Cell, Vol. 101, 307–317, April 28, 2000, Copyright ©2000 by Cell Press

A Chromodomain Protein, Swi6, Performs Imprinting Functions in Fission Yeast during Mitosis and Meiosis

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Summary

Inheritance of stable states of gene expression is essential for cellular differentiation. In fission yeast, an epigenetic imprint marking the mating-type (*mat2/3*) region contributes to inheritance of the silenced state, but the nature of the imprint is not known. We show that a chromodomain-containing Swi6 protein is a dosage-critical component involved in imprinting the *mat* locus. Transient overexpression of Swi6 alters the epigenetic imprint at the *mat2/3* region and heritably converts the expressed state to the silenced state. The establishment and maintenance of the imprint are tightly coupled to the recruitment and the persistence of Swi6 at the *mat2/3* region during mitosis as well as meiosis. Remarkably, Swi6 remains bound to the *mat2/3* interval throughout the cell cycle and itself seems to be a component of the imprint. Our analyses suggest that the unit of inheritance at the *mat2/3* locus comprises the DNA plus the associated Swi6 protein complex.

Introduction

Cells of an organism, containing exactly the same genetic information, can selectively express or repress a subset of genes to give rise to vastly different cell types. Maintaining a committed cell type depends upon a memory system that is essential to produce stable patterns of gene expression over the course of multiple cell divisions. While gene-specific control mechanisms participate in this process, a surprising degree of control occurs via epigenetic modifications; that is, changes that do not alter the DNA but nevertheless cause Mendelian-like chromosomal inheritance of the gene expression state. Such changes can be stably propagated through mitosis and, remarkably, even through meiosis (Grewal and Klar, 1996; Cavalli and Paro, 1998; Morgan et al., 1999). Examples of epigenetic control of gene expression include X chromosome inactivation (reviewed by Riggs and Porter, 1996), parental imprinting in mammals (reviewed by Ainscough and Surani, 1996), and position effect variegation (PEV) in *Drosophila* (reviewed by Wallrath, 1998). Similar effects can be seen in stable expression of homeotic genes in *Drosophila* (Paro, 1993) and heritable gene inactivation in plants (Martienssen, 1998). It has been shown that epigenetic alterations have profound effects in mammalian development, as they appear to regulate the expression of developmentally important genes (Reik and Walter, 1998).

Among the diverse classes of epigenetic phenomena, some are regulated by reversible modifications of DNA and some depend upon posttranscriptional events, but a surprising majority seems to involve chromatin structure as an integral component of the gene repression mechanism (Wolffe and Matzke, 1999). For example, studies of PEV in *Drosophila*, whereby heterochromatin variably but stably silences nearby genes, have suggested that the spreading of repressive chromatin silences nearby genes (Wallrath, 1998). Similarly, in the distantly related unicellular eukaryotes, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, silencing at domains such as the mating-type loci, telomeres, and centromeres is also associated with altered chromatin packaging (Allshire, 1996; Grunstein, 1998). Unlike *S. cerevisiae*, silencing at the mating-type region of fission yeast extends to a large 15 kb chromosomal domain. In addition to silencing of *mat2* and *mat3* loci, which are used as donors of genetic information to switch the *mat1* locus, an 11 kb interval between donor loci, called the *K* region, also exhibits transcriptional and recombinational suppression (Grewal and Klar, 1997). Several *trans*-acting factors are required for complete repression at the *mat2/3* interval (see Klar et al., 1998). Interestingly, Swi6 (Lorentz et al., 1994) and Clr4 (Ivanova et al., 1998) contain the chromodomain, a motif found in proteins associated with higher-order chromatin packaging such as polycomb group (PcG) proteins and heterochromatin protein HP-1 from *Drosophila*, mouse, and humans (Singh, 1994). Furthermore, Clr3 and Clr6, which also affect silencing, share homology to histone deacetylases (Grewal et al., 1998). That a similar mechanism operates at the centromeres and at *mat2/3* is suggested by findings that mutations in *trans*-acting factors essential for silencing at the *mat2/3* region also affect silencing of markers inserted within centromeres (Allshire et al., 1995). Moreover, a part of the *K* region sequence shows extensive homology to centromeric repeat sequences (Grewal and Klar, 1997).

Previous studies showed that expression of the *ura4*$^+$ marker gene inserted in the *K* region and the efficiency of mating-type switching are controlled by an epigenetic mechanism (Grewal and Klar, 1996; Thon and Friis, 1997). Furthermore, these effects are correlated with each other, defining two functional states: cells containing repressed *ura4*$^+$ (*ura4-off*) switch mating-type at a frequency comparable to their wild-type counterparts, whereas cells containing transcriptionally active *ura4*$^+$ (*ura4-on*) switch inefficiently. More importantly, *ura4-off* and *ura4-on* epigenetic states are inherited in *cis*, suggesting that an epigenetic imprint marking the *mat2/3* region promotes inheritance of the silenced state. Since no DNA modifications have been observed in *S. pombe* (Antequera et al., 1984), DNA methylation is an unlikely candidate for this imprinting event. Instead, we advanced a "chromatin-replication" model, in which the *ura4-off* and *ura4-on* states represent "closed" and

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"open" chromatin structures, respectively, and where propagation of each state occurs by self-templated assembly of chromatin in the *mat2/3* region (Grewal and Klar, 1996). During DNA replication, the preexisting nucleoprotein complexes are hypothesized to contribute significantly toward assembly of the chromatin to propagate the parental state onto both daughter chromatids.

Here, we addressed the question of whether modifiers of PEV affect the initial generation of the imprint or simply the maintenance of the imprint. We demonstrate that a chromodomain-containing protein Swi6 acts as a dosage-critical factor whose recruitment to the *mat2/3* region is a limiting step in establishment of the epigenetic imprint. We also show that Swi6 remains associated with the mating-type region throughout the cell cycle and might itself be an important component of the imprint. This study strongly supports the involvement of chromatin proteins in imprinting and cellular memory.

Results

Transient Presence of *swi6* Multiple Copies Causes Efficient Heritable Conversion of *ura4-on* to *ura4-off* Epigenetic State

The chromatin replication model makes several testable predictions. First, introduction of multiple copies of the *trans*-acting genes, such as *clr1-clr4* or *swi6*, some of which may encode limiting chromatin factors, should lead to an enhanced conversion of *ura4-on* to *ura4-off* state. To test this prediction, we employed a *KΔ::ura4*$^+$ strain carrying replacement of 7.5 kb of the *K* region with *ura4*$^+$ gene (Figure 1A) (Grewal and Klar, 1996). *ura4-on* derivative of *KΔ::ura4*$^+$ cells were transformed with the plasmid pWH5 (Wright et al., 1986) carrying either *clr1, clr3, clr4, clr6*, or *swi6*. Assaying the *KΔ::ura4*$^+$ expression and the efficiency of mating-type interconversion showed that multiple copies (~3–4 copies per cell) of *clr1*, *clr3* (Figures 1B and 1C), or *clr6* (data not shown) did not affect the rate of *ura4-on* to *ura4-off* conversion. However, multiple copies of either *clr4* or *swi6* produced a significant increase in the proportion of cells exhibiting the *ura4-off* state. This was indicated by increased growth on FOA counterselective medium and increased intensity of iodine staining (Figures 1B and 1C). In comparison with *swi6*, the phenotype caused by *clr4* was subtle. Since Swi6 localization to the mating-type region is dependent upon the *clr4* gene product (Ekwall et al., 1996), it is possible that Clr4 acts indirectly by enhancing the recruitment of Swi6 to the *mat* locus.

We also tested whether multiple copies of *clr4* or *swi6* can enhance *ura4-on* to *ura4-off* conversion even in cells carrying a mutation in *clr1, clr2, clr3, clr4,* or *swi6*. Except for the self-complementation, no significant enhancement was seen (data not shown). Thus, extra copies of chromodomain proteins Clr4 and Swi6 enhance the establishment of the *ura4-off* state, but this change requires the products of other *trans*-acting loci.

The second, perhaps most critical, prediction of the model is that multiple copies of *swi6* should induce a heritable change in the imprint. That is, the newly established *ura4-off* state should maintain itself after

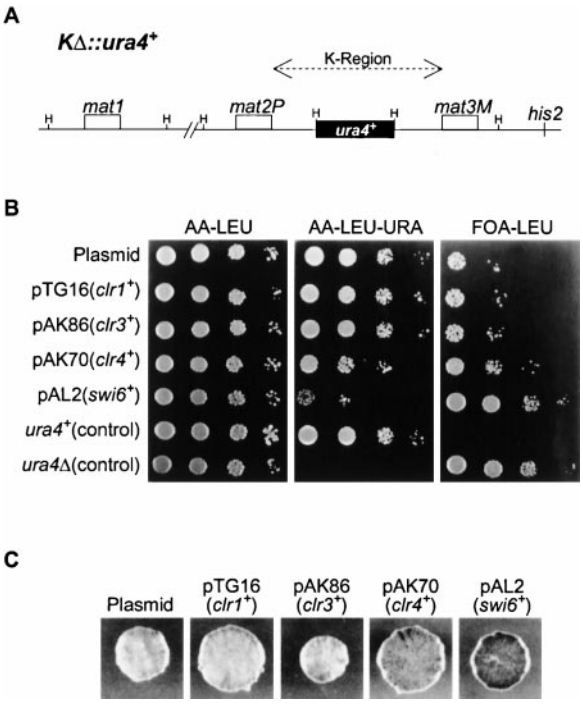

Figure 1. Effect of Multiple Copies of *clr1, clr3, clr4,* or *swi6* on *ura4-on* to *ura4-off* Transition

(A) A physical map of the mating-type region in *KΔ::ura4*$^+$ strains. The locations of the *mat1, mat2, mat3,* and the tightly linked *his2* loci are shown. H indicates the HindIII site. (B and C) A Leu$^-$ *KΔ::ura4*$^+$ (SPG27) strain in the *ura4-on* state was transformed with the *LEU2*-based plasmid pWH5 alone or containing the indicated genes. Transformants were grown on AA-LEU medium for several generations. For assaying *KΔ::ura4*$^+$ expression, 10-fold diluted cultures were plated onto the indicated media and grown for 3–4 days at 33°C (B). FOA-LEU selects for the growth of Ura$^-$ Leu$^+$ cells. A strain containing the functional *ura4* (*ura4*$^+$; SP819) at its normal chromosomal location and a strain in which *ura4* was deleted (*ura4Δ*; SP837) were transformed with pAL2 and used as controls. The iodine-staining phenotype of transformants indicating the efficiency of mating-type interconversion was determined by replicating colonies onto PMA$^+$-LEU medium (C).

the extra copies of *swi6* are removed (Figure 2A). Remarkably, this prediction proved to be correct, since the *ura4-off* state was stable for more than 30 generations after loss of the *swi6*-containing plasmid from >50 independent transformants (Figure 2B, top). Fluctuation analysis showed equivalent rates of epigenetic reversion in induced and spontaneous *ura4-off* epialleles (7.1×10^{-4} and 5.6×10^{-4} per cell division, respectively). Sequencing of the *K* region from cells carrying *ura4-on* or induced *ura4-off* epialleles revealed that the observed effect is not due to a change in the DNA sequence (data not shown). However, it was possible that the heritable effect was due to the long half-life of the Swi6 protein produced by the multiple copies. We ruled out this possibility by transforming *KΔ::ura4*$^+$ *swi6*$^-$ *ura4-on* cells with a *swi6*-containing plasmid. A significant increase in *ura4-off* cells was observed, as expected. If Swi6 encoded by the plasmid was stable for many generations, the *ura4-off* state should be propagated when the plasmid is lost. Instead, removal of the plasmid led to

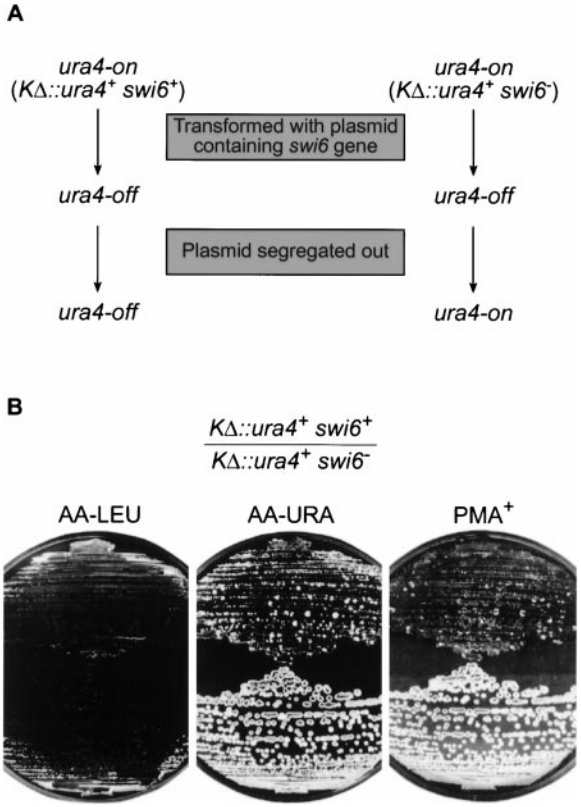

Figure 2. Transient Presence of the Multiple Copies of *swi6* Induces Heritable Conversion from *ura4-on* to *ura4-off* Epigenetic State

Strains SPG27 (*swi6*$^+$) and SPG16 (*swi6*$^-$), both originally of *ura4-on* state, were converted to *ura4-off* state by transforming with the *swi6*-containing pAL2 plasmid carrying the *LEU2* selectable marker (A). The plasmid was then segregated out by growing cells on nonselective (N/S) YEA medium and its loss was confirmed by Southern analysis (data not shown). The resulting Leu$^-$ colonies were spread on YEA medium for single cell isolations and replicated onto indicated media. The majority of *swi6*$^+$ colonies (top) maintained the *ura4-off* state but *swi6*$^-$ cells (bottom) efficiently reverted back to *ura4-on* state (B).

full reversion of the induced *ura4-off* state to *ura4-on* state (Figure 2B, bottom). In conclusion, these results suggest that Swi6 affects both the establishment and maintenance of the imprint required for propagation of the *ura4-off* state.

Swi6-Induced *ura4-off* Epigenetic State Is Inherited In *cis*

To determine whether the Swi6-induced heritable change in *KΔ::ura4*$^+$ expression reflected a change in the chromosomally inherited imprint at the *mat* locus, we crossed a strain (SPG27, *KΔ::ura4*$^+$ *his2*$^-$) carrying *ura4-off* epiallele, produced by the transient overexpression of Swi6 in originally *ura4-on* cells, to a *ura4-on* strain (SPG51, *KΔ::ura4*$^+$ *his2*$^+$). The resultant diploid was sporulated and subjected to tetrad analysis. If the heritable effect of multiple copies of *swi6* is localized to the mating-type region, a 2 Ura$^+$:2 Ura$^-$ segregation pattern should be observed and each state should cosegregate with the respective alleles of *his2*, a marker tightly

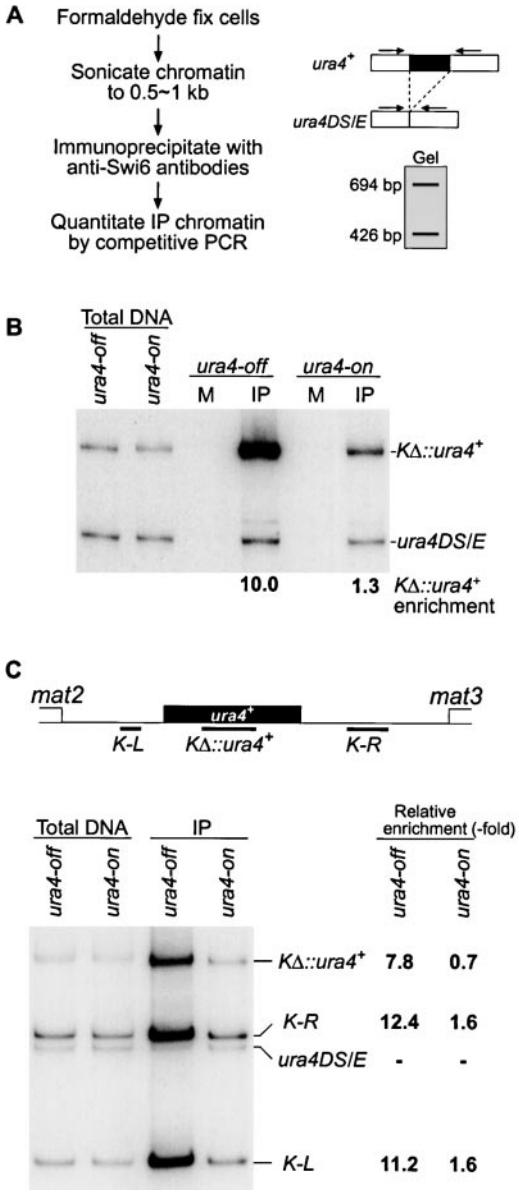

Figure 3. *ura4-off* Cells Have Significantly Higher Levels of Swi6 at Their Mating-Type Region Than the *ura4-on* Cells

(A) A brief description of CHIP protocol and the competitive PCR strategy for quantitating the amount of mating-type region *ura4*$^+$ versus endogenous *ura4* (*ura4DS/E)* locus are shown.

(B) Quantitative estimation of Swi6 levels at the *mat* region of *ura4-on* and *ura4-off* cells. DNA isolated either from crude extracts, mock-immunoprecipitated chromatin fraction (M), or anti-Swi6 immunoprecipitated chromatin fraction (IP) was used as a template for PCR amplification. PCR products of 426 bp and 694 bp from the *ura4DS/E* and *KΔ::ura4*$^+$, respectively, were resolved on a polyacrylamide gel and then quantified using a phosphoimager. The relative enrichment of *KΔ::ura4*$^+$ sequences is shown underneath each lane.

(C) To determine the distribution of Swi6 at the *K* region, multiplex PCR method was employed. Thick bars (top) show positions of DNA fragments examined.

linked to the *mat* locus. Indeed, this segregation pattern was observed in all 20 tetrads analyzed (data not shown). Therefore, the Swi6-promoted heritable effect

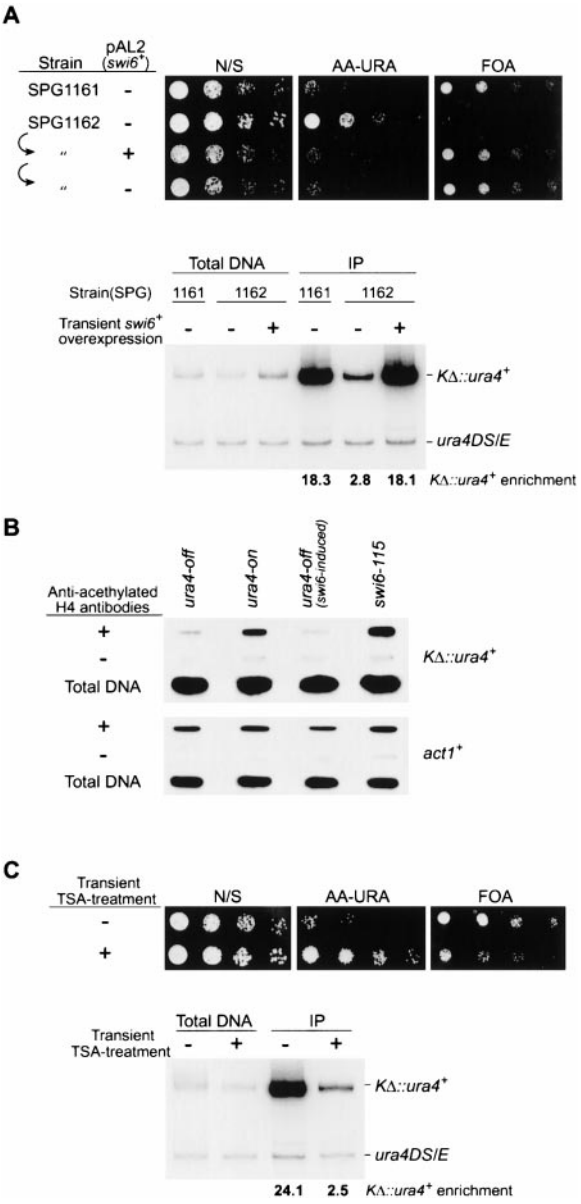

Figure 4. The Establishment and Maintenance of the Epigenetic Imprint Is Coupled to Swi6 Recruitment and Its Persistence at the Mating-Type Region, and Correlates with Changes in Chromatin Organization

(A) Transient overexpression of Swi6 causes stable increase in its levels at the *mat2/3* region. The *ura4-on* derivative of SPG1162 was converted to *ura4-off* state by transforming cells with the *swi6*-containing pAL2 plasmid. The plasmid was then segregated out by growing cells on YEA medium. Cells from each step were serially diluted onto indicated medium to determine their *ura4* phenotype (top panel). For comparison, a spontaneous *ura4-off* derivative of SPG1161 strain is shown. The quantitative measurement of Swi6 levels at the *mat* locus was carried out using CHIP analysis (bottom panel).

(B) Swi6-induced establishment of imprint correlates with changes in chromatin organization at the *KΔ::ura4*$^+$. Histone acetylation levels at the *KΔ::ura4*$^+$ locus were analyzed by CHIP method using anti-acetylated H4 antibodies, as described previously (Braunstein et al., 1993). DNA recovered from immunoprecipitated fractions was immobilized to nitrocellulose by using slot blot apparatus and hybridized to the indicated probes. Strains used were SPG1077 (*swi6*$^+$) and SPG1093 (*swi6-115*). Both strains carry deletion of the endogenous *ura4* gene.

localizes to the mating-type region and, remarkably, survives through meiosis.

Differences in Localization of Swi6 at the Mating-Type Region of *ura4-off* and *ura4-on* Cells

The experiments described above suggest that Swi6 is a dosage-critical component required for establishing and maintaining the silenced state and might be differentially localized at the *mat2/3* interval of *ura4-on* and *ura4-off* cells. That is, the mating-type region of *ura4-off* cells might contain higher levels of Swi6 than *ura4-on* cells. To test this idea, we directly analyzed the spontaneously generated *ura4-on* and *ura4-off* variants for the presence of Swi6 at *KΔ::ura4*$^+$ locus by using chromatin immunoprecipitation (CHIP) methodology. In brief, total chromatin fraction prepared from yeast cells fixed with formaldehyde was either mock-immunoprecipitated or immunoprecipitated (IP) with affinity-purified anti-Swi6 antibodies. The DNA recovered from both fractions was analyzed quantitatively using a competitive PCR strategy. This approach employs a strain bearing a *KΔ::ura4*$^+$ allele and a *ura4* minigene (*ura4DS/E*) with a 268 bp deletion at the endogenous *ura4* locus (Figure 3A) (Ekwall et al., 1997). By using primers designed to give products of 426 and 694 bp from the *ura4DS/E* and *ura4*$^+$, respectively, the relative enrichment of *KΔ::ura4*$^+$ sequences in the immunoprecipitated fractions can be accurately determined. Remarkably, we detected only 1.3-fold enrichment of *KΔ::ura4*$^+$ sequences in IP fractions of *ura4-on* cells, but 10-fold enrichment was observed in chromatin immunoprecipitated from *ura4-off* cells (Figure 3B). Therefore, significantly higher levels of Swi6 are present at the *KΔ::ura4*$^+$ locus of *ura4-off* than those of the *ura4-on* cells.

We also analyzed the Swi6 levels in other parts of the *mat2/3* region. In addition to the *KΔ::ura4*$^+$ locus, chromatin fractions immunoprecipitated with anti-Swi6 antibodies were simultaneously analyzed for the presence of *K* region sequences, by using multiplex PCR. As shown in Figure 3C, *ura4-off* cells have notably more Swi6 present at all locations tested within the *mat2/3* interval when compared with *ura4-on* cells. This result further suggests that *ura4-on* and *ura4-off* cells exhibit differential Swi6 localization pattern throughout the *mat2/3* region.

The Establishment of the Imprint Is Associated with a Stable Increase in Swi6 Levels at the *mat* Locus and Changes in Chromatin Organization

We next determined whether recruitment of Swi6 to the *mat* locus is an important factor in establishment of the *ura4-off* state. A *KΔ::ura4*$^+$ strain in the *ura4-on* state was transformed with pAL2 plasmid carrying the *swi6*$^+$ gene. As expected, the presence of multiple copies (3–4

(C) TSA-induced change in imprint correlates with dissociation of Swi6 from the *KΔ::ura4*$^+$. *ura4-off* derivative of SPG1161 was treated with TSA (10 μg/ml) for ~10 generations and then grown further to assay for the *ura4* expression state (top panel) and the presence of Swi6 at the *KΔ::ura4*$^+$ (bottom panel). (−), untreated parental cells; (+), transiently TSA-treated cells.

copies per cell) of *swi6* resulted in efficient *ura4-on* to *ura4-off* conversion (Figure 4A, top panel). Furthermore, the Swi6-induced *ura4-off* state was stably maintained when plasmid was segregated away. CHIP analysis revealed that establishment of the *ura4-off* state was correlated with a stable increase in Swi6 levels at the *KΔ::ura4*$^+$ locus when compared with the original *ura4-on* cells (Figure 4A, bottom panel). These data strongly suggest that the recruitment of Swi6 to the *mat2/3* region and establishment of the epigenetic imprint are functionally related. Moreover, Swi6 might itself be a component of the imprint.

Chromosomal regions that are actively transcribed often contain hyperacetylated histones while silenced regions are hypoacetylated (Braunstein et al., 1993). To determine possible differences in chromatin organization at the mating-type region of *ura4-off* and *ura4-on* cells, we compared the levels of histone acetylation at the *KΔ::ura4*$^+$ locus. Antibodies specific for acetylated histone H4 were used to immunoprecipitate chromatin from *ura4-off* and *ura4-on* cells in the CHIP assay (see Braunstein et al., 1993). The DNA in IP fractions were analyzed for *ura4*$^+$ and control *act1* sequences. Histone H4 was acetylated in *ura4-on* cells but underacetylated in *ura4-off* cells (Figure 4B). Furthermore, a mutation in *swi6* (*swi6–115*) caused an increase in acetylation. These data suggest that the silenced state results from assembly of a closed heterochromatin-like structure and Swi6 protein participates in this process. To further establish that transient expression of *swi6*$^+$ multiple copies alters the chromatin imprint at *mat2/3* region, we determined the level of histone acetylation in Swi6-induced *ura4-off* cells. Remarkably, establishment of the silenced state was accompanied by decrease in acetylation (Figure 4B). Therefore, the change in imprint is accompanied by alterations in chromatin organization.

Transient Inhibition of Histone Deacetylation "Erases" the Imprint and Alters Swi6 Localization at the Mating-Type Locus

The transient inhibition of histone deacetylation by Trichostatin A (TSA) alters the epigenetic imprint at the *mat2/3* region and heritably converts the majority of cells from the *ura4-off* state to the *ura4-on* state (Grewal et al., 1998). Here we tested whether the TSA-induced change of imprint is associated with a stable decrease in levels of Swi6 at the *mat* locus. *KΔ::ura4*$^+$ *ura4-off* cells cultured in the presence of TSA for ~10 generations were harvested, grown further in absence of the drug for ~30 generations, and then subjected to CHIP analysis with anti-Swi6 antibodies. First, confirming our previous result, the transient exposure to TSA resulted in a dramatic increase in *ura4-off* to *ura4-on* conversion when compared with the untreated control cells (Figure 4C, top panel). Second, in cells treated with TSA, the Swi6 localization at the *KΔ::ura4*$^+$ locus was severely disrupted (Figure 4C, bottom panel). Therefore, inhibition of histone deacetylation by TSA adversely affects propagation of the epigenetic imprint at the mating-type region and, remarkably, the loss of imprint correlates with the disruption of Swi6 localization.

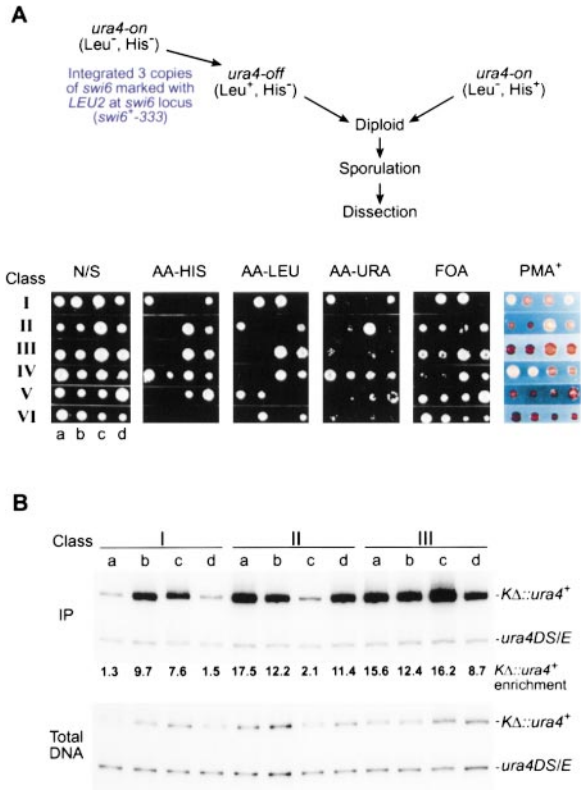

Figure 5. The Presence of Multiple Copies of *swi6* Causes Novel non-Mendelian Segregation of *ura4-on* and *ura4-off* Epialleles through Alterations in the Epigenetic Imprint at the *KΔ::ura4*$^+$ Locus

(A) Different segregation patterns produced by multiple copies of Swi6 in genetic crosses. Two copies of the *LEU2*-based plasmid containing *swi6*$^+$ were integrated in tandem at the *swi6* locus (*swi6*$^+$-*333* allele) of a *ura4-on* strain. The resultant *ura4-off* strain SPG1232 was crossed to the *ura4-on* derivative of SPG1162 to construct several independent diploids. Diploids were sporulated and subjected to tetrad analysis. Segregation of the *swi6*$^+$*-333* allele was followed by its linkage to *LEU2*. Similarly, segregation of *KΔ::ura4*$^+$ epialleles was followed by their linkage to the respective *his2* marker. Examples of the different segregation patterns obtained are shown. Among 80 tetrads analyzed, 14 class I, 32 class II, 12 class III, 7 class IV, 10 class V, and 5 class VI tetrads were observed. As expected, an increased frequency of classes IV–VI was observed when diploids were allowed to grow for a longer time, allowing more opportunities for homozygosis and Swi6 action. Growth on AA-URA or FOA indicates *KΔ::ura4*$^+$ expression, and iodine staining intensity (PMA$^+$ panel) indicates efficiency of mating-type switching.

(B) Analyses of Swi6 levels at the *mat2/3* region of class I, II, and III tetrads. Segregants from all three classes were grown further in YEA medium and subjected to CHIP analysis with anti-Swi6 antibodies.

Multiple Copies of *swi6* Cause Non-Mendelian Segregation of *ura4-off* and *ura4-on* States

The role of Swi6 in establishment of the imprint is important because it suggests that the Mendelian gene comprises more than a DNA moiety. We independently confirmed this result by using a strain (SPG1232) constructed to contain exactly three copies of *swi6* at its normal endogenous location (*swi6*$^+$*-333* allele). *ura4-off* cells of SPG1232 (*his2*$^-$) were crossed to *ura4-on* cells of SPG1162 (*his2*$^+$; 1 copy of *swi6*) and subjected to tetrad analysis. Of the six different segregation patterns observed for this cross (Figure 5A), we found that

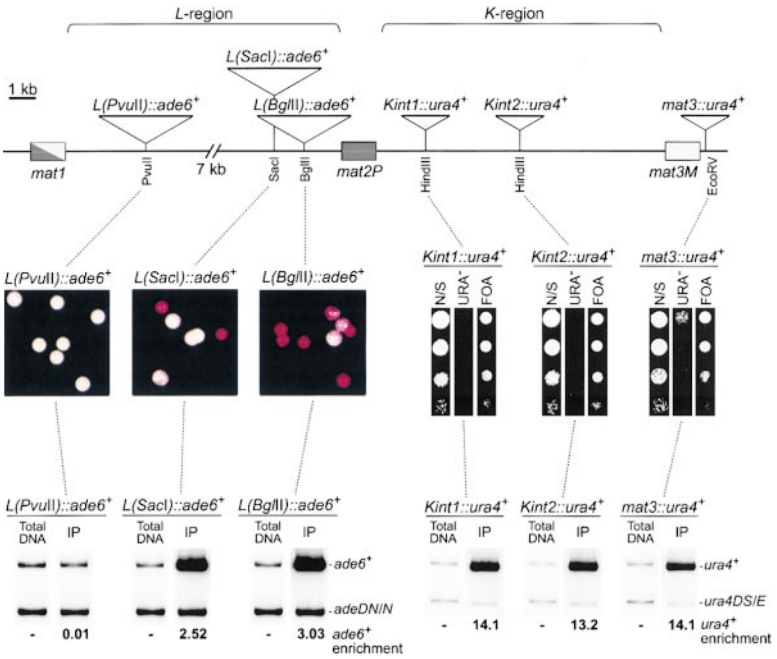

Figure 6. Swi6 Is Present throughout the *mat2/3* Interval and in Neighboring Regions

The maps of mating-type region and restriction enzyme positions where prototrophic markers were inserted are shown (top). For cultures carrying *ade6*$^+$ insertion at *L*(PvuII), *L*(SacI), and *L*(BglII), cells were plated on adenine-limiting YE medium and incubated at 33°C for 2–3 days before photography. The representative colonies of each strain are shown (left three panels in the middle). The red or white color of colonies implies Ade$^-$ or Ade$^+$ phenotypes, respectively. The expression of *ura4*$^+$ marker inserted at *Kint1*, *Kint2*, and *mat3* locations was examined by plating assay (right three panels in the middle). CHIP assay with anti-Swi6 antibodies was employed to determine the presence of Swi6 at each marker gene location. Strains used were: SPG1216, *L*(PvuII)::*ade6*$^+$; SPG1217, *L*(SacI)::*ade6*$^+$; SPG1218, *L*(BglII)::*ade6*$^+$; SPG1235, *mat3M::ura4*$^+$; SPG1236, *Kint2::ura4*$^+$; and SPG1237, *Kint1::ura4*$^+$.

classes II-V were novel, and were not observed among standard *swi6*$^+$-containing strains (Grewal and Klar, 1996). Whenever *swi6*$^+$*-333* segregated with *his2*$^-$, linked to the *ura4-off* epiallele, the usual 2 Ura$^+$:2 Ura$^-$ segregation was seen (class I), suggesting that epigenetic states are stably maintained during meiosis and inherited in *cis*. However, in tetrads in which *swi6*$^+$*-333* segregated with *his2*$^+$, originally linked to the *ura4-on* epiallele, remarkably, 1 Ura$^+$:3 Ura$^-$ (class II) or 0 Ura$^+$:4 Ura$^-$ (class III) non-Mendelian segregation patterns were observed (Figure 5A). The gain of the imprint in classes II and III confirmed that as few as three copies of *swi6*$^+$ convert *ura4-on* to *ura4-off* by changing the imprint at the *mat* locus. Moreover, once established, the *ura4-off* epistate is stable even in the absence of the *swi6*$^+$*-333* modifier. The effect of *swi6*$^+$ multiple copies was also observed during the diploid's mitotic growth, causing 0 Ura$^+$:4 Ura$^-$ segregation pattern (class V).

Diploid cells carrying a break at *mat1* undergo homozygosis of all markers located centromere-distal to the break site in ~2% of cell divisions (Egel, 1981). Interestingly, we observed that *ura4-on* and *ura4-off* epigenetic states also undergo homozygosis. As indicated by 4$^+$:0$^-$ and 0$^+$:4$^-$ segregation of *his2* marker (Figure 5A), segregation patterns observed in classes IV and VI are the result of homozygosis phenomenon. The cohomozygosing of epigenetic states with their respective *his2* alleles further reinforces the conclusion that these states are chromosomally inherited.

We next investigated whether different levels of Swi6 at the mating-type region of *ura4-on* and *ura4-off* cells are maintained during meiosis. In addition, we verified the generality of our conclusions that establishment of the epigenetic imprint in *ura4-off* cells is coupled to the recruitment of Swi6 to the *mat* locus. The meiotic segregants from classes I–III, described above, were subjected to CHIP analysis to assess the levels of Swi6 present at the *KΔ::ura4*$^+$ locus. Remarkably, different levels of Swi6 corresponding to *ura4-on* and *ura4-off* states were maintained both during the diploid's mitotic growth as well as meiosis (Figure 5B). Additionally, the gain of imprint in classes II and III was found to correlate with increased levels of Swi6 at *KΔ::ura4*$^+$, suggesting that the recruitment of Swi6 to the *mat* locus is a crucial step in establishment of the silenced state. More importantly, Swi6-containing nucleoprotein complex is maintained both during mitosis and meiosis.

Distribution of Swi6 throughout the *mat2/3* Interval and in Neighboring Regions

In addition to the ~15 kb silent *mat2–K–mat3* interval, silencing also extends into the neighboring sequences including an interval between *mat1* and *mat2*, called the *L* region, but is alleviated with increase in distance from donor loci (Ayoub et al., 1999). Marker genes introduced at different places within the silent domain are also subject to transcriptional silencing, which is dependent on the functional *clr1, clr2, clr3, clr4,* and *swi6* gene products (Thon et al., 1994; Grewal and Klar, 1997; Ayoub et al., 1999). That is, *swi6* and *clr* gene functions act upon the entire silent domain to maintain it in the repressed state. Therefore, considering the global effect of these *trans*-acting factors, it was of interest to investigate whether Swi6 protein was distributed throughout the silent domain. CHIPs analysis was used to determine the presence of Swi6 at the marker genes inserted at different locations within the silent domain. Strains bearing *ura4*$^+$ or *ade6*$^+$ gene integrated at the mating-type region, and which carry a small deletion in their corresponding endogenous gene (*ura4DS/E* or *ade6DN/N*, respectively) were employed. Data presented in Figure 6 showed that Swi6 is distributed throughout the silent domain encompassing the *mat2/3* region and the surrounding areas. Moreover, the relative abundance of Swi6 in the *L* region gradually decreases with increase

in distance from the *mat2* locus and correlates well with the stringency of repression at a particular location (Figure 6). These data are also consistent with the idea that silencing of heterologous promoters introduced at the mating-type region occurs because the heterochromatin protein complexes spread into the marker gene.

Swi6 Is Associated with the Mating-Type Region throughout the Cell Cycle

A key feature of the chromatin replication model is that the preexisting nucleoprotein complexes that are stably distributed to sister chromatids serve as an epigenetic imprint. Therefore, maintenance of the protein–DNA interactions throughout the cell cycle including the DNA replication process is crucial. Since Swi6 seems to be an important component of the imprint at the *mat2/3* region, we examined its association with the mating-type region at different stages of the cell cycle. We first analyzed the levels of total Swi6 present throughout the cell cycle. Temperature-sensitive *cdc25–22* mutant cells were arrested at the G_2/M boundary at the restrictive temperature and then allowed to grow synchronously. Cell cycle progression was followed by septation index, which peaks during the late G_1/S phase (Figure 7B). Cells were sampled every 20 min and analyzed for the presence of Swi6 and the Orp1-HA protein used as a control. As shown previously by Grallert and Nurse (1996), Orp1-HA levels remain constant at all phases of the cell cycle. Likewise, Western blotting analysis showed that Swi6 levels remain constant throughout the cell cycle (Figure 7A).

We next tested whether Swi6 was associated with the *mat2/3* region throughout the cell cycle. Synchronously growing *KΔ::ura4*$^+$ *ura4-off* cells were sampled every 20 min and subjected to CHIP analysis with anti-Swi6 antibodies. Remarkably, the *KΔ::ura4*$^+$ sequences were preferentially enriched at all time points examined (Figure 7C), indicating that the Swi6 protein was bound to the silent mating-type region throughout the cell cycle. Interestingly, the amount of Swi6 present at the *KΔ::ura4*$^+$ locus peaked during the G_2 phase but decreased slightly during passage through mitosis. Although the biological significance of increase in Swi6 levels during G_2 is not yet known, the localization of *mat2/3* region to a nuclear peripheral compartment enriched for Swi6 may play a role in this process. In any case, significant amounts of Swi6 remain bound to the mating-type region throughout the cell cycle. It is therefore possible that Swi6 itself is a crucial component of the epigenetic imprint.

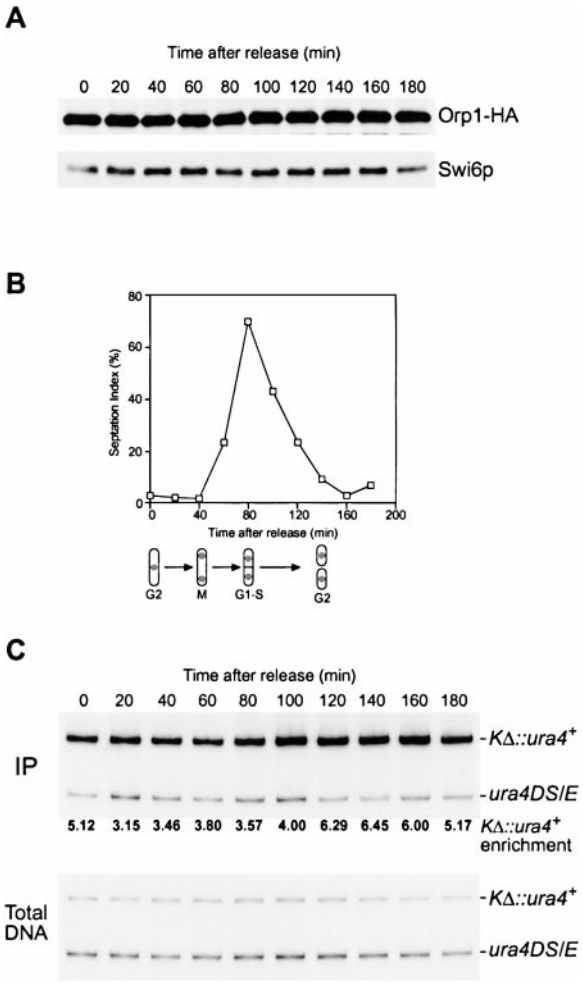

Figure 7. Swi6 Protein Levels and Association with the *mat2/3* Region through the Cell Cycle

(A) Swi6 is present throughout the cell cycle. Orp1-HA *cdc25–22* (SPG1234) cells were synchronized at the G_2/M transition by incubation for 4 hr at the restrictive temperature (36°C), released to the permissive temperature (25°C), and then sampled every 20 min. Whole cell extracts were fractionated on an SDS-PAGE gel and subjected to Western blotting analysis with anti-HA (12CA5) antibodies (top panel) or anti-Swi6 antibodies (bottom panel).

(B) The septation index is shown along with schematic illustrations of corresponding phases of the cell cycle. The septation peak roughly coincides with S phase.

(C) Swi6 is associated with the mating-type region throughout the cell cycle. *KΔ::ura4*$^+$ *ura4-off* (SPG1231) cells were synchronized and the septation index is shown in (B). Samples were collected every 20 min and subjected to CHIP analysis. Overall enrichment values in this experiment were slightly lower due to the variations in fixation conditions (see Experimental Procedures).

Discussion

The inheritance of stable chromosomal imprints helps to maintain specific patterns of gene expression. Although development of higher eukaryotes is highly complex, heritable transcriptional repression has often been hypothesized to be mediated by self-templating chromatin structures. This study addresses the molecular mechanism responsible for the establishment and inheritance of a chromosomally inherited epigenetic imprint at the mating-type region of fission yeast, which controls both silencing and the efficiency of mating-type interconversion (Grewal and Klar, 1996). We investigated whether components of the chromatin machinery, mutations in which adversely affect silencing at *mat2/3* region, act as modifiers of the epigenetic imprint. A remarkable finding is that, indeed, chromatin proteins are important determinants of the imprint. Specifically, we demonstrate that a chromodomain protein Swi6 remains bound to the mating-type region throughout the cell cycle and plays a key role in the establishment and maintenance of

Table 1. *S. pombe* Strains Used in This Study

Strain	*ura4*$^+$/*ade6*$^+$ Insertion	*mat1*	Genotype
SP819		h^{90}	*leu1-32 ade6-210*
SP837		h^{90}	*leu1-32 ura4-D18 ade6-216*
SPG16	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4 his2 ade6-210 swi6-115*
SPG27	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4 his2 ade6-210*
SPG51	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4 ade6-216*
SPG1161	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4DS/E ade6-210*
SPG1162	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4DS/E ade6-216*
SPG1077	*KΔ::ura4*$^+$	*mat1-Msmt-o*	*leu1-32 ura4-D18 ade6-216*
SPG1093	*KΔ::ura4*$^+$	*mat1-Msmt-o*	*leu1-32 ura4-D18 ade6-210 swi6-115*
SPG1216	*L*(PvuII)::*ade6*$^+$	*mat1-Msmt-o*	*leu1-32 ura4-D18 his2 ade6DN/N*
SPG1217	*L*(SacI)::*ade6*$^+$	*mat1-Msmt-o*	*leu1-32 ura4-D18 his2 ade6DN/N*
SPG1218	*L*(B*glII*)::*ade6*$^+$	*mat1-Msmt-o*	*leu1-32 ura4-D18 his2 ade6DN/N*
SPG1231	*KΔ::ura4*$^+$	*mat1-Msmt-o*	*leu1-32 ura4DS/E ade6-210 cdc25-22*
SPG1232	*KΔ::ura4*$^+$	h^{90}	*leu1-32 ura4DS/E his2 ade6-210 swi6*$^+$*-333*
SPG1234		h^+	*cdc25-22 orp1-HA*
SPG1235	*mat3M::ura4*$^+$	h^{90}	*leu1-32 ura4DS/E ade6-216*
SPG1236	*Kint2::ura4*$^+$	h^{90}	*leu1-32 ura4DS/E his2 ade6-216*
SPG1237	*Kint1::ura4*$^+$	h^{90}	*leu1-32 ura4DS/E his2 ade6-216*

the imprint. That is, the persistence of macromolecular protein complexes associated with the mating-type region DNA presumably contributes to the maintenance of the silenced state during mitosis and meiosis. Therefore, the unit of inheritance, that is, the "gene", at the mating-type region comprises DNA plus the associated chromatin proteins, such as Swi6.

Imprinting Cellular Memory into the Chromatin: Role of a Chromodomain Protein Swi6 in Epigenetic Inheritance

The Swi6 protein is an essential structural component of centromeres, telomeres, and the mating-type locus (Allshire, 1996). Mutations in Swi6 alleviate recombination suppression and silencing at the *mat2/3* region and disrupt nonrandom utilization of donor loci during mating-type switching (Klar et al., 1998). A proposed interpretation of its multiple roles is that Swi6 participates in formation of heterochromatin-like structures essential for silencing and mating-type switching. We found that Swi6 is present throughout the silent domain in *mat2/3* region and spreads into neighboring sequences. More importantly, Swi6 is a dosage-critical component required for establishing and maintaining an epigenetic imprint, which controls propagation of the silenced state. Remarkably, transient presence of multiple copies of *swi6* altered the structural and functional imprint at the *mat2/3* region, resulting in a heritable change from an inefficiently switching expressed state (*ura4-on*) to an efficiently switching repressed state (*ura4-off*). This Swi6-induced change is inherited in *cis* and is remarkably stable during mitosis and meiosis. Supporting our genetic data, biochemical analysis revealed that *ura4-off* cells contain several-fold higher levels of Swi6 at their mating-type region when compared with *ura4-on* cells. Moreover, establishment of the *ura4-off* epigenetic state is tightly coupled to the recruitment of Swi6 at the *mat2/3* interval. Once recruited, however, Swi6 remains associated with the mating-type region throughout the cell cycle (Figure 7), providing a molecular bookmark to clonally propagate a specific chromatin configuration, hence maintaining the silenced state during cell division.

Previously, we have shown that when strains showing *ura4-off* and *ura4-on* states are crossed to construct a diploid, each chromosome maintains its respective epigenetic state (Grewal and Klar, 1996). In this regard, we found that despite sharing the same nuclear environment, *ura4-off* and *ura4-on* epialleles maintained differential Swi6 localization patterns during the diploid's mitotic growth as well as during meiosis (Figure 5). Therefore, the persistence of Swi6-containing nucleoprotein complex assembled at the *mat* region, rather than diffusible factors, was likely to serve as a cellular memory. Since Swi6 localizes to the mating-type region presumably through protein–protein interactions (Ekwall et al. 1996), it is unlikely, however, that it alone is responsible for the maintenance of the imprint.

Recently, it was observed that transient inhibition of histone deacetylases by TSA treatment "erases" the epigenetic imprint at centromeric and *mat2/3* regions and induces a heritable increase in histone acetylation (Ekwall et al., 1997; Grewal et al., 1998). Therefore, the maintenance of the hypoacetylated chromatin-state and the presence of histone deacetylase at these silenced regions were postulated to be critical in propagation of the imprint. In this regard, we found that histone deacetylases Clr3 and Clr6 (Grewal et al., 1998) are essential but not limiting components for assembly of the silenced state; instead, Swi6 seems to be a dosage-critical factor essential for resetting the epigenetic memory (Figure 1). Besides, TSA-induced change in imprint seems to be mediated through stable change in Swi6 localization pattern at the *mat* locus (Figure 4C). We propose that a self-perpetuation mechanism might be in place, in which recruitment of Swi6 to chromatin is dependent upon its association with deacetylated histones. Once recruited, however, it might stabilize the localization of other factors including histone deacetylases, promoting the maintenance of the silenced state. Consistent with this hypothesis, we found that mutation in Swi6 results in elevated levels of histone acetylation at the mating-type region (Figure 4B).

In *S. cerevisiae*, the silencer-mediated repression of silent mating-type loci is dependent on their proximity

to telomeres. It has been suggested that unequal distribution of silencing factors (e.g., Sir3 and Sir4) at the telomeric repeats creates a specialized silencing compartment within the nucleus, increasing the access of silencer sequences to the pool of Sir factors (Maillet et al., 1996). Since Swi6 protein is mainly concentrated at two to three foci at the periphery of haploid nuclei (Ekwall et al., 1996), its concentration may be limiting in other parts of the nucleus. Therefore, the establishment of the silenced state might involve localization of the *mat* locus to a specific nuclear compartment, such as an environment enriched for heterochromatin proteins, by a stochastic event. Furthermore, the overexpression of Swi6 might increase its concentration throughout the nucleoplasm, causing efficient conversion of expressed state to repressed state.

We found that Swi6 remains bound to the mating-type region throughout the cell cycle, including S phase. Although the biochemical basis of Swi6 association with the *mat2/3* region remains to be further explored, our recent work suggests that DNA polymerase α (Polα) might play a crucial role in this process (J.-i. N., unpublished data). A mutation in Polα affects both recruitment and maintenance of Swi6 at the mating-type region and causes a 45-fold increase in spontaneous transition from the *ura4-off* state to the *ura4-on* state. Genetic analysis showed that Swi6 and Polα affect silencing through the same pathway. Furthermore, we found that Swi6 shows direct physical interaction with Polα in vitro. Therefore, DNA replication machinery might be directly involved in replicating the epigenetic imprint by maintaining Swi6 protein at the imprinted locus. In *Drosophila*, a homolog of Swi6, the HP-1 protein, is found in a complex with the origin recognition complex (ORC) (Pak et al., 1997). Since, ORC homologs are also found in fission yeast and are present at replication origins throughout the cell cycle (Ogawa et al., 1999), it is possible that Swi6 might also interact with these proteins, providing an independent mechanism for its persistence at the *mat* locus. Alternatively, it can be imagined that chromatin assembly factor 1 (CAF-1), which physically associates with the DNA replication machinery (Shibahara and Stillman, 1999) and HP-1 family members (Murzina et al., 1999), might be involved.

Chromatin Assembly and Epigenetic Inheritance in Other Systems

Although DNA methylation and chromatin structure have been proposed to act in concert to govern stable inheritance of gene expression patterns in higher eukaryotes, chromatin-mediated mechanisms alone seem to be sufficient to control heritable repression of transcription in short-lived organisms. In addition to the enzymes modifying the histones, several *trans*-acting genes that encode nonhistone chromatin proteins have been identified in different model systems, which function in epigenetic control of gene expression. For instance, Sir3 and Sir4, which are essential components of heterochromatin in *S. cerevisiae*, participate in epigenetic gene silencing (Grunstein, 1998), but these factors do not have any obvious orthologs in other eukaryotes. In contrast, silencing factors in *S. pombe* closely resemble similar proteins from multicellular organisms. In particular, genetic screens in *Drosophila* have revealed several chromosomal proteins that are involved in regulation of position-dependent gene activity (Wallrath, 1998). Among these, chromodomain-containing proteins HP-1 and *Suvar3–9*, which share structural and functional similarities with heterochromatin proteins from vertebrates (Aagaard et al., 1999), represent homologs of *S. pombe* Swi6 and Clr4, respectively. Interestingly, HP-1 and *Suvar3–9* display dosage dependent modification of PEV (reviewed in Singh, 1994). However, it is not known whether their effect persists even in the absence of the modifier allele. In this regard, our results showing the persistence of Swi6-induced change in the epigenetic imprint, which is stably propagated even in the absence of modifier allele, are novel. In another closely related example, chromodomain containing polycomb proteins appear to be central component in heritable inactivation of homeotic genes in *Drosophila* (Paro, 1993), but the quantitative differences in their association with active and inactive chromatin states remains to be shown. Since chromodomain-containing proteins are also found in other evolutionarily unrelated systems, it is possible that protein-based mechanisms of epigenetic inheritance might be responsible for phenotypic variations or the increased risk to diseases within populations. The meiotic stability of the Swi6-containing protein complex in *S. pombe* (this study; Grewal and Klar, 1996) and PcG-mediated transcriptional states in *Drosophila* (Cavalli and Paro, 1998) further support this possibility. Moreover, the meiotic inheritance of epigenetic modifications has also been observed in the mouse (Morgan et al., 1999).

Apart from transcription, epigenetic events have been shown to affect other aspects of chromosome architecture, such as inter- or intrachromosomal recombination and chromosome segregation (Grewal and Klar, 1997; Karpen and Allshire, 1997; Maloisel and Rossignol, 1998), and they have implications for the maintenance of genome integrity. The importance of these secondary roles should not be underestimated. In general, homology encourages recombination. Nevertheless, despite the presence of interspersed repetitive sequences throughout the intergenic regions of higher eukaryotic genomes, recombination is limited to gene-rich regions (SanMiguel et al., 1996), avoiding potentially deleterious effects on the integrity of the genome. Recent studies have suggested that abnormal gene expression caused by epigenetic changes also play a causal role in cancer progression as exemplified by studies on Wilm's tumor (reviewed by Jones and Laird, 1999). In fact, certain carcinogens and environmental factors might act by altering epigenetic imprints rather than causing genetic mutations (Klein and Costa, 1997). A deeper understanding of the mechanisms of epigenetic inheritance is therefore of critical importance. Since silencing factors in fission yeast closely resemble proteins from higher eukaryotes, any conclusions drawn from these studies are expected to have direct implications for human biology and disease.

Experimental Procedures

Strains

The genotypes of the *S. pombe* strains used in this study are listed in Table 1. The *swi6*$^{+}$*-333* allele was constructed by integrating two

additional copies of *swi6*$^+$ marked with *S. cerevisiae LEU2* gene at the *swi6* locus. The strains containing *ura4*$^+$ or *ade6*$^+$ at the *mat2/3* region were made as part of previous studies (Thon and Klar, 1992; Grewal and Klar, 1996, 1997; Ayoub et al., 1999). The construction of *orp1-HA, ura4DS/E*, and *ade6DN/N* alleles was described previously (Grallert and Nurse, 1996; Ekwall et al., 1997). The *mat1M-smto* allele, which abolishes the break at the *mat1* locus, is described in Engelke et al. (1987). Standard genetic crosses were used to construct all other strains.

Iodine Staining Assay

Efficiency of mating-type switching was analyzed by the iodine staining assay. Individual colonies were replicated onto sporulation (PMA) medium and then grown for 3 days at 25°C, before being exposed to iodine vapors. The dark staining indicates efficient mating-type switching, while streaks/sectors or light staining indicates inefficient switching.

Antibodies

Anti-Swi6 antisera were raised against peptides corresponding to the N and C termini of the Swi6 protein. The crude rabbit antisera were affinity purified against purified recombinant Swi6 protein by using NHS-activated HiTrap system (Amersham Pharmacia). The purified antibodies against the N and C termini of Swi6 were mixed and used for CHIP and Western blotting analyses.

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed as described in Ekwall and Partridge (1999) with some modifications. Exponentially growing yeast cells (5×10^8 cells at 1×10^7 cells/ml) were incubated at 18°C for 2 hr and then fixed for 30 min in 3% paraformaldehyde. In the cell cycle experiments with *cdc25–22* mutants, the incubation time at 18°C was reduced to 5 min and crude lysates from a half number of the cells (2.5×10^8) were used. DNA from anti-Swi6 immunoprecipitated fractions was isolated and subjected to PCR analysis. PCR products were labeled by adding 0.25 μl of 10 mCi/ml of [α-^{32}P]dCTP (Amersham Pharmacia), separated on a 4% nondenaturing polyacrylamide gel, and then quantified using a phosphoimager (Fuji BAS 2000, Fuji Medical Systems). The primer sets used were: for *ura4*$^+$/*ura4DS/E*, ura4#1 (5′-GAGGGGATGAAAAATC CCAT-3′) and ura4#2 (5′-TTCGACAACAGGATTACGACC-3′); for *ade6*$^+$*/ade6DN/N*, ade6#1 (5′-TGCGATGCACCTGACCAGGAAAGT-3′) and ade6#2 (AGAGTTGGGTGTTGATTTCGCTGA-3′); for *K-L*, 17868 (5′-GATGCGATCTTTTATGCATC-3′) and NSA423 (5′-CGTTTCGGA GTTCAGACTGATCTCGC-3′); and for *K-R*, 11557 (5′-GTATGTG GAACAAGAGAAG-3′) and 17879 (5′-CTCGCCTGCTTACATTTTA AGG-3′). To calculate the enrichment of *ura4*$^+$, the ratio of *ura4*$^+$ and *ura4DS/E* signals present in total DNA prepared from crude extract was used to account for nonspecifically precipitated background signal.

Acknowledgments

We thank A. Cohen, R. Allshire, and P. Nurse for providing strains, H. Schmidt for providing *swi6*-containing plasmid and C. D. Allis for histone antibodies. We are grateful to R. Allshire and J. Partridge for help with Swi6 CHIP methodology and R. Martienssen for critical reading of the manuscript. G. Thon constructed plasmid pTG16. This research was sponsored in part by Ellison Medical Foundation, National Institutes of Health (R01 GM59772-01A1), and by the National Cancer Institute.

Received January 7, 2000; revised March 21, 2000.

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