A role for DNA polymerase α in epigenetic control of transcriptional silencing in fission yeast

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In the fission yeast Schizosaccharomyces pombe, transcriptional silencing at the mating-type region, centromeres and telomeres is epigenetically controlled, and results from the assembly of higher order chromatin structures. Chromatin proteins associated with these silenced loci are believed to serve as molecular bookmarks that help promote inheritance of the silenced state during cell division. Specifically, a chromodomain protein Swi6 is believed to be an important determinant of the epigenetic imprint. Here, we show that a mutation in DNA polymerase α (polα) affects Swi6 localization at the mating-type region and causes a 45-fold increase in spontaneous transition from the silenced epigenetic state to the expressed state. We also demonstrate that polα mutant cells are defective in Swi6 localization at centromeres and telomeres. Genetic analysis suggests that Polα and Swi6 are part of the same silencing pathway. Interestingly, we found that Swi6 directly binds to Polα in vitro. Moreover, silencing-defective mutant Polα displays reduced binding to Swi6 protein. This work indicates involvement in DNA replication protein, Polα, in heterochromatin assembly and inheritance of epigenetic chromatin structures.

Keywords: DNA polymerase/epigenetic/fission yeast/heterochromatin/silencing

Introduction

During cellular differentiation, the fate of individual cells is determined by specifying unique patterns of gene expression. Once established, these expression patterns are faithfully maintained through multiple rounds of cell division. It is believed that differentiated cells rely on an epigenetic cellular memory system, whereby non-mutational reversible alterations of the chromosomes promote stable transmission of determined states to progeny cells. Self-templating chromatin structures and/or DNA methylation-mediated mechanisms have been suggested to be important for the inheritance of epigenetic states through DNA replication and chromosome duplication (reviewed in Riggs and Porter, 1996; Grewal, 2000).

Epigenetic control of higher order chromatin assembly has been observed in fission yeast at the mating-type (mat2/3) region, centromeres and telomeres (Allshire, 1996; Grewal, 2000). Prototrophic markers artificially inserted at these loci are subject to transcriptional silencing and exhibit variegated expression patterns (Thon et al., 1994; Allshire et al., 1995; Grewal and Klar, 1996; Ayoub et al., 1999). Several trans-acting factors that affect silencing at the 15 kb mat2/3 interval and centromeres are either structural components of heterochromatin or histone-modifying enzymes. Among these factors, clr3 and clr6 belong to the family of histone deacetylases (HDACs; Grewal et al., 1998). Swi6; Clr4, Chp1 and Chp2 proteins contain a chromodomain, an evolutionarily conserved motif initially identified in heterochromatin-associated protein HP1 and polycomb in Drosophila (Lorentz et al., 1994; Ivanova et al., 1998; Eisenberg and Elgin, 2000; Thon and Verhein-Hansen, 2000). Clr4, which possesses intrinsic histone methyltransferase activity (Rea et al., 2000), recently was shown to methylate Lys9 preferentially on histone H3 tail at the mat2/3 region and centromeres (Nakayama et al., 2001). It was also demonstrated that Clr4 cooperates with a histone H3-specific HDAC Clr3 to establish a ‘histone code’ that is essential for higher order chromatin assembly (Nakayama et al., 2001). Moreover, the localization of Swi6 to the silenced loci is mediated through its binding to the H3 Lys9-methyl mark (Bannister et al., 2001; Nakayama et al., 2001). The Swi6 protein is present throughout the silent mat2/3 interval but its presence at centromeres is confined to the outer repeat sequences (Nakayama et al., 2000; Partridge et al., 2000).

How the silenced chromatin state is faithfully maintained during cell division remains unclear. Several lines of evidence have indicated a connection between the DNA replication machinery and silencing. For example, mutations in subunits of the origin recognition complex (ORC) cause derepression of silent mating-type loci in Saccharomyces cerevisiae (Micklem et al., 1993; Bell et al., 1995; Fox et al., 1995) and suppress position effect variegation (PEV) in Drosophila (Pak et al., 1997). Furthermore, chromatin assembly factor 1 (CAF-1), which physically associates with proliferating cell nuclear antigen (PCNA) and HP1 family members (Murzina et al., 1999; Shibahara and Stillman, 1999), participates in inheritance of epigenetic chromatin states (Enomoto and Berman, 1998; Zhang et al., 2000).

Previously, we showed that Swi6 is an integral component of the epigenetic cellular memory mechanism and might serve as a molecular bookmark to propagate the silenced chromatin state during cell division (Nakayama et al., 2000). Here, we show that a mutation in an essential gene, swi7 (Singh and Klar, 1993), encoding the catalytic subunit of DNA polymerase α (Polα; Damagnez et al., 1991), adversely affects silencing and Swi6 localization at the mating-type region and centromeres. We also demonstrate that Swi6 directly interacts with Polα in vitro.
This study supports involvement of Polα in epigenetic control of higher order chromatin assembly.

Results

Mutation in polα suppresses PEV at the mating-type region

swi7/polα and two other genes (swi1 and swi3), which genetically interact with swi7, were shown previously to be crucial for establishment of an imprint at mat1 that is essential for mating-type switching (reviewed in Klar et al., 1998; Grewal, 2000). We tested whether mutations in these genes also affect transcriptional repression of the KΔ::ural* marker gene at the mat locus. The KΔ::ural* cells containing substitution of 7.5 kb of the K region with ura4* (see Figure 1A) exhibit variegation of ura4* expression through an epigenetic mechanism (Grewal and Klar, 1996). Furthermore, ura4-off and ura4-on epigenetic states representing ‘closed’ and ‘open’ chromatin structures, respectively, are inherited in cis during both mitosis and meiosis (Grewal and Klar, 1996; Nakayama et al., 2000). By genetic crosses, the mutations in swi1, swi3 and swi7/polα were combined with the ura4-off derivative of a non-switching (mat1-Mmnto; Styrkarsdottir et al., 1993) KΔ::ural* allele. When replicated onto medium lacking uracil (AA-URA), we found that colonies formed by ura4-off cells carrying the swi mutations showed significantly higher levels of Ura*
Fig. 2. Mutation in *swi7/pola* causes *mat2-P* derepression. (A) A schematic diagram of the *mat2/3* region. The trans-acting loci, *clr1-clr4* and *swi6*, affect silencing in the entire *mat2/3* interval and define one silencing pathway, while the cis-acting sequences between *BglII* and *BsaHI* sites, indicated by the black box, define a second pathway specific for *mat2-P* silencing. (B) Effect of *swi* mutations and Δ(Bg-Bs)-*mat2-P* deletion on *mat2-P* expression. RT–PCR analysis of *mat2-P* transcripts was performed as described in Materials and methods. Strains used were as follows: *mat1-Mssto* *mat2-P* derivatives, SPI125 (WT), SPI814 (swi1) SPI815 (swi3) and SPI151 (swi7-1); *mat1-Mssto* Δ(Bg-Bs)-*mat2-P* derivatives, SPI152 (WT), SPIG153 (swi1), SPIG154 (swi3) and SPIG152 (swi7-1). (C) Analysis of *mat2-P* derepression by iodine staining. The colonies of *mat1-Mssto* or *mat1-Mssto* Δ(Bg-Bs)-*mat2-P* cells carrying the wild-type (swi7+) or mutant *swi7* (swi7-) allele were sporulated and exposed to iodine vapor before being photographed. Because *mat1-Mssto* strains fail to switch, their intensity of brown staining indicates the level of ‘haploid meiosis’, a phenotype caused by expression of both *P* and *M* information in haploid cells, thus reflecting the level of *mat2-P* derepression.

sectors as compared with their wild-type counterpart (Figure 1B), indicating an increase in *ura4*-off to *ura4*-on transition in mutant backgrounds. Serial dilution analysis and a fluctuation test to measure the effect of mutations quantitatively revealed that the *swi7/pola* mutant had the strongest (45-fold) effect (Figure 1C). Further genetic analysis revealed that the *swi7*-induced *ura4*-on state was segregating with the *mat* region (data not shown), suggesting that the mutation altered the imprint at the *mat* locus.

It has been shown that the silencing domain extends across the interval between *mat1* and *mat2*, called the *L* region (Ayoub et al., 1999). An ade6+ reporter gene inserted on the left side of the *mat2* locus is subject to PEV. To test whether *swi7/pola* mutation globally affects silencing at the mating-type region, we also analyzed its effect on the expression of the ade6+ marker integrated at two sites in the *L* region: *L(BglIII):ade6+* and *L(SacI):ade6+* located 1.5 and 2.5 kb from the *mat2* locus, respectively (Figure 1A). The expression of ade6+ was monitored by plating cells on low-adenine (YE) medium. The occurrence of red or white colonies on this medium implies ade6-off and ade6-on phenotypes, respectively. In the wild-type background, expression of *L(BglIII):ade6+* and *L(SacI):ade6+* was variegated, resulting in a mixture of red and white colonies, as shown previously (Ayoub et al., 1999). Mutation in *swi7/pola* suppressed variegation of marker gene expression at both locations, resulting in formation of white colonies by the entire population of cells (Figure 1D). Based on these data, it was concluded that *swi7/pola* is essential for silencing at the *mat2/3* interval and in the neighboring regions.

The *pola* mutation affects donor mating-type loci silencing

We next tested whether mutations in *swi7/pola* as well as *swi1* and *swi3* also affect donor loci silencing. Previously, it has been shown that silencing of the *mat2-P* and *mat3-M* loci is controlled by at least two overlapping or redundant mechanisms. Trans-acting factors *clr1-clr4* and *swi6*, which have a weak effect on donor loci silencing but alleviate repression of markers at different locations, are involved in silencing the entire *mat2/3* region and neighboring sequences (see Figure 2A). The second pathway is defined by *cis*-acting sequences located near *mat2* (Thon et al., 1994) and *mat3* (Thon et al., 1999). Unlike the global effect of *clr1-clr4* and *swi6*, the effect of these repressor elements seems to be specific to sequences around *mat2* and *mat3*. Consistent with redundant silencing mechanisms, the chromosomal deletion of a 1.5 kb *BglIII–BsaHI* fragment on the centromere-proximal side of *mat2-P* [Δ(Bg-Bs)-*mat2-P*], which removes *cis*-acting elements, does not derepress *mat2* noticeably. However, the same deletion in combination with a mutation in *clr1-clr4* or *swi6* causes cumulative derepression (Thon et al., 1994). To test whether *swi7/pola* is involved in specific or global silencing, or both, we constructed pairwise combinations of mutations in *clr1, clr2, clr3, clr4* and *swi6* or a Δ(Bg-Bs)-*mat2-P* deletion with the *swi7-1* mutant allele. RT–PCR analysis and/or an iodine staining assay (see Materials and methods for details) were used to measure
the level of mat2-P expression and haploid meiosis, a phenotype caused by concurrent expression of both P and M information in haploid cells. We found that mutation in swi7/polα did not show a cumulative effect in combination with clr1-5, clr2-760, clr3-735, clr4-681 and swi6-115 alleles (data not shown). However, when combined with the Δ(Bg-Bs)-mat2-P deletion, a significant cumulative derepression was seen (Figure 2B and C). Therefore, swi7/polα also affects mat2-P silencing and it appears to act in the same pathway as clr1-clr4 and swi6. We also tested the effect of swi1 and swi3 mutations on the expression of mat2-P. Although mutations alone did not cause significant derepression, a cumulative derepression of mat2-P was observed when swi3-146 and swi1-282 mutations were combined with the Δ(Bg-Bs)-mat2-P allele (Figure 2B)

Mutation in swi7/polα affects Swi6 localization at the mat2/3 region

Genetic and biochemical evidence suggests that Swi6 is an essential structural component of heterochromatin at the mating-type region (Lorentz et al., 1994; Grewal and Klar, 1997; Nakayama et al., 2000). Given that Polα and Swi6 seem to be part of the same silencing pathway, it was possible that swi7/polα mutant cells might be defective in Swi6 localization at the mat locus. We directly addressed this possibility by comparing Swi6 levels at the mating-type region of wild-type and mutant cells using the chromatin immunoprecipitation (CHIP) technique. Briefly, chromatin fractions prepared from formaldehyde-treated cells were fragmented and then immunoprecipitated (IPed) with affinity-purified anti-Swi6 antibodies (Nakayama et al., 2000). The DNA recovered from IPed fractions was quantitated using a competitive PCR strategy, whereby one primer pair amplifies 694 and 426 bp products from full-length ura4+ inserted at the mat locus and the control ura4DS/E mini-gene at the endogenous location, respectively (Figure 3A). This approach allows the relative levels of the ura4+ sequences, in comparison with the ura4DS/E DNA, in IPed fractions to be determined accurately. The levels of Swi6 present at ura4+ inserted adjacent to mat2-P (mat2-P::ura4+) were assayed. Data presented in Figure 3B revealed that swi7/polα mutant cells are defective in silencing as well as Swi6 localization at mat2-P::ura4+. Thus, mutation in polα affects silencing by altering Swi6 localization and chromatin organization at the mating-type region.

We recently showed that the presence of multiple copies of swi6+ results in efficient conversion of the ura4+ on state to the ura4-off state in a KΔ::ura4+ strain, and this change correlates with a stable increase in Swi6 levels at the mat2/3 region (Nakayama et al., 2000). To investigate further the involvement of Polα in silencing and Swi6 localization, we tested whether Swi6-induced transition from the expressed state to the silenced state is affected by the swi7/polα mutation. Three copies of swi6+ inserted at its endogenous chromosomal location (swi6+·333 allele; Nakayama et al., 2000) were combined with the ade6+ on derivative of a KΔ::ade6+ strain by using genetic crosses. As expected, the presence of the swi6+·333 allele resulted in efficient ade6+ on to ade6-off transitions in swi6+ cells but no transition was observed in swi6− cells (Figure 4A). These data were confirmed further by using KΔ::ura4+ strains. Again, the presence of three copies of swi6 caused a significant increase in ura4+ to ura4-off transition in swi7+ cells but no change in state was observed in swi7− cells (Figure 4B, top panel). More importantly, CHIP analysis showed that the overexpression of Swi6 results in a notable increase in its levels at the mating-type region of swi7+ cells, as observed previously, but no detectable increase in Swi6 levels was observed at the mating-type region of swi7− cells (Figure 4B). Therefore, the swi6+·333-promoted increase in Swi6 levels at the mat2/3 region requires a functional swi7/polα gene product. Taken together with the results described above, these data suggest that swi7/polα mutant cells are defective in Swi6 localization at the mating-type region.
et al., 1995; Grewal et al., 1998). We investigated whether swi7/pola mutation affects silencing of markers artificially inserted into centromeric sequences. For this purpose, the swi7-1 allele was combined with ura4+ integrated at three different locations within cen1: one each at outer repeat regions otr and imr and one at the central domain cnt1 (Allshire et al., 1995; Figure 5A, top panel). Dilution analysis revealed that a mutation in swi7/pola caused derepression at all three locations tested though its effect at the cnt location was subtle (Figure 5A, middle panel).

We next used CHIPS to test whether the swi7/pola mutant cells are defective in Swi6 localization at cen1. An ~5-fold decrease in Swi6 levels at the otr and imr repeats was observed in swi7- cells, when compared with their swi7+ counterparts (Figure 5A, bottom panel). In contrast to the otr and imr repeats, Swi6 levels at the cnt region were found to be almost negligible (Figure 5A; Partridge et al., 2000). Hence, the possible effect of swi7/pola mutation on Swi6 localization in this region could not be ascertained. We also analyzed the effect of swi7/pola mutation on Swi6 localization at telomeres. Data presented in Figure 5B indicate reduced levels of Swi6 at telomeres of swi7- cells when compared with swi7+ cells. These results suggest that in addition to the mat2/3 region, swi7/ pola mutant cells are also deficient in Swi6 localization at the centromeres and telomeres.

Direct interaction between Polα and Swi6
Polα might affect Swi6 localization at heterochromatic loci by interacting with it directly. To test this, protein extracts from wild-type or swi7/pola mutant cells were loaded onto a Swi6 affinity column or a blank column as described in Materials and methods. Analysis of the eluted fractions showed that Polα binds to a His6-Swi6 affinity column but not to a control blank column (Figure 6A). Significantly, the swi7-1 mutation in Polα that adversely affects silencing and Swi6 localization at heterochromatic domains also affects interaction between these proteins (Figure 6A). To characterize further the interaction of Polα with Swi6 protein, we tested whether these proteins interact directly. In vitro translated deletion derivatives representing the N- (amino acids 1–635) or C-terminal (amino acids 1032–1405) part of Polα were incubated with glutathione S-transferase (GST)–Swi6 or GST alone, and then subjected to GST pull-down assays. As shown in Figure 6B, the C-terminal region of Polα bound directly to the GST–Swi6 fusion protein, whereas luciferase protein, used as a non-specific binding control, or the N-terminal region of Polα did not bind to Swi6. Therefore, the C-terminal region of Polα, the domain containing the G1116E change in swi7-1 mutant cells (Singh and Klar, 1993), is sufficient for its interaction with Swi6 protein. As further confirmation of this result, we found that MBP–Polα, containing amino acids 635–1405 of Polα fused to maltose-binding protein, bound to His6-Swi6 (data not shown). This interaction seems to be mediated through Polα as MBP alone did not bind to Swi6. In summary, our results argue that Polα physically interacts with Swi6 protein in vitro and that this interaction might be mediated, at least in part, through the C-terminal region of Polα.

The role of Polα in Swi6 localization was also investigated using immunofluorescence. As observed

The polα mutation also affects Swi6 localization at centromeres and telomeres
In addition to the mat2/3 region, fission yeast centromeres are also assembled into silent heterochromatin-like structures. Moreover, factors affecting silencing at the mating-type region also affect centromeric silencing (Allshire A. 4. swi6-333-mediated ‘on’ to ‘off’ state transition and the corresponding increase in Swi6 levels at the mating-type region require swi7/pola: (A) Mutation in polα affects ade6-on to ade6-off transition. Three copies of swi6- inserted at its normal endogenous location (swi6-333 allele) were combined with ade6-on derivatives of Kα:ade6+ swi7- or Kα:ade6+ swi7+ strains by using genetic crosses. The resulting strains were grown on adenine-limiting YE medium before being photographed. Formation of red colonies by swi7+ cells indicated efficient ade6-on to ade6-off transition. In contrast, no change in ade6+ expression state was observed in swi7- cells as suggested by formation of white colonies by all cells. Strains used were SPG1304 (WT) and SPG1305 (swi7). (B) The Kα:ura4+ ura4-on derivatives of the swi7+ and swi7- strains were combined with swi6-333. The resultant cultures were subjected to CHIP analysis and serial dilution plating assay to assess the levels of Swi6 at the mat locus and Kα:ura4+ expression, respectively. Strains used were SPG1301 (swi7) and SPG1302 (WT).
Fig. 5. Mutation in swi7/pola affects Swi6 localization at centromeres and telomeres. (A) A map of the cenl indicating marker gene insertion sites (not drawn to scale) and results from serial dilution plating analysis are shown (top and middle panel). CHIP analysis was used to quantify Swi6 levels at cen, imr and otr sites within cenl (bottom panel). Strains used were: cen1:ura4+, FY336 (WT) and SPG1060 (swi7); imr1:ura4+, FY498 (WT) and SPG1062 (swi7); otr1:ura4+, FY468 (WT) and SPG1067 (swi7). (B) Multiplex PCR was used to examine Swi6 levels at telomeres. DNA isolated from crude extracts or anti-Swi6 IPed fractions was analyzed for telomere-associated sequences (TAS) as well as mat2−P:ura4+ and ura4DS/E sequences, used as controls. A diagram of a telomere indicating TAS, short repeats (gray triangles) and PCR primer-binding sites (black triangles) is shown (top panel). The PCR product marked with an asterisk resulted from primers binding to an additional site within TAS, as confirmed by its cloning and sequencing. Strains used were SPG1228 (WT) and SPG1229 (swi7).

Previously (Ekwall et al., 1996), wild-type cells showed normal Swi6 staining with 2–4 foci at the nuclear periphery (Figure 7). In comparison, Swi6 localization was disrupted in swi7/pola mutant cells, resulting in a diffuse/punctate pattern of nuclear staining in the majority of cells, while a small proportion of cells showed three or more faint spots. Taken together with the in vitro binding experiments, these data support our genetic and CHIP analysis showing a decrease in silencing and Swi6 levels at heterochromatic regions in swi7/pola mutant cells.

Discussion

Chromatin-based epigenetic imprints marking the mating-type region and centromeres contribute to maintenance of the higher order chromatin structure, which controls
transcriptional silencing at these loci (Grewal and Klar, 1996; Ekwall et al., 1997; Nakayama et al., 2000). A key component of heterochromatin in fission yeast, Swi6, is believed to be an important determinant of the epigenetic imprint at the mat locus (Nakayama et al., 2000). We demonstrate that a mutation in DNA replication factor Polα suppresses variation of marker gene expression at the mat2/3 interval and centromeres. Moreover, biochemical analyses revealed that Polα interacts with Swi6 and that swi7/polα mutant cells are defective in Swi6 localization at the mating-type region, centromeres and telomeres. Taken together, these observations indicate that Polα might participate in higher order chromatin assembly at the silenced chromosomal domains.

However, the requirement for Polα in silencing is coupled to its role in DNA replication in general remains to be explored. It seems unlikely, however, because the swi7-1 mutation does not seem to increase UV sensitivity significantly as expected for reduced primase activity (Schmidt et al., 1989). Also, the mutation is not located in any of the regions conserved in the DNA polymerases of other species (Singh and Klar, 1993). In budding yeast, mutations in Polα cause lengthened telomeres (Smith et al., 1999; Adams Martin et al., 2000). It was therefore possible that variations in telomere length in the swi7/polα mutant might influence silencing by redistributing a limited pool of Swi6 protein to the telomere compartment, causing a decrease in Swi6 levels at the mating-type region and centromeres. However, we found that in addition to the mat2/3 region and centromeres, Swi6 levels at the telomeres of swi7/polα mutant cells were also significantly decreased. We suggest a model whereby a distinct role for Polα, in conjunction with other factors including Clr4 and Rik1 that affect Swi6 localization (Ekwall et al., 1996; Bannister et al., 2001; Nakayama et al., 2001), may be to recruit and maintain Swi6 at the heterochromatic loci. That is, there may be different factors contributing to nucleation and/or maintenance of heterochromatin structures, but each may feed into a common pathway leading to heterochromatin assembly. SIR proteins in budding yeast were shown recently to move to new locations inside the nucleus in response to DNA damage (Martin et al., 1999; Mills et al., 1999). Similarly, it can be imagined that the delocalization of Swi6 in swi7/polα mutant cells might occur in response to signals sent by changes in DNA. Another possibility is that the changes in replication timing of the silenced chromosomal domains in mutant cells affect heterochromatin assembly. In this model, Swi6 protein, through its association with Polα, might control the timing of replication and, hence, epigenetic marking at mat and cen loci.

Epigenetic inheritance of silencing has several links to the DNA replication machinery in other organisms. Apart from components of the ORC complex (Micklem et al., 1993; Bell et al., 1995; Fox et al., 1995), mutations in PCNA, RF-C, Polε and Polα affect silencing in budding yeast (Ehrenhofer-Murray et al., 1999; Smith et al., 1999; Zhang et al., 2000). However, the precise role of the replication factors in silencing is not clear. The role of the ORC in silencing was shown to be independent of its role as a replication initiator (Fox et al., 1997), suggesting that some replication factors might have dual functions. Furthermore, recent evidence suggests that establishment of the silenced state can be uncoupled mechanistically from the passage of a replication fork (Kirchmaier and Rine, 2001; Li et al., 2001). A more direct connection between DNA replication and chromatin assembly is suggested by a recent study showing that the marking of newly replicated DNA molecules by PCNA helps promote nucleosome assembly by the chromatin assembly factor CAF-1 (Shibahara and Stillman, 1999). Interestingly, similarly to polα, mutations in CAF-1 or PCNA affect inheritance of repressed chromatin states in S. cerevisiae (Enomoto and Berman, 1998; Zhang et al., 2000) and the PCNA mutant allele mus209 is a dominant suppressor of PEV in Drosophila (Henderson et al., 1994). Although our data suggest that Polα participates directly in heterochromatin assembly through its interaction with Swi6, it remains a possibility that mutation in polα impairs the
CAF-1 interaction with other proteins at the replication fork.

Recent analyses showed that the *Drosophila* HP1 protein, which shares structural and functional similarities with Swi6, binds a consensus pentamer peptide sequence in which proline predominates at position 1, valine at position 3 and a hydrophobic residue at position 5 (Smothers and Henikoff, 2000). This consensus sequence, referred to as MIR peptide (MOD1-interacting region), is present in the HP1-interacting regions of TIF1-α, TIF1-β and CAF-1 (Murzina et al., 1999; Ryan et al., 1999). We found that a putative MIR peptide (residues 1129–1133) lies within the C-terminal part of Polt, 13 amino acids downstream from the swi7-1 mutation site, in a region that binds to Swi6 in vitro (Figure 6C). Although further work is required, it is tempting to speculate that an evolutionarily conserved mechanism, mediated through MIR peptide, might promote interaction between Polt and Swi6.

In summary, the data presented here implicate the replication protein Polt in higher order chromatin assembly and Swi6 localization at the silenced chromosomal locations. Considering that proteins related to Swi6 are also found in higher eukaryotes, similar interactions in other species might be involved in the assembly of specific chromatin structures.

**Materials and methods**

**Strains and culture conditions**

Standard conditions were used for growth, sporulation, tetrad analysis and construction of diploids from haploid strains. The *swi6*−333 allele was constructed by integrating two additional copies of *swi6*, marked with the LEU2 gene, at the *swi6* locus. The proper integration and number of copies of *swi6* were confirmed by Southern blot analysis. The construction of KΔ::ura4Δ, KΔ::ade6Δ, Δ(Bg-B)m2-Pint::ura4Δ, L(BgII)::ade6Δ, L(SacI)::ade6Δ or insertions of ura4Δ at different *cen*1 locations are described elsewhere (Thon et al., 1994; Allshire et al., 1995; Grewal and Klar, 1996; Ayoub et al., 1999). Genetic crosses were used to construct all other strains. The complete genotypes of strains are listed in Table 1.

**Iodine staining**

The iodine staining assay was used to assay the derepression of donor loci. Individual colonies grown on sporulation (PMA') medium for 3 days were exposed to iodine vapors to stain a starch-like compound produced by sporulating cells. In mating-type switching-defective strains, such as *mat1-MSm1*, the intensity of brown/black staining indicates the level of ‘haploid meiosis’, a phenotype resulting from concurrent expression of both *P* and *M* information in haploid cells.

**RT–PCR analysis**

RNA prepared from the nitrogen-starved non-switching (*mat1-MSm1*) cells was treated with DNase to remove contaminating DNA. Reverse transcription was done using the Gibco Superscript™ Pre-amplification system, utilizing oligo(dt)15 to prime cDNA synthesis. The cDNAs were then amplified in logarithmic phase, using primers 5′-GAATAT-AGTATGCGCTCTAATCTCAG-3′ and 5′-ATGATGATACGTTAAGG-GACGCACC-3′ for *mat2-P* transcripts, and 5′-TCTTCCTTGGTGG-TGAAGGC-3′ and 5′-TCCTTAATGTAACCTTCA-3′ for *act1* control transcripts. The cDNA products were resolved by agarose gel (1.2%) electrophoresis and subjected to Southern analysis using the 0.9 kb HindI−MluI fragment of the *mat2-P* cassette and the 0.6 kb PCR product of *act1* as probes.

**Plasmid constructions**

All cloning steps were performed according to standard protocols. A 2.7 kb XbaI−PstI fragment was excised from the plasmid containing the full-length *polar* gene (Singh and Klar, 1993) and cloned into pMALT2 (New England Biolabs) to produce recombinant C-terminal *Polar* protein (amino acids 635–1405) fused to MBP. Similarly, a 1.6 kb Spol−PstI fragment containing the 3′ end of the *polar* gene was cloned into pBluescript KS to construct pBK-polar-C. The PCR-amplified 5′-terminal fragment (450 bp) was cloned and ligated to a 1.5 kb EcoRI−XhoI fragment to construct pBK-polar-N. pBK-polar-N and pBK-polar-C plasmids were used for the T7 promoter-based in vitro translation–transcription reaction (Promega) to synthesize N- (amino acids 1–635) or C-terminal (amino acids 1032–1405) Polt protein. The Swi6 gene was amplified using primers swi6/FW 5′-GGGATCCAAAGAAGGAGGG-TTGGTTCG-3′ and swi6/RV 5′-AAGATTCTTAACTTCAATTTCTTCCAGG-3′. The resultant PCR product was cloned into pRE30 (Invitrogen) to construct the pRE30/swi6 plasmid used to express His6-tagged Swi6.

**Formaldehyde cross-linked CHIP**

The crude rabbit antisera raised against full-length Swi6 protein were affinity purified using His6-tagged Swi6. Purified antibodies were used for CHIP and immunofluorescence as described previously (Ekwall and Partridge, 1999; Nakayama et al., 2000). The primer sets used were: for ura4 ura4D5/E, ura4H1 (5′-GAGGGATGAAAAATCCCAT-3′) and ura4H2 (5′-TTCGCACAGAGGATTGACAAGGACCG-3′); and for telomeres, TEL-FW (5′-GGATGAAAAATTTAACTCCACC-3′) and TEL-RV (5′-CTCGGTTCGCTCGTGTCG-3′).

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**Fig. 7.** Mutation in swi7/polar disrupts the Swi6 localization. Wild-type (A and B) or swi7/polar mutant cells (C and D) were stained with affinity-purified anti-Swi6 antibody. DAPI staining of each cell is also shown (E–H). Strains used were SP976 (WT) and SP15 (swi7).
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**Protein affinity chromatography**

Purified Hsp90-tagged Swi6 protein was cross-linked to NHS-activated HiTrap (Amersham Pharmacia). The column was washed successively with 10 column volumes of phosphate-buffered saline (PBS), 10 column volumes of buffer E (50 mM Tris–HCl pH 7.5, 75 mM NaCl, 1 mM dithiothreitol (DTT), 2.5 M urea) and 10 column volumes of buffer A (50 mM Tris–HCl pH 7.5, 75 mM NaCl, 0.1% NP-40, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Boehringer)). A blank column cross-linked without ligand was used as negative control. One liter of yeast culture was grown to an A₅₉₀ of 0.5, harvested by centrifugation, washed with ice-cold STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN₃ pH 8.0) and stored at −70°C. The frozen cell pellet (1 × 10^10 cells) was resuspended in 1 ml of ice-cold HB buffer (25 mM MOPS pH 7.2, 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 15 mM MgCl₂, 15 mM EGTA, 1 mM DTT, 0.1 mM sodium vanadate, 1% Triton X-100, 1 mM PMSF, supplemented with protease inhibitor cocktail) and disrupted by agitation with glass beads at 4°C. The glass beads were washed with 10 ml of ice-cold HB buffer and the cell lysate was centrifuged at 10 000 r.p.m. for 20 min at 4°C. The supernatant was diluted with 1 vol. of buffer A and loaded onto a Swi6 affinity column or blank column at a flow rate of −10 ml/h. The columns were washed with 20 column volumes of buffer A, and the bound proteins were eluted using eight column volumes of buffer E. The peak fractions were precipitated by 2 vol. of ice-cold ethanol, resolved on 6% SDS–polyacrylamide gels and subjected to western analysis with affinity-purified anti-Pol7 antibodies. The binding of Swi6 to Pol7 was performed essentially as described previously (Murzina et al., 1999). Pol7 protein samples were produced by in vitro translation in the rabbit reticulocyte lysate and incubated with recombinant GST-tagged Swi6 bound to glutathione–agarose beads. After extensive washes, the proteins that remained bound to GST–Swi6 were separated using SDS–PAGE and detected by fluorography.

**Immunofluorescence analysis**

Immunofluorescence was performed as previously described (Ekwall et al., 1996). Cells were grown to mid-log phase in YE medium. An equal volume of YEA + 2.4 M sorbitol was added and the culture was incubated further at 18°C for 5 min. Cells were fixed by adding paraformaldehyde to a final concentration of 3.8% and incubating at 18°C for 30 min. After the fixation was stopped by adding 2.5 M glycine to 0.125 M, the cell pellet was washed twice with PEM (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM MgSO₄) and once with PEMS (PEM + 1 M sorbitol). Cells were incubated in PEM/S + 1 mg/ml of zymolysate 100T (ICN) for 60–120 min at 37°C to digest the cell wall, washed sequentially with PEM/S, PEM/S + 1% Triton X-100, PEM/S and PEM, and then incubated with PEBMAL [PEM/S + 1% bovine serum albumin (Sigma A6738) 0.1% sodium azide, 0.1 M l-lysine] for 1 h. Cells were incubated overnight with affinity-purified Swi6 antibody in PEBMAL, washed three times in PEBMAL and incubated with Alexa-conjugated anti-rabbit immunoglobulin G (Molecular Probe) at a 1/2000 dilution in PEBMAL for 3 h. After extensive washing, cells were stained with 4’,6-diamidino-2-phenylindole (DAPI), mounted in Vectashield mounting medium (Vector laboratories) and analyzed by a fluorescence microscope.

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**References**


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