An imprinting function of DNA polymerase α in establishment of mating type silencing in *Schizosaccharomyces pombe*

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Parental imprinting is a well-known phenomenon in higher eukaryotes. Similar epigenetic phenomena occur in budding yeast and fission yeast as represented by silencing. In *Schizosaccharomyces pombe*, the same genetic information is expressed at one locus *mat1*, but silenced at two other loci, *mat2* and *mat3* (and also the cases of centromere and telomere loci). Studies in both yeasts have suggested an integral role of DNA replication or passage through S phase in establishment of the silent state. Deletion of *K* region lying between the *mat2* and *mat3* loci establishes two alternative, stable epigenetic states of silencing, which are stably inherited during mitosis and meiosis. Recently, we have shown that in *S. pombe* the mating type silencing is abrogated at permissive temperature in a novel, dominant and temperature-sensitive mutant of DNA polymerase α. Here, we show that this mutant exhibits two alternative states, the repressed and the derepressed states, which are stably inherited during passage through mitosis. During meiosis, these alternate states segregate in a Mendelian fashion, indicating that these represent imprinted chromosomal *mat* loci. These alternate states are associated with different chromatin structures and in the derepressed state the chromodomain protein swi6 is delocalized from the *mat* loci. These results show that DNA polymerase α, the enzyme required for lagging strand synthesis during DNA replication, plays a direct and integral role in establishing an imprinted chromatin structure by recruiting the heterochromatin-associated protein swi6, thus coupling DNA replication with establishment of silencing. We speculate that such a mechanism may be widely conserved among higher eukaryotes.

GENE silencing in *Schizosaccharomyces pombe* and *S. cerevisiae* have served as good model systems for epigenetic mechanisms of gene regulation[1,2]. The key events involved in this process are the establishment and propagation of the silent state. In *S. cerevisiae*, the Sir1p is the rate-limiting factor[3], whose recruitment to the silent loci *HMR* and *HML* through the ORC complex[4], which is assembled at the *cis*-acting silencers, establishes the commitment to silencing. Other factors like Sir2–Sir4 are then recruited to the locus, which interact with and modulate the activity of histones[5], thus leading to establishment and propagation of the inactive heterochromatin structure. Similarly, in *S. pombe*, the *K* region spanning the *mat2-mat3* interval affects the efficiency of establishment of the silent state[6], for which the *trans*-acting factors, namely swi6, clr1–clr4 are important[6–10]. Recently, swi6 was shown to function in a dose-dependent manner in enhancing the efficiency of the establishment of the silent state, indicating that it is a rate-limiting factor required for establishment of the imprint[11]. We have shown that *swi7H4*, a novel temperature-sensitive mutant of DNA polymerase α (ref. 12) is defective in silencing at the *mat*, centromere and telomere loci (Ahmed *et al.*, submitted). Specific genetic and biochemical interactions were observed between polα and the *trans*-acting factors like swi6 and clr1–clr4. Furthermore, we found that in the *swi7H4* mutant, swi6 is delocalized away from the *mat* loci (Ahmed *et al.*, submitted). Accordingly, we proposed a recruitment model for silencing, wherein DNA polymerase α recruits swi6 during lagging strand synthesis to the *mat*, *cen* and telomere loci (Ahmed *et al.*, submitted) which, in turn, interacts with the histone H3 methylated at Lys 9 position[14,15], thus initiating the assembly of heterochromatin.

The silencing phenotype in this mutant is best manifested when the *cis*-acting silencer linked to *mat2P* locus is deleted in the non-switching background *Msmto* (Ahmed *et al.*, submitted; Figure 1 *a*), which is similar to the effect of *swi6* and *clr1–clr4* mutants[9]. These results indicate a genetic interaction between polα and swi6/clr1–clr4. The resulting colonies give dark staining with iodine and haploid meiosis, because the simultaneous expression of the expressed *mat1M* and the silent *mat2P* alleles, due to the loss of silencing, triggers meiosis in haploid cells (called haploid meiosis) and sporulation in haploid cells. The spore cell wall contains a starch-like compound which stains dark with iodine[16]. Such dark staining is not observed in the *swi7H4* mutant if the silencer is intact, but a high level of haploid meiosis (~ 41%) and dark iodine staining is observed if the silencer is deleted (Ahmed *et al.*, submitted), indicating that polα functions through the silencer element with its associated ARS function (autonomous replication sequence, Dubey and Singh, unpublished). Moreover, the expression of the linked *ura4* marker gene (Figure 1 *a*) is enhanced in the *swi7H4* mutant compared to the parental strain (Figure 1 *b*), as indicated by enhanced growth on plates lacking uracil and reduced growth on FOA plates[16].

Interestingly, the *swi7H4* mutant exhibited unique semidominant phenotype with respect to growth at 36°C, as the *ts* phenotype was not complemented by an integrative duplication of *polα* gene (data not shown).

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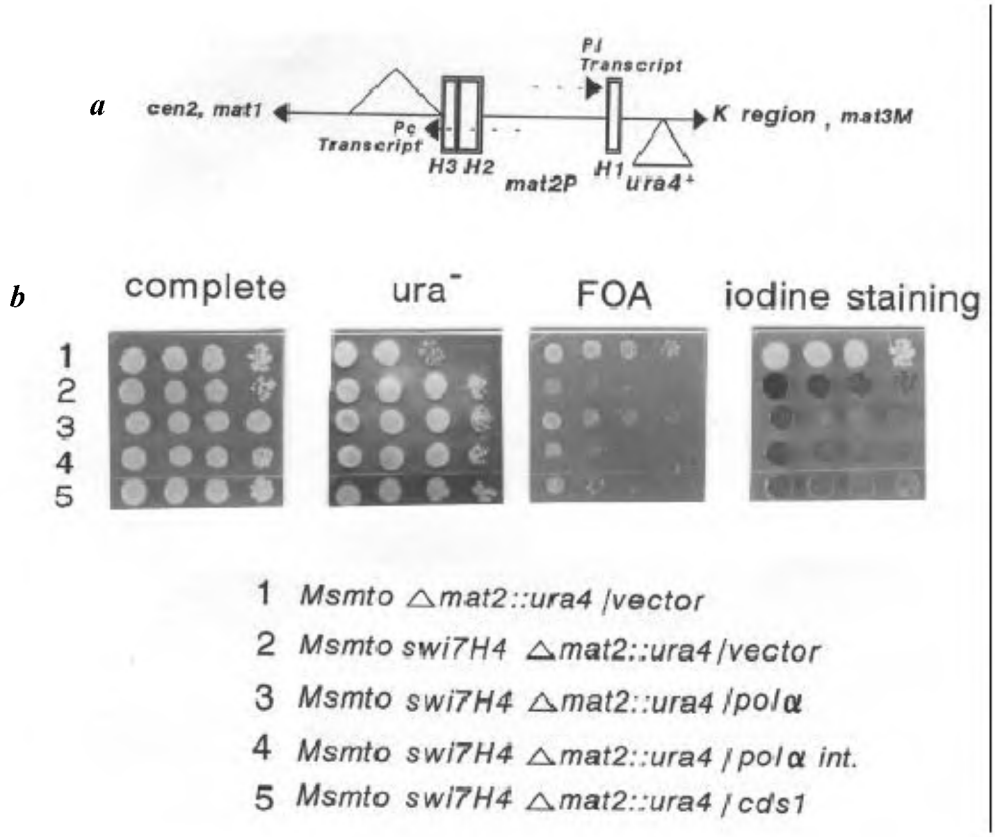


Figure 1. Dominance of the silencing phenotypes of *swi7H4* mutant. ***a***, Organization of the mating type loci in fission yeast, depicting the sites of insertion of *ura4* marker gene at the *mat2* and the site of deletion of the centromere-proximal silencer element shown as a triangle. The *K* region and *mat3* are located distally and *cen2* and *mat1* are indicated as located proximally to *mat2*. Also shown are the two transcripts *Pc* and *Pi* that are divergently transcribed from the *mat2P* locus. ***b***, *swi7H4* mutation causes derepression of the *mat2*-linked *ura4* gene and the dominance of the silencing defect of *swi7H4* mutation. Serial dilutions of cultures of the parental strain (genotype: *Msmto* Δ*mat2::ura4*$^+$ *ade6–210*) containing the control vector and the *swi7H4* strain (D), transformed with either the control vector, integrated copy of *polα* gene, *polα* gene on a low copy vector or *cds1* gene on a high copy vector, were spotted on complete, PMA-leu, PMA-leu-ura and FOA-leu[16] (for selection of plasmid). The PMA-leu plates were stained with iodine after 4 days of growth at 30°C.

This is unlike the *swi7–1* mutation, which is complemented by a targeted integration of the *polα* gene[17]. A similar dominance was observed for the silencing defect as well. The *polα* gene on high copy vector, when transformed into the mutant, did not reduce the extent of iodine staining (Figure 1 *b*). Furthermore, the *cds1* gene[12], which suppresses the checkpoint defect of the *swi7H4* mutant, also does not reduce the iodine staining of the *swi7H4* mutant (Figure 1 *b*). Moreover, the mutant exhibits similar time of passage through S phase as wild type cells when analysed by FACS analysis (data not shown), ruling out the possibility that a prolonged S phase might elicit the silencing defect in the *swi7H4* mutant.

The silencing defect of the *swi7H4* mutation was also observed at the *cen* and telomere regions (Ahmed *et al.*, submitted), but no effect was observed on the expression of thiamine-regulated gene *nmt1* and cell cycle regulated gene *H2B* (Ahmed and Singh, data not shown). Thus, polα specifically affects silencing at the heterochromatin loci in *S. pombe*.

Interestingly, upon streaking the dark colonies of the *swi7H4* mutant, (Figure 2 *a*, lower panel) a few light staining colonies with reduced level of haploid meiosis (~ 1%; Figure 2 *a*, top panel) were observed. Southern analysis showed that these colonies had an intact mating type organization (data not shown), ruling out the possibility that a rearrangement caused by the *swi7H4* mutation was responsible for the silencing defect. To assess the levels of the silent copy transcript directly, quantitative RT-PCR analysis was carried out. The results showed that light colonies had a low but detectable level of *mat2Pc* transcript (Figure 2 *b*, lanes 1 and 2), which was similar to that in non-switching *swi7H4* mutant strain with intact silencer (not shown). This level was further enhanced by ~ 9-fold in dark colonies (Figure 2 *b*, lanes 3 and 4). Furthermore, a greater fraction of cells have a stably derepressed *mat2-*

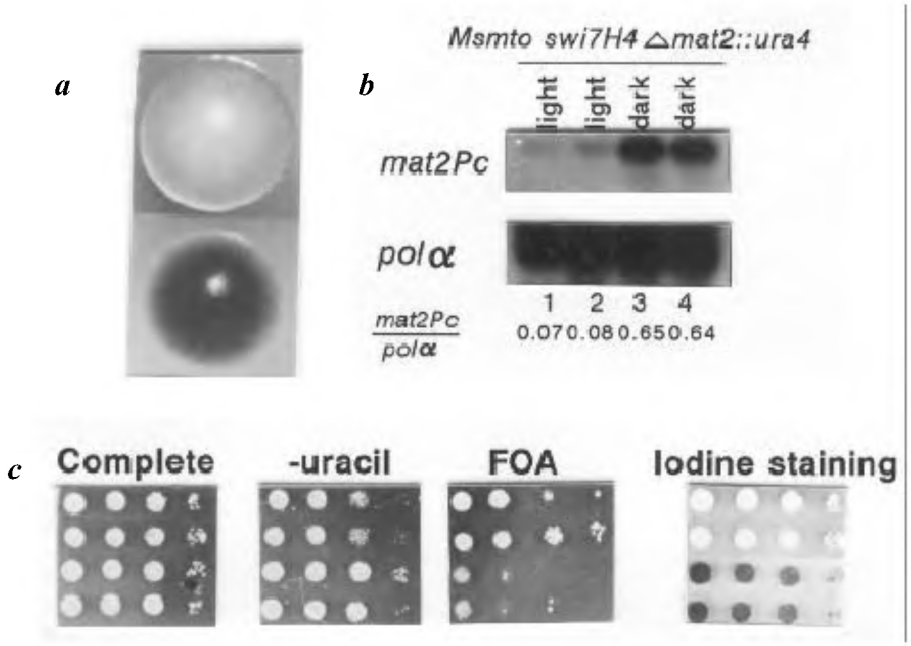


Figure 2. Two alternate stable silencing phenotypes displayed by *swi7H4* mutant. ***a***, Light colony (top) and dark colony (bottom) of the *swi7H4* mutant strain (*Msmto ura4D18 Δmat2::ura4*$^+$ *leu1–32 ade6–216 swi7H4*). ***b***, RT-PCR analysis to quantitate the level of *mat2Pc* transcript in cells from light and dark colonies. RNA derived from two independent light and dark colonies of strain SPA233 was subjected to RT-PCR analysis to detect *mat2Pc* and *pol*α transcript as described earlier[20]. The relative ratios of *mat2Pc/pol*α transcript were estimated by densitometric scanning. ***c***, Derepression of *mat2*-linked *ura4* marker in the dark and light colonies of the *swi7H4* mutant. Serial dilutions of cells from two independent light (top two rows) and dark colonies (bottom two rows) grown overnight in YEA medium[16], were spotted on complete plate and plate lacking uracil or containing FOA (third panel) or PMA$^+$ plate (last panel). The PMA$^+$ plates were stained with iodine after 3–4 days of growth at 30°C.

linked *ura4*$^+$ marker in the dark colonies (better growth on ura$^-$ plates and lack of growth on FOA plates) than in the light colonies (Figure 2 *c*).

We checked the mitotic stability of the dark and light colonies and found that these colonies interconvert at a very low rate[18] (< 10^{-4}/generation; Tables 1 and 2), which, however, is much higher than the spontaneous mutation rate of 10^{-8}. Thus, these colonies represent two alternative states of silencing that are mitotically stable. The stability is also reflected in the uniformly dark and light staining of serial dilution of cells from the dark and light colonies with iodine, respectively (Figure 2 *c*, last panel). To test the meiotic stability of the dark state we crossed the dark strain (genotype: *Msmto Δmat2::ura4 swi7H4*) with another strain, which contains a *his2*$^-$ marker linked distally to *mat3* locus (genotype: *PΔ17::LEU2 ura4D18 Δmat2::ura4*$^+$ *his2*$^-$ *swi7H4*) and gives light staining with iodine (Figure 3). All the 30 tetrads produced the dark and light segregants in 2 : 2 ratio – all the dark segregants were *his*$^+$ and the light segregants *his*$^-$ (Figure 3). (Some *his*$^+$ segregants exhibited a grey staining, but they all exhibited higher levels of haploid meiosis than the light segregants; Figure 3). These results indicate that the dark and light represent alternative metastable epigenetic states that are stably marked and propagated through meiosis and mitosis. Thus, DNA polα is required for establishing an imprint at *mat2*, which behaves like an epigenetic locus and can propagate itself not only during mitotic cell division, but also in a meiotic cross.

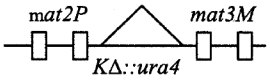
To check whether the dark and light states are associated with different chromatin structures, we used the *in vivo* expressed *E. coli dam* methylase approach. Earlier, we showed that the *E. coli dam* methylase, when expressed in the budding and fission yeast (both of which lack any DNA methylation), preferentially methylates the *Sau3*AI sites in active genes[19,20]. A low level of methylation of *Sau3*AI sites was observed in the *mat2P* locus in *Msmto* strain, which was not significantly altered in the *swi7H4* mutant (data not shown). However, in the silencer-deleted strain (genotype : *Msmto ura4D18 Δmat2::ura4*$^+$), several sites flanking the *mat2P* locus (two sites each in the centromere-distal *K* region and the *ura4*$^+$ marker; Figure 4) show significant methylation even when the *mat2* locus is repressed (Figure 2 *b*, lane 2). It has been noted earlier that deletion of silencer does cause a slight increase in growth on uracil$^-$ plates[9]. Thus, the centromere-proximal silencer may exert a subtle long-range effect on folding of *mat2P* and the centromere-distal *K* region without affecting the expression of mating type transcripts. Most importantly, methylation of the specific 3.4 kb site, located within the region encoding the Pi transcript, is increased by ~ 2.5 fold in the light state (1.4%) and

Table 1. Stability of two epigenetic states of silencing in *swi7H4* mutant in silencer deletion and *K*-deletion background

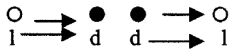
Strain	Genotype	Nature of switch	Rate/div.	Rate/% div.
SP1152	*Msmto, Δmat2ura4*	ND*	ND*	ND*
SPA233D	*Msmto, Δmat2ura4 swi7H4* (dark)	Dark to light	3.5×10^{-5}	3.5×10^{-5}
SPA233L	*Msmto, Δmat2ura4 swi7H4* (light)	Light to dark	8.0×10^{-5}	8.0×10^{-5}

*Not determined because no dark staining colonies were observed.

Table 2. *swi7H4* mutation influences the rate of switching between the alternate switching/silencing states generated by the *K* deletion



switching rate /generation



	I	II	Ratio I/II	Fold difference
KΔ::ura4	9.2×10^{-4}	8.0×10^{-2}	1.0×10^{-2}	–
KΔ::ura4 swi7H4	4.6×10^{-3}	8.7×10^{-3}	0.5	50

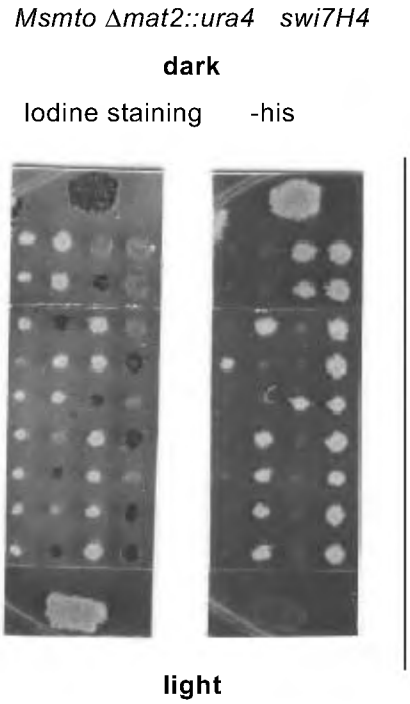


Figure 3. Derepressed epigenetic state generated by *swi7H4* mutation is stably inherited during meiosis and is chromosomally marked. Tetrads derived from a diploid between strains SPA233-D (genotype: *Msmto leu1-32 ura4D18 Δmat2::ura4*$^+$ *ade6–216 swi7H4*) and SPA319 (*PΔ17::LEU2 leu1–32 ura4D18 Δmat2::ura4*$^+$ *his2 ade6–210 swi7H4*) were sporulated and dissected on YEA plates. After growth for 4 days at 30°C, the plates were replica-plated on to PMA$^+$ plates and plates lacking histidine. The replicas were allowed to grow for another 4 days at 30°C and stained with iodine.

~10-fold in the dark state (5.8%) compared to parental strain (0.6%, Figure 4, compare lanes 3 and 4 with lane 2).

From these results we infer that DNA polα acts in concert with the silencer to efficiently establish an inactive folded chromatin structure in all the cells. The silencer region may affect the efficiency since in the presence of the silencer no such epigenetic states were observed. A similar role may be performed by the *K* region whose deletion has been shown to generate two alternate states of silencing[6]. To test whether polα is required for the stability of the dark and light states generated by the deletion of *K* region, we checked whether *swi7H4* mutation affects the rate of switching between the light and dark states in the *K* deletion strain (Table 2). Interestingly, we find that the light to dark (from expressed to repressed in this case) switching is enhanced by 50-fold in the *swi7H4* mutant. Thus, polα is also required for the efficient generation of the epigenetic switch brought about by deletion of *K* region.

The above results indicate a mechanism of action of polα that operates in the same pathway as swi6 and clr1–clr4. It has been shown that the swi6 protein becomes delocalized from the heterochromatin loci, *mat*, cen and telomeres, in the *clr4* and *rik1* mutants[21]. It has been recently shown that swi6 is localized at roughly constant level at *K* and L regions throughout the cell cycle[11]. Therefore, we checked whether the *swi7H4* mutation might affect the localization and binding of swi6 to *mat* loci. Fluorescence microscopy of normal strains containing gfp-swi6 construct showed 1–3 spots/cell corresponding to its localization to centromere, te-

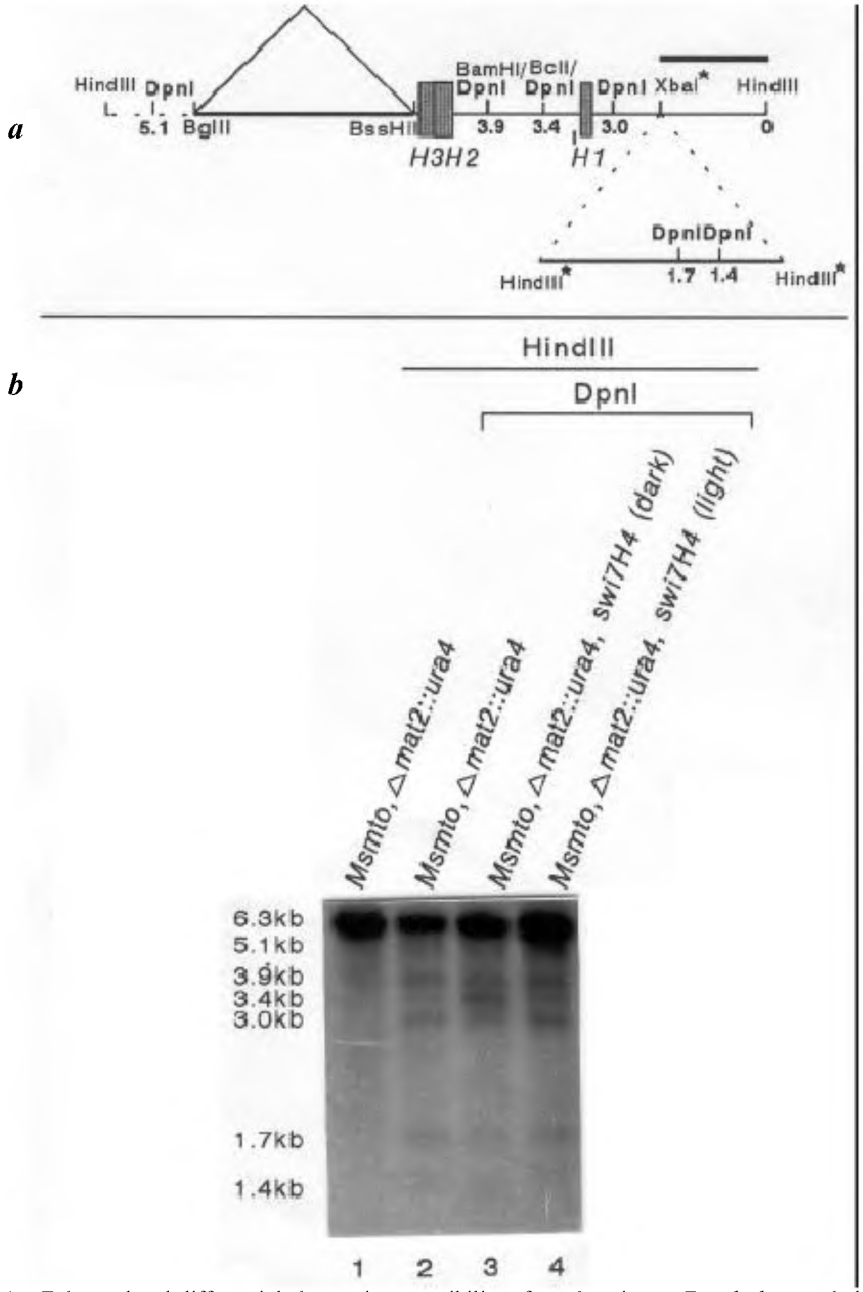


Figure 4. Enhanced and differential chromatin accessibility of *mat2* region to *E. coli dam* methylase in the dark and light states of the *swi7H4* mutant. ***a***, Molecular organization of the *mat2* locus with deletion of centromere-proximal silencer and insertion of 1.8 kb *ura4*$^+$ *Hin*dIII fragment at the centromere-distal *Xba*I site. The location of *Sau*AI/DpnI sites and their distance from the centromere distal *Hin*dIII site are shown. Also shown as a horizontal filled bar is the *Xba*I–*Hin*dIII fragment which is used as a probe for indirect end labelling. ***b***, Southern hybridization. 1 µg of DNA from strain SP1152 (genotype: *Msmto ura4D18 Δmat2::ura4*$^+$ *ade6–210*; lanes 1 and 2), strain SPA233-D (genotype: *Msmto leu1–32 ura4D18 Δmat2::ura4 swi7H4*; lane 3) and strain SPA233-L (genotype: *Msmto leu1–32 ura4D18 Δmat2::ura4 swi7H4*; lane 4) was digested with *Hin*dIII. Subsequent digestion was with *Dpn*I for strain SP1152 (lane 2), strain SPA233-D (lane 3) and SPA233-L (lane 4). The digested samples were subjected to electrophoresis in agarose gel, followed by Southern blotting and hybridization with the *Xba*I–*Hin*dIII fragment under conditions described earlier[20].

lomere and *mat* loci as shown earlier[22]. On the other hand, in the dark colonies of the *swi7H4* mutant significantly fewer cells show 2–3 spots and with more cells having one spot (Figure 5 *a*; Table 3). Interestingly, in the light colonies the localization to 2 and 3 spots was restored (Figure 5 *a*; Table 3). Results of chromatin immunoprecipitation (ChIP) experiments using mouse anti HA-antibody with strains containing HA-tagged swi6 plasmid in place of the endogenous *swi6* gene indicate that swi6p is localized at the *K* region in wild type (Figure 5 *b*, lane 2), but is not detectable in the dark colony of the *swi7H4* mutant (Figure 5 *b*, lane 5).

Table 3. Epigenetic effect of *swi7H4* mutation on localization of swi6-gfp plasmid

No. of loci	Wild type (%)	*swi7H4* mutant (dark) (%)	*swi7H4* mutant (light) (%)
1	2 (1.1)	37 (15.1)	15 (5.9)
2	51 (28)	122 (49.8)	80 (31.6)
3	129 (70.9)	86 (35.1)	168 (66.4)

Only up to 3 loci were counted. Cells with fainter 4 and 5 foci were not counted.

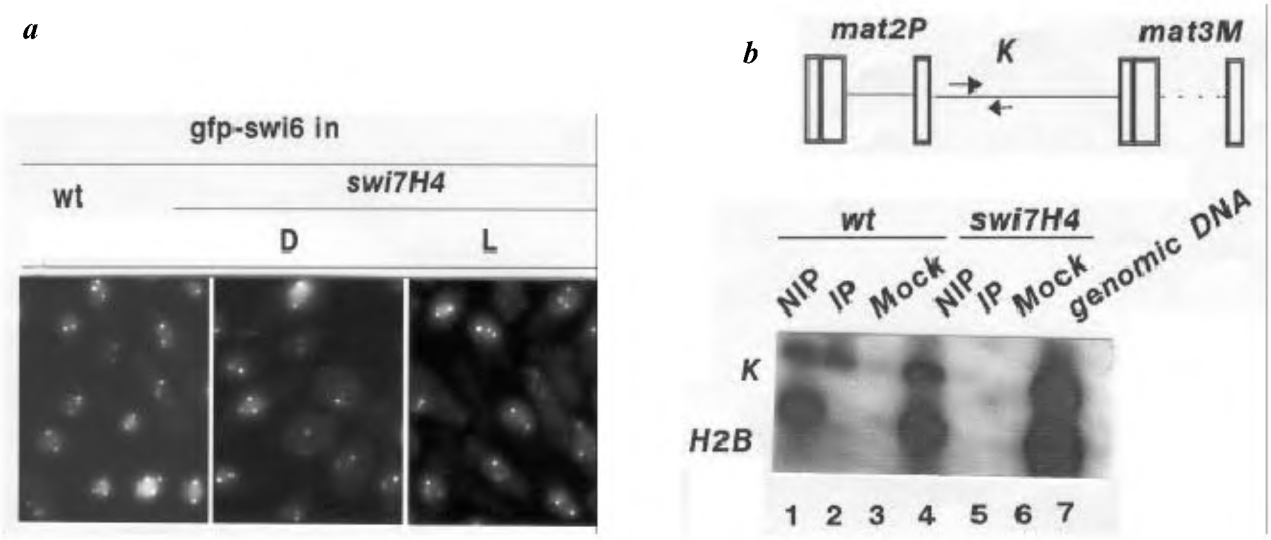


Figure 5. Epigenetic effect of DNA polymerase α on the localization of swi6 to *mat* loci. ***a***, Cells of wild type and the dark and light forms of the *swi7H4* mutant strain containing gfp-swi6 in place of the endogenous copy of *swi6* gene were monitored by fluorescence microscopy at 1000 × magnification. The number of cells containing 1, 2 and 3 fluorescent spots was counted. ***b***, ChIP analysis of wild type and *swi7H4* strain harbouring HA-swi6 construct. DNA isolated either from crude extract (NIP), mock immunoprecipitated (Mock), or anti HA immunoprecipitated (IP) chromatin fraction was used as template for PCR amplification as described. Primers used for the CHIP analysis were TGACAAAGCTTTTGTGG and TGTTAAAGCTTTTCTTCC for *K* region (700 bp) and GTCAGGATCCGCTGCTGAAAAGAAA CC and ACTGGAATTCCTGAGGAGAAGAAGAATAC for *H2B* (395 bp). Upper panel shows the position of primers used for PCR. Histone H2B was used as a negative control. PCR products were separated on 1.5% agarose gel and subjected to Southern hybridization with the radiolabelled homologous probes. Wild type (lanes 1–3): nonimmunoprecipitated (NIP; lane 1) and immunoprecipitated (IP; lane 2); where the latter lane suggests binding of swi6 to the *K* region, but not to histone H2B and lane 3 represents mock immunoprecipitation; *swi7H4* (dark) chromatin samples (lanes 4–6): lane 4, NIP control; lane 5, treated with anti HA antibody; lane 6, mock immunoprecipitated. Lane 7 represents the PCR products obtained with genomic DNA. The ChIP methodology described by Ekwall and Partridge[34].

Our recent data also show that the association of swi6p with K region is substantially restored in the light colony of the *swi7H4* mutant (not shown).

Recently, we showed that polα interacts with swi6 both *in vitro* and *in vivo* (Ahmed *et al.*, submitted) and proposed a recruitment model for coupling of heterochromatin assembly to DNA replication. DNA polα may progressively recruit the *trans*-acting factors like swi6, at the replication forks initiated from the proximal and distal silencers/ARS elements flanking the silent locus *mat2* (refs 23 and 24) during lagging strand replication. This may bring about a convergent, bidirectional and cooperative spreading of heterochromatin at both *mat2P* and *mat3M* loci as well as in the direction of *mat1* (although at a reduced level, data not shown). The *swi7H4* mutant protein may have a weaker interaction with swi6. As a result, in the absence of the proximal silencer the mutant polα functions less efficiently and stochastically, utilizing only the centromere-distal silencer and recruits the *trans*-acting factors like swi6 only in a sub-population of the cells. However, once these states are established, they have the intrinsic memory to propagate themselves. Evidence of stable chromosomal states of switching of mating type and expression of a *ura4*$^+$ marker have been reported recently[6]. Moreover, overexpression of swi6 was shown to reduce the stability of the derepressed state by associating with the *mat* region and an imprinting function of swi6 has been suggested recently[11]. The results of this study demonstrate that DNA polα carries out an 'imprinting' function in establishment of silencing temporally prior to swi6, but not in its propagation (Figure 6). We propose that swi6 may be involved in the re-establishment after every round of DNA replication, presumably through fresh recruitment by polα and propagation of the silent chromosomal state as an integral component of silent chromatin (Figure 6). Since the chromodomain and SET domains present in swi6 (ref.

25) and clr4 (ref. 26) are widely conserved among heterochromatin-associated proteins in mice and humans[27] and the mutations in polα that cause silencing defect map to essential conserved regions, the mechanism involving interaction between DNA polα and heterochromatin-associated proteins in establishment of heterochromatin may also be conserved.

Recent reported examples of epigenetic phenomena include generation of a derepressed epigenetic state in *Drosophila* which is inherited during mitosis and meiosis even after the activating signal is withdrawn[28,29]. A recent elegant study showed that marking of replicating SV40 DNA molecules by PCNA helps in proper nucleosome assembly by the chromatin assembly factor CAF1 (ref. 30) and interaction between the mouse CAF1 and mouse heterochromatin protein HP1 (ref. 31) which also contains the chromodomain[27]. DNA replication is known to perturb the chromatin structure and, therefore, it is intuitively appealing that reassembly of chromatin structure should be coupled to DNA replication machinery[32]. More recently, PCNA, which interacts with polδ during leading strand synthesis, has also been shown to be involved in silencing at the *HMR* and telomere loci in the budding yeast[33]. Therefore, we speculate that the replication enzymes may closely interact with the chromatin proteins and chromatin assembly factors and play an important role in epigenetic phenomena. Further genetic, biochemical and molecular biology studies will help us understand the mechanisms better.

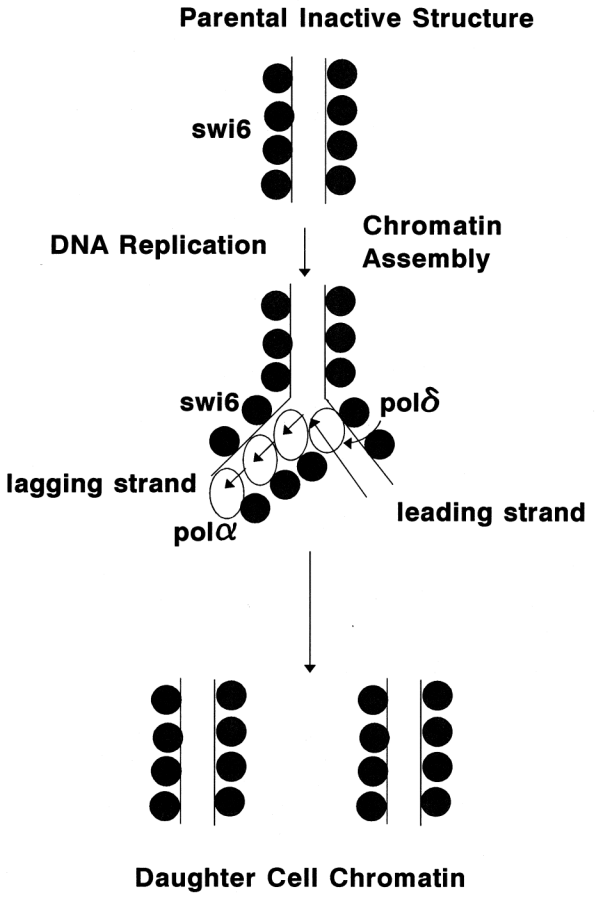


Figure 6. Schematic model depicting the role of DNA polα in recruiting swi6 during lagging strand synthesis and reassembling the transcriptionally inactive parental chromatin configuration in the daughter cells.

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