strain, were mated to *KΔ::ura4*$^+$ *his2*$^+$ Ura$^-$ (SPG51) to construct diploids. The diploids were allowed to grow for at least 30 generations, sporulated, and subjected to tetrad analysis. The meiotic segregants were analyzed for linkage of the Ura$^+$ state to *his2*, a marker tightly linked (<1 cM) to the *mat* locus. The 2 Ura$^-$His$^+$: 2 Ura$^+$ His$^-$ segregation pattern in each tetrad suggested that the TSA-induced Ura$^+$ state is inherited in *cis* as a marker linked to the mating-type region. Closed and open boxes indicate the Ura$^-$ and Ura$^+$ states of *KΔ::ura4*$^+$ or the mutated and wild-type alleles of *his2*, respectively.

TSA for 1 day exhibited the haploid meiosis phenotype when transferred to sporulation medium in the absence of the drug, suggesting that the drug treatment causes heritable derepression of donor loci as well. Similarly, cells displaying the Ura$^+$ state were also exposed to TSA and, as expected, the treatment did not change them to the Ura$^-$ phenotype. Briefly, these data show that transient exposure to TSA causes conversion of the transcriptionally repressed state to the expressed state, and that the expressed state is stably propagated in the progeny for many generations in the absence of TSA.

In principle, the TSA effect (transition from Ura$^-$ to Ura$^+$) could be caused either by stable changes in the expression of *trans*-acting loci that are essential for silencing or by alteration in the chromosomally inherited imprint at the *mat* locus (Grewal and Klar 1996). We differentiated between these possibilities by the following cross (see Figure 6). A strain carrying the Ura$^+$ epillele (SPG27, *KΔ::ura4*$^+$ *his2*), obtained by TSA treatment of Ura$^-$ cells, was crossed to a Ura$^-$ (SPG51, *KΔ::ura4*$^+$ *his2*$^+$) strain. The resultant diploid was sporulated and subjected to tetrad analysis. If the effect of TSA is localized to the mating-type region, two Ura$^-$ and two Ura$^+$ segregants should be found in each tetrad, and these states should cosegregate with respective alleles of *his2*, a marker that is closely linked to the mating-type region. This segregation pattern was indeed observed in all 30 tetrads analyzed. Thus, inhibition of histone deacetylase activity adversely affects propagation of the stable chromosomal imprint, which determines gene activity, at the *mat2-mat3* locus. Similarly, a heritable change from Ura$^-$ to Ura$^+$ linked to the mating-type region was also observed in nonswitching *mat1-Msmto KΔ::ura4*$^+$ (SPG32) strain (data not shown).

The *clr6* mutation affects *ura4*$^+$ repression at three sites within *cen1*: Assembly of the repressive chromatin structure is also believed to be essential for the proper functioning of fission yeast centromeres (Clarke *et al.* 1993). As mentioned above, *trans*-acting elements originally identified as essential for silencing in the mating-type region also affect the silencing of markers artificially inserted into the centromeric sequences (Allshire *et al.* 1995). Therefore, it was conceivable that *clr6* might also affect centromeric silencing. To test this, we combined the *clr6-1* mutation with *ura4*$^+$ integrated at three sites within *cen1*: one in the central domain, *cnt1*/TM1, and one in each of the outer flanking repeats, *imr1*R and *otr1*R (Allshire *et al.* 1995). Interestingly, the mutation showed contrasting effects on *ura4*$^+$ integrated at different centromeric domains. Dilution analysis of strains carrying *imr1*R(*Nco*I)*::ura4*$^+$ and *otr*R (*Sph*I)*::ura4*$^+$ insertions showed that the *clr6-1* mutation significantly increased their ability to grow on URA$^-$ medium, but there was no growth difference between

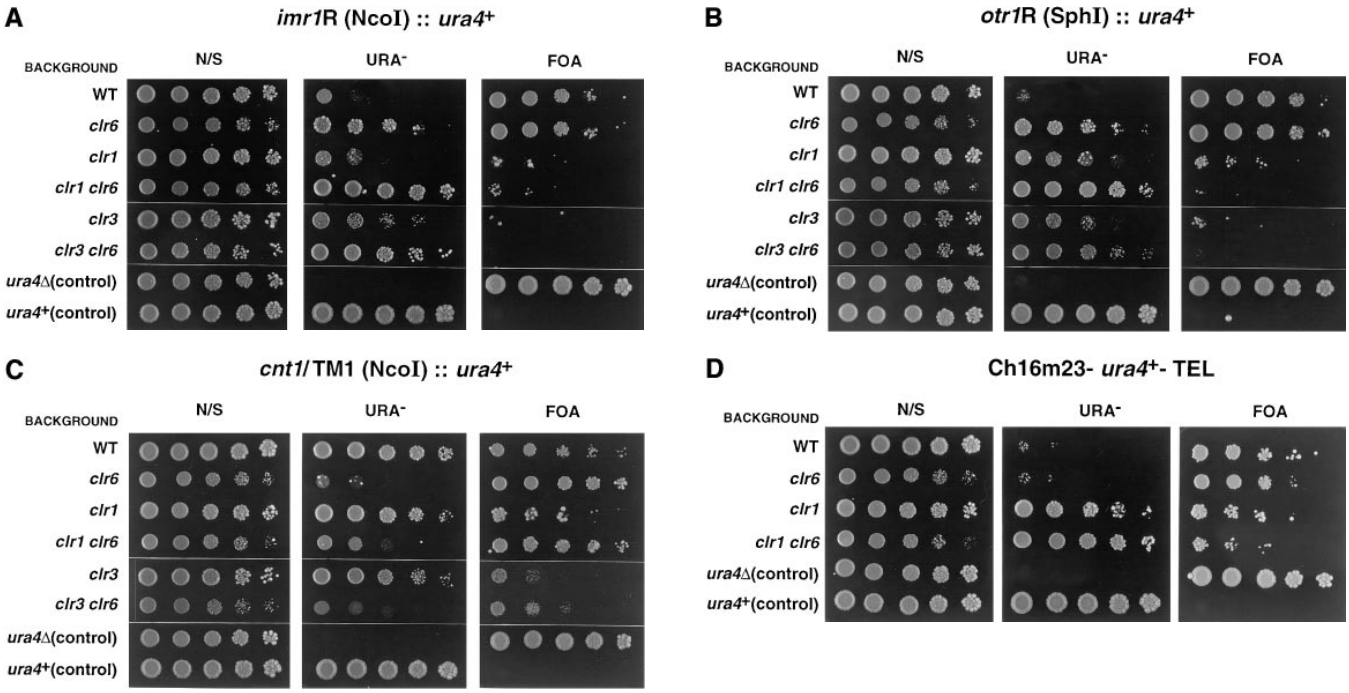

Figure 7.—The effect of *clr6* mutation on silencing of *ura4*$^+$ inserted at three different locations within *cen1* and adjacent to a telomere. Cultures with *imr1*R(*Nco*I)::*ura4*$^+$ (A), *otr1*R(*Sph*I)::*ura4*$^+$ (B), *cnt1*/TM1::*ura4*$^+$ (C), or Ch16m23-*ura4*$^+$-TEL (D) in N/S medium were suspended in water. Fivefold serial dilutions of cell suspensions were spotted onto N/S, URA$^-$, and FOA media, and the plates were incubated at 30° for 3–4 days before photography. A strain containing the functional *ura4*$^+$ gene at the wild-type chromosomal location (SP412) and another with the *ura4* deletion (SP837) were used as controls. The strains used were as follows: (A) FY498, WT; SPG141, *clr6-1*; SPG142, *clr1-5*; SPG143, *clr1-5 clr6-1*; SPG158, *clr3-735*; and SPG159, *clr3-735 clr6-1*; (B) FY648, WT; SPG144, *clr6-1*; SPG145, *clr1-5*; SPG146, *clr1-5 clr6-1*; SPG160, *clr3-735*; and SPG161, *clr3-735 clr6-1*; (C) FY336, WT; SPG138, *clr6-1; SPG139, clr1-5*; SPG140, *clr1-5 clr6-1*; SPG162, *clr3-735*; and SPG163, *clr3-735 clr6-1*; and (D) FY520, WT; SPG147, *clr6-1*; SPG148, *clr1-5*; and SPG149, *clr1-5 clr6-1*. All strains containing *ura4*$^+$ at *cen1* or a telomere carried a mutation of the indigenous *ura4*. WT indicates the *clr*$^+$ genotype.

clr6-1 and wild-type cells on FOA medium (Figure 7, A and B). This result suggests that the level of expression of *ura4*$^+$ inserted at both locations was increased, but that the mutation did not completely abolish repression, with *ura4*$^+$ expression presumably remaining below the level required to cause FOA sensitivity. In contrast and paradoxically, the mutation resulted in increased repression of *cnt1*/TM1(*Nco*I)::*ura4*$^+$ insertion at the central domain of *cen1*, as indicated by the impaired ability of cells to form colonies on URA$^-$ medium and the corresponding increased growth on FOA plates (Figure 7C).

We also measured the expression of *cen1 ura4*$^+$ constructs in the *clr1-5 clr6-1* and *clr3-735 clr6-1* double-mutant backgrounds. As expected, this double-mutant combination resulted in additive derepression of *ura4*$^+$ in the *imr1*R(*Nco*I)::*ura4*$^+$ and *otr1*R(*Sph*I)::*ura4*$^+$ strains (Figure 7, A and B). Additionally, *clr1*$^-$ promoted derepression was reduced by the *clr6* mutation at the *cnt1*/TM1(*Nco*I)::*ura4*$^+$ insertion (Figure 7C). We also tested the *clr6* mutation for its effect on telomere-mediated repression of *ura4*$^+$ (Ch16m23-*ura4*$^+$-TEL), but found that it had no visible effect alone or in combination with *clr1-5* (Figure 7D).

In summary, these results indicate that there are distinct domains within *cen1* and that *clr6* differentially affects these domains, presumably by participating in their assembly into heterochromatin-like structures. Furthermore, the observed additive derepression in double mutants further suggests that Clr6 might perform an overlapping function with Clr1 plus Clr3 activity.

The *clr6* mutation reduces the fidelity of chromosome segregation: It was previously shown that mutations affecting centromeric silencing also disrupt chromosome segregation (Allshire *et al.* 1995). Therefore, it was formally possible that the *clr6-1* allele could also impair centromere function. We tested the effect of the *clr6-1* mutation on the mitotic stability of the minichromosome Ch16, a 530-kb derivative of chromosome *III* (Niwa *et al.* 1986), as described in materials and methods. As shown in Table 3, strains bearing a lesion at *clr6* missegregated the minichromosome at a 26-fold higher rate compared with their wild-type counterpart. This result further indicated that *clr6* gene function might also be involved in the formation of fully functional centromeres.

It was recently demonstrated that mutations in *clr4*, *rik1*, and *swi6* also cause elevated rates of chromosome loss, whereas *clr1*, *clr2*, and *clr3* mutations have a negligi-

TABLE 3

Effect of *clr* mutations on loss of 530-kb minichromosome

Strain	Genotype	Loss rate (per cell division)	Relative loss rate[a]
HM248	Wild type	1.2×10^{-3}	Control
SPG134	*clr1-5*	3.4×10^{-3}	3-fold
SPG155	*clr3-735*	2.2×10^{-3}	2-fold
SPG135	*clr6-1*	3.1×10^{-2}	26-fold
SPG136	*clr1-5 clr6-1*	7.9×10^{-2}	66-fold
SPG156	*clr3-735 clr6-1*	5.4×10^{-2}	45-fold

[a]Increase in rate compared with that of the wild-type control.

ble effect on chromosome segregation (Allshire *et al.* 1995). Because Clr6 acts in an overlapping manner with Clr1 and Clr3 (this study), we investigated chromosome loss in the *clr1 clr6* and *clr3 clr6* double mutants. The data presented in Table 3 suggest that the *clr6* mutation further stimulates *clr1*- and *clr3*-promoted chromosome loss. Likewise, we also studied the effect of TSA treatment on the fidelity of chromosome segregation. Remarkably, transient exposure of minichromosome-containing cells to TSA resulted in elevated levels of missegregation in subsequent generations (Table 4). The effect of transient exposure to TSA clearly persists over many generations.

Clr3 is related to histone deacetylases: The partially redundant function of the putative histone deacetylase Clr6 and proteins encoded by the *clr1* and *clr3* genes suggests that the latter might also encode components of the histone deacetylase machinery. In this regard, Clr1 was found to contain the DNA-binding zinc finger motifs (G. Thon and A. Klar, unpublished results), but Clr3 remains to be characterized. To define the mechanism of action of Clr3, we cloned the gene by transforming a h^{90} *mat3-M::ura4*$^+$ *clr3-735* strain (SP1359) with a partial *Sau*3A library. The transformants were screened for repression of *mat3-M::ura4*$^+$ by selecting for their growth on FOA-containing medium. An 8.0-kb insert resulted in complementation of the *ura4*$^+$ expression phenotype and of the sporulation defect caused by derepression of donor loci in the *clr3-735* background. Subcloning and sequencing analysis identified an ORF that is capable of encoding a protein of 687 amino acids.

TABLE 4

Effect of length of TSA treatment on fidelity of minichromosome segregation

	Loss rate (per cell division)	
Days	DMSO	DMSO+TSA
1	1.31×10^{-3}	1.48×10^{-2}
3	1.41×10^{-3}	4.90×10^{-2}
5	1.46×10^{-3}	5.80×10^{-2}

The encoded Clr3 protein sequence was compared with the sequences in the GenBank database. The best scores were obtained for a group of proteins that include known histone deacetylases, such as Hda1 (Rundlet *et al.* 1996) and Rpd3 (Vidal and Gaber 1991) from *S. cerevisiae* and HDAC1 (TAunton *et al.* 1996) from humans. Furthermore, we noticed that similarity between Clr3 and Hda1 (41% identity, 65% similarity; Figure 8) was greater than that between Clr3 and *S. cerevisiae* Rpd3 (27% identity, 49% similarity) or human HDAC1 (26% identity, 49% similarity). In addition to eukaryotic sequences, Clr3 also shares a high degree of similarity (41–52%) to acetylpolyamine hydrolase proteins from Synechocystis, *Mycoplasma ramosa*, and *Methanococcus janaschii*. Further comparison of Clr3 to other *S. pombe* sequences revealed that it shares only 26% (51% similarity) and 28% (52% similarity) identity to Clr6 and Hoc1 (see above) sequences, respectively, but was found to be homologous to the subsequently reported Hoc2 protein (see above) identified by Sanger Genome Sequencing Center (cosmid SP41410). Physical mapping of the *clr3* gene confirmed that it is the same gene as *hoc2*, which maps to the short arm of chromosome *II*, which is located centromere distal to *rad11*. Cloning and sequencing analysis of the *clr3-735* mutant allele identified a single G-to-A transition in codon 232, resulting in the D232N change (Figure 8).

These data suggest that the putative Clr3 protein belongs to the family of histone deacetylases. Unlike Clr6, which is more similar to *S. cerevisiae* Rpd3 and its human homolog, HDAC1, Clr3 shares greater similarity to *S. cerevisiae* Hda1. Therefore, it is possible that Clr6 and Clr3 proteins are fission yeast homologs of Rpd3 and Hda1, respectively.

DISCUSSION

In fission yeast, there are three known domains that exhibit position-effect control, namely the silent mating-type region, centromeres, and telomeres. It has been suggested that repression at these loci is mediated through assembly of a heterochromatin-like structure. Consistent with this model, we report the identification

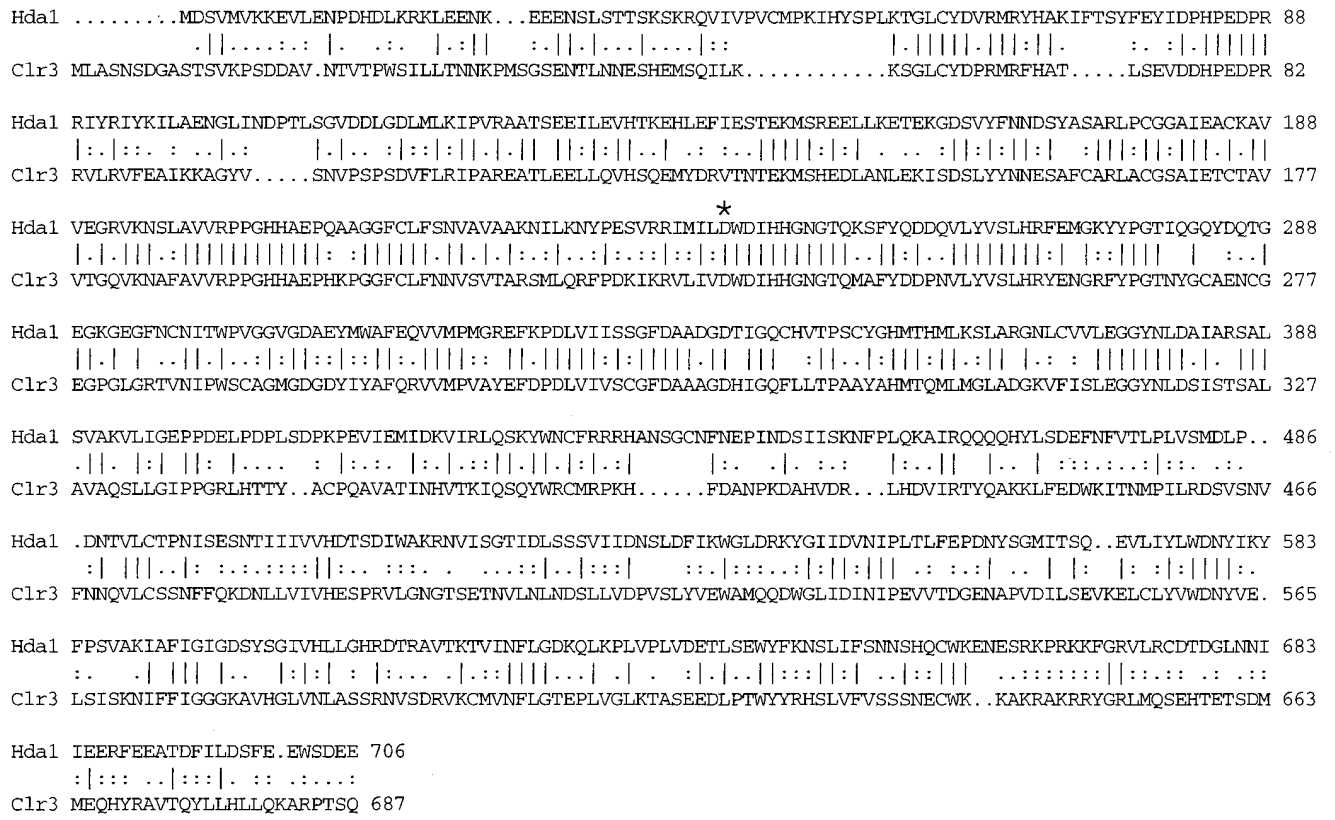

Figure 8.—Alignment of the predicted Clr3 and *S. cerevisiae* Hda1 amino acid sequences. The sequences were aligned using the GAP program. Identical amino acids are marked by vertical lines, and similar amino acids are indicated by colons. The asterisk indicates the residue that is changed to asparagine [N] in the *clr3-735* mutant allele.

of an essential gene, *clr6*, encoding a putative histone deacetylase, which, when mutated, affects centromeric as well as donor loci silencing and reduces the fidelity of chromosome segregation. Furthermore, the *clr6* mutation affects propagation of the epigenetic states regulating expression of a marker gene integrated in the silent mating-type region, recombination suppression, and directionality of mating-type switching.

Genetic evidence suggests that Clr6 function is partially redundant to the function of Clr1/Clr3. In this respect, we find that the putative Clr3 protein also shares similarity to histone deacetylases. Our earlier study indicated that *clr1* and *clr3* work together, either in the same pathway or as a complex (Thon *et al.* 1994). Taken together, these data suggest that silencing at above-mentioned loci might be regulated by two distinct yet functionally overlapping histone deacetylation mechanisms: Clr1 and Clr3 might be components of one such system, whereas Clr6 is a component of a second system. Likewise, the budding yeast proteins Hda1 and Rpd3, which share high similarity to Clr3 and Clr6, respectively, have been found to be associated with the distinct histone deacetylase complexes HDA and HDB, respectively (Rundlett *et al.* 1996).

On the basis of recent studies (Braunstein *et al.* 1993; Brownell and Allis 1996; Wolffe 1996; Hartzog and Winston 1997; Pazin and Kadonaga 1997), it has been suggested that histone deacetylation leads to assembly of "compact" chromatin, which prevents access to transcription factors, whereas acetylation makes it "open," allowing access to the transcription machinery. In contradiction to this model, mutations in *RPD3* and related genes were recently shown to enhance silencing in the heterochromatic regions of both budding yeast and Drosophila (Derubertis *et al.* 1996; Rundlet *et al.* 1996; Vannier *et al.* 1996). Similarly, mutation in *clr6* causes a significant (20-fold) increase in Ura^+ to Ura^- transition of *KΔ::ura4*$^+$ epialleles and silencing at the central domain (*TM1*) of *cen1*. However, the effects of *clr6-1* are not uniform across all sites tested, and the marker gene integrated at either of two outer flanking repeats (*imr1* and *otr1*) of *cen1* was weakly derepressed (this study). In contrast to increased repression and consistent with the proposed role of histone deacetylases, a mutation in *clr3* adversely affects silencing both at the silent mating-type region (Thon *et al.* 1994; Grewal and Klar 1997) and at centromeres (Allshire *et al.* 1995). Also, functional *clr3*$^+$ is required for propagation of the repressed (Ura^-) epigenetic state (Grewal and Klar 1996). To accommodate these seemingly contradictory results, we propose a hypothetical "competition model." We suggest that, as in budding

yeast, where the subtelomeric region and silent mating-type loci compete for silencing proteins (Buck and Shore 1995; Maillet *et al.* 1996), different heterochromatin domains in fission yeast might compete for a defined pool of heterochromatic proteins (HPs) such as Swi6. Furthermore, the affinity of a particular chromosomal domain for HPs may depend on its state of histone acetylation. If, for example, HPs preferentially bind to deacetylated histone H3 and H4, regional differences in acetylation state could ensure their targeting to specific chromosomal domains. In turn, differences in acetylation pattern could depend on region-specific preferential recruitment of different combinations of Clr3, Clr6, Hoc1, or other deacetylating proteins. In this way, mutation in a particular deacetylase might result in relocalization of HPs from one chromosomal domain to another. For example, mutation in *clr6* might delocalize HPs from the *imr* and *otr* regions of centromeres. The free HPs could then be recruited to the mating-type and central centromeric regions, leading to contrasting effects on corresponding loci. Consistent with this competition model, we see that the increase in repression at the *mat2-mat3* region observed in *clr6* mutants is dependent on the presence of functional Clr1 and Clr3 (this study). Also, the *clr6-1*-mediated increased silencing is sensitive to TSA treatment (our unpublished data), indicating the probable involvement of other deacetylases.

As an alternative to the competition model, we imagine that the *clr6* mutation might affect silencing indirectly by altering the regulation of *trans*-acting genes, including *clr1-clr4*, *swi6*, and *rik1*. It has been suggested that enhanced repression in mutant cells might be caused by a specific pattern of histone acetylation (such as increased acetylation of H4 lysine 12; Rundlett *et al.* 1996) or by the assembly of a specialized chromatin structure (Deruber tis *et al.* 1996). Supporting the latter argument, it was previously observed that the central centromeric domain in fission yeast is packaged into an unusual chromatin structure (Polizzi and Clarke 1991; Takahashi *et al.* 1992). These three models are not mutually exclusive, and further work is needed to differentiate among them.

During development and differentiation, propagation of the stable states of gene expression through numerous cell divisions is essential (Felsenfeld 1992). It is possible that the histone acetylation pattern of chromosomal domains acts as a "cell memory" and helps to maintain the respective states of gene expression. Consistent with this idea, we observed that transient inhibition of histone deacetylation by TSA causes a remarkable heritable change in the expression of a marker gene integrated in the silent mating-type region, converting the epigenetically repressed state to the expressed state in the majority of cells. The expressed state is inherited in *cis* as a marker linked to the mating-type locus and, as observed previously (Grewal and Klar 1996), correlates with reduced efficiency of mating-type interconversion. In our chromatin-replication model, we previously suggested that silencing, efficiency of mating-type switching, and recombination suppression in the *mat2-mat3* interval are regulated by a heterochromatin structure that, once assembled, is capable of self-replicating for many generations through cooperative binding of HPs (Grewal and Klar 1996, 1997). It is possible that inhibition of histone deacetylation compromises the assembly of the proposed chromatin structure. It remains to be determined whether the change in imprint is caused by direct alteration of the acetylation state at the silent mating-type region or by an indirect transitory change in the expression of *trans*-acting factors required for silencing, which, in turn, cause a change in the imprint. There is also a possibility that TSA treatment affects the timing of replication and/or nuclear localization of the *mat* region, causing changes in the structural and functional imprint. Regardless of whether the TSA effect is direct or indirect, the final effect is shown to cause a heritable change in the imprint at the *mat2-mat3* region. Interestingly, Ekwall *et al.* (1997) recently showed that transient TSA treatment induces a heritable hyperacetylated state in centromeric chromatin, which correlates with functionally defective centromeres, suggesting a link between histone acetylation and the assembly of functional centromeres.

Unlike histone deacetylase from *S. cerevisiae* (Vidal and Gaber 1991), *S. pombe clr6* is an essential gene. We believe that lethality is at least partly related to a reduced fidelity of chromosome segregation in mutant strains. In addition, the possible disruption of chromatin structure throughout the genome in the *clr6* mutant may result in deleterious deregulation of gene expression. We note that *clr6-1* mutant is UV sensitive, a phenotype common in mutants of genes controlling the assembly of chromatin structure (Kaufman *et al.* 1997) and the DNA repair process (Reynolds *et al.* 1990). In conclusion, this study implicates putative histone deacetylases in the assembly of heterochromatin-like structures at the silent mating-type region and at centromeres. Supporting our chromatin replication model, we demonstrate that inhibition of histone deacetylase activity can compromise the propagation of the stable chromosomal imprint at the *mat* locus.

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