The fission yeast DNA polymerase α has been shown to play an important role in setting up imprint at mat1 locus but the mechanism is still not known. This study is an attempt to address the mechanism of DNA polα in setting up imprint at mat1 locus. It involves the site directed and random mutagenesis approaches of mutating the polα gene in search of switching defective mutants.

The outcome of this study is that, the putative homing endonuclease and Restriction enzymes-like motifs present in polα play no role in imprinting at the mat1 locus. Interestingly, the catalytically dead polα is proficient in imprinting, implying that the catalytic function of polα is not required for imprinting.

The heterochromatin structure perturbation in polα/δ mutants at mating type and centromeres and detection of sharp bent DNA structures at mat1 locus are some other interesting findings of this study.
Chapter 3. Role of DNA polymerase α in setting up Imprint and chromatin Organization.

3.1 Role of DNA polymerase α in setting up imprint at matl locus

3.1.1 Putative LAGLIDADG endonuclease motif in fission yeast DNA Polα

The imprinting event at matl locus requires three genes (Egel et al., 1984): swi1, swi3 (Dalgaard and Klar, 2000) and swi7/pola (Singh and Klar, 1993). Swi1 and Swi3 were shown to create a replication pause site near the imprint and block replication forks proceeding to matl from the centromere side (Dalgaard and Klar, 2000); defective pausing in swi1 and swi3 mutants is correlated with their imprinting deficiency (Dalgaard and Klar, 2000). swi7-1/pola mutant, however, showed a normal pause and normal replication block and is defective at some other undefined step in imprinting, which is independent on Swi1 and Swi3 (Dalgaard and Klar, 2000). Earlier genetic screen revealed that a mutation in swi7 gene affects the level of double strand break at mating type locus (Egel et al., 1984). swi7-1, the only known mutation in this gene that affects the imprinting was mapped to the catalytic subunit of DNA polymerase α and shown to be complemented by the wild type copy pola gene (Singh and Klar, 1993).

In order to study the role of DNA Polα in setting up imprint at matl locus it was hypothesized earlier that Polα might function as an endonuclease and thus generate the double strand break at matl region. Multiple sequence alignment of fission yeast DNA Polα with the known homing endonucleases, performed using ClustalX software, showed that Polα contains a LAGLIDADG homing endonuclease-like motif at its C-terminal region. In most of the LAGLIDADG endonucleases the active site constitutes Asp (D) or Glu (E) residues. The DAD domain of budding yeast HO endonuclease, VMA and oxi4 show alignment with fission yeast DNA Polα (Figure 10).

3.1.2 The LAGLIDADG endonuclease motif of Polα is dispensable for imprinting

Presence of putative LAGLIDADG homing endonuclease domain in Polα encouraged us to check its role in setting up imprint at matl locus. The Asp (D) acid residue of LAGLIDADG motif has been shown to play a role in catalysis (Galburt and Stoddard, 2002). With the help of site directed mutagenesis the corresponding residues 1158 Asp (D) and 1160 Asp (D) were mutated to 1158 Ala (A) and 1160 Ala (A), respectively (Figure 11). Mutations were generated in the pola gene and cloned in to the expression vector pART1. The mutant clones were named as pART1polaA1(Asp1158Ala), pART1polaA2(Asp1160Ala) and pART1polaA1A2 (Asp1158/1160Ala). To check the effect of these mutants on switching, swi7-1 strain was chosen because a single point mutation from Gly (G) to Glu (E) at 1116 codon of the swi7 strain makes it
LAGLIDADG motif in Pol α

Fig. 10. ClustalX alignment of Fission yeast DNA polymerase α with known LAGLIDADG motif containing proteins. The sequence of *S. pombe* DNA polymerase α was analyzed against known LGLIDADG homing endonuclease motif containing proteins. Results show the presence of putative DADG domain in *S. pombe* DNA polymerase α, depicted by (Black) box.

Fig. 11. The diagrammatic representation of fission yeast DNA polymerase α gene with DADG mutation. The *S. pombe*, DNA polymerase α was 4218 bp long gene codes for 159.3 kDa protein with 1405 amino acids. The relative position of *swi7-1* and LAGLIDADG domain mutations has been shown. The putative DADG domain in, which residues, 1158 Asp (D) and 1160 Asp (D) have been mutated to Ala (A).
defective in switching (Singh and Klar, 1993) and gives light colonies on iodine staining. To check whether the LAGLIDAGD domain mutated polα plasmids complement the defect of swi7-l mutation, all three single and double Asp to Ala mutant plasmids were linearised with BglII and integrated into resident swi7-l allele by homologous duplicative recombination (Figure 12). The Leu+ transformants were streaked on selective PMA leu- plates to follow the switching of the transformant colony by iodine staining. Results showed that, like intact polα, both single and double Asp to Ala mutants of polα were able to restore the switching to the swi7-l mutant (Figure 13), as indicated by iodine staining of the transformant colonies. These results showed that the putative DADG region is not involved in switching.

3.1.3 Effect of catalytically dead Polα in switching

Next we asked whether it is only the physical presence of Polα in the replication complex or the catalytic function of Polα is necessary for setting up the imprint. We checked a catalytically dead but structurally intact polα mutant known as polα D984N (Bhaumik and Wang, 1998). This mutation involves a critical residue in region-1, the most conserved region of the α-like DNA polymerases. Similar mutation of the second Asp residue of human Polα Asp (D) 1004 to Asn (N) completely abolishes the catalytic activity. This mutation, however, does not alter either the protein structure of Polα or the ability of the mutant Polα protein to assemble into the Polα/primase complex (Copeland and Wang, 1993a; Copeland and Wang, 1993b). The corresponding Asp (D) residue in fission yeast is located at position Asp (D) 984 (Figure 14A) (Bhaumik and Wang, 1998). The physically intact but catalytically dead polα mutant, polα (D984N) was cloned under control of thiamine-repressible nmtl promoter. Both swi7-l and wild type strains were transformed with polα (D984N) mutant along with wt polα gene and vector control. Transformants were plated on selective medium in presence of thiamine to keep the deleterious effect of mutant polα (D984N) to a minimum level. Under these conditions, polα gene has been shown to complement the swi7-l mutation (Ahmed and Singh, unpublished), likely due to leaky expression of nmtl promoter (Forsburg, 1993).

3.1.4 The (D984N) mutation in polα abolishes switching in swi7-l mutant

The wild type and swi7-l mutant strains were transformed with the polα D984N mutant plasmid and transformants were checked microscopically for the presence of ascus, on each day after the appearance of the transformants on the selective plate (the day, when the transformants first appeared on the selective plate was counted as day one). Interestingly, it was observed that, in the presence of catalytically dead polα, switching in swi7-l mutant is completely abolished, as
Fig. 12. The schematic representation of DADG domain mutated polα plasmid integration into chromosome. The DADG domain mutated pARTpolα and wild type polα was linearised with the help of Bg/II and the linearised DNA was transformed into swi7-1 strain. The diagram is depicting the integration of mutant polα into chromosome by double cross over.

Fig. 13. The DADG domain mutated polα complements the switching defect of swi7-1 mutation. All three mutants pART polα A1, A2 and A1A2 are integrated into swi7-1 strain along with wt polα plasmid and vector control and The transformants were checked for polα complementation by iodine staining. Even in presence of mutated DADG domain the swi7-1 defect was complemented, suggests that LAGLIDADG domain has no role in switching.
indicated by absence of zygotic asci even after three days no sign of switching was observed (Figure 14B, upper panel). In wild type cells, catalytically dead pola transformants showed a reduced level of switching on the first day. (Possibly due to deleterious effect of the mutation) but were elevated on 2nd and 3rd days (Figure 14B, lower panel).

Percentages of switching (spo+ colonies (giving dark staining with iodine) were also counted among the transformants. Results show that, in swi7-l mutant containing catalytically dead (D984N) pola the number of spo+ colonies were almost reduced to zero level, while the vector control and wt pola transformants show normal extent of switching as expected (Figure 14C, left graph). In the wild type strain transformed with catalytically dead (D984N) pola almost negligible effect of (D984N) pola was observed in comparison to vector control and wt pola (Figure 14C, right graph).

3.1.5 The pola (D984N) mutation does not affect the imprinting in swi7-l mutant strain

To check the effect of (D984N) mutation on imprinting at molecular level, the double strand break was checked in transformants. Since pola (D984N) mutant was under the control of nmtl promoter, which gives leaky expression in presence of thiamine and is toxic to cells when fully expressed in absence of thiamine (Bhaumik and Wang, 1998), the cells were grown in presence of thiamine upto OD 595 of 0.5-0.6, followed by inoculation into medium lacking thiamine. The 0hr sample was taken before thiamine removal, and after thiamine removal samples were collected at each hour up to 6 hrs and the last sample was collected after 24hrs interval to ensure maximum level of expression of catalytically dead pola. Southern blots of the HindIII digests of the genomic DNA were hybridized with 10.4kb mat1M HindIII fragment. Interestingly in swi7-l mutant transformed with (D984N) mutant pola the break level is restored even at 0hr. This observation was quite unexpected (Figure 15, left panel, Lane 4). Similar break level was observed in case of wild type strain transformed with (D984N) mutant pola Figure 15, right panel, Lane 4). The apparently different behavior of swi7-l mutant transformed with (D984N) pola plasmid at cellular and molecular level was explained by the following results.

3.1.6 Donor deletion in swi7-l mutant in presence of (D984N) mutant pola

Careful analysis of Southern blots showed an interesting difference in wild type and swi7-l Southern blots. In case of swi7-l mutant transformed with pola (D984N) plasmid the donor copy of mat3M appeared to be deleted (Figure 15, left panel, lanes 4 to 9). The deletion of mat2P
Fig. 14. The effect of Catalytically dead polα (D984N) mutation on switching. (A) Diagrammatic representation of fission yeast DNA polα gene with catalytically dead (D984N) mutation. (B) Microscopic study of wild type and swi7-1 mutant transformed with catalytically dead pol α. The (D984N) mutation does not complement the switching defect of swi7-1 mutation. The effect was so deleterious that not even a single switching cell was observed under microscope in swi7-1 strain transformed with (D984N) polα, while it has a very short lasting effect on wild type strain. (C) The percentage of switching colonies were counted in wild type and swi7-1 strain transformed with mutant (D984N) polα. In swi7-1 strain (D984N) mutation completely abolishes switching and number of switching colonies was reduced to negligible level while in wild type strain. This effect (D984N) mutation last only for one day. The day on which transformants first appeared on the plate was counted as day one.
Fig. 15. The effect of catalytically dead pola (D984N) mutation on switching at molecular level. The wild type and swi7-1 strain transformed with control plasmid, wt pola and (D984N) pola plasmid and transformants were grown in thiamine containing selective media. After growth for 6 hrs, the 0 hr sample was taken and thiamine was removed. Cells were inoculated into fresh media followed by pelleting of cells at each hour up to 6 hrs and last sample was taken after 1 day ensuring complete induction of (D984N) plasmid in absence of thiamine. DNA was prepared from all samples and digested with HindIII followed by Southern blotting and hybridization with 10.4 kb mat1M fragment. The Southern blot shows restoration of double strand break even at 0 hr in swi7-1 strain containing (D984N) plasmid (left pannel, Lane 4) as well as in wild type strain (right pannel, Lane 4). Interestingly, in swi7-1 transformants the donor (mat3M) locus of 4.2 kb was deleted selectively in the (D984N) transformants (left panel, Lane 4-9).

Fig. 15, A. The deletion of donor cassettes by catalytically dead pola (D984N) mutation. The swi7-1 strain transformed with control plasmid, wt pola and (D984N) pola plasmid and transformants were grown in selective media with thiamine and genomic DNA was prepared before and after removal of thiamine. The DNA samples were digested with HindIII followed by 0.8% agarose gel electrophoresis and Southern blotting with 10.4 kb mat1M fragment. The Southern blots shows deletion of both mat2P and mat3M donor cassettes in transformants harboring catalytically dead pola (Lanes 4-7).
donor cassette was also confirmed by repeating the experiment (Figure 15A, compare lane 1 and 3 with lanes 4 to 7). The switching defect at cellular level can now be ascribed to donor deletion in swi7-1 transformants while in case of wild type transformants such effect was not observed (Figure 15, right panel, lanes 4 to 9).

These observations point towards two possibilities either the catalytically dead pola complements switching defect of swi7-1 strain, implying that merely the physical presence of pola is sufficient for imprinting or the (D984N) mutation gets repaired inside the cell to cope up with its deleterious effect on replication, thus providing a wt pola gene that complements the swi7-1 mutation.

3.1.7 Complementation of swi7-1 mutation by catalytically dead pol α is donor independent

To investigate the effect of donor deletion on swi7-1 complementation by (D984N) pola mutant. A donor deleted swi7-1 mutant strain was transformed with vector control, wt pola and pola (D984N) plasmids. Earlier work by Klar showed that the imprint is generated even in donor-deleted strains (Klar and Miglio, 1986). The swi1 and swi3 mutant strains were also taken to check whether D984N mutant could restore the imprint in these mutants.

Southern blotting result showed complete restoration of double strand break by (D984N) mutant pola even in donor deleted strain (Figure 16, lane 12) while, no restoration of DSB was observed in swi3-157 and swil-111 mutants (Figure 16, lanes 1 to 8).

3.1.8 Catalytically dead Pola (D984N) generates a Plus allele at matl locus

The apparent deletion of donor cassettes in pola (D984N) transformants of swi7-1 strain encouraged us to check the mating type predominance at matl locus. The predominance of a particular mating type within a single colony of h90 strain can be determined by mating with the tester strains which contains either P or M stable mating types. Interestingly, the spo transformants of h90 swi7-1 showed mating preference with Minus strain but not with Plus strain (Figure 17, upper panel). This result indicates that the matl locus in the (D984N) transformants of swi7-1 may contain predominantly the P-type information.

This result was confirmed by multiplex PCR using primers that specifically determine the presence of either mat2 (mat1P) or mat3 (mat1M) information at the expressed matl locus (Jia et
Fig. 16. The effect of Catalytically dead pola (D984N) mutation on switching was independent of donor loci. To confirm the donor dependence of (D984N) mutation in swi7-1 strain, donor deleted swi7-1 strain along with swi1 and swi3 mutant as negative control were transformed with (D984N) plasmid and double-strand break was checked. Southern blots shows that effect of (D984N) mutation on break restoration was independent of donor status (Lanes, 10-12). swi1 and swi3 mutants show no effect of catalytically dead pola (D984N) plasmid.
Fig. 17. The Catalytically dead polα (D984N) exerts a strong directionality effect. Analysis of the effects on mating-type switching of polα (D984N) in a homothallic $h^{00}$ strain was performed by mating with tester strains (Msmt0) and (PΔ17). The polα (D984N) mutation exhibits a strong P mating type as shown by dark staining colonies when crosses with msmt0 tester strain. The multiplex PCR determination of the predominant mating type PCR reactions were performed using appropriate primers to amplify matIM and matIP sequences simultaneously. The polα (D984N) mutation shows a predominant Plus mating type in both episomal as well as in integrated form (Fig. 17, Lanes 4 and 5).
3. Role of DNA polymerase α in setting up Imprint and chromatin Organization

The results show that in wild type h^90 cells, efficient mating type switching results in equal utilization of mat2 or mat3 as a donor, so that bands representing mat1P and mat1M cells are roughly of the same intensity. In contrast, in pola (D984N) transformed swi7-1 strain only mat1P mating type was visible and mat1M information was entirely absent (Figure 17, lower panel). These results indicate that the D984N transformant of swi7-1 mutant harbor only the plus allele at mat1.

3.1.9 Effect of catalytically dead Pola on mating type silencing

polα has been reported to affect mating type silencing in fission yeast (Ahmed et al., 2001; Ahmed and Singh, 2001). The DNA elements adjacent to the mat2P and mat3M cassettes of fission yeast participate in the repression of transcription of mat2P and mat3M loci. These elements are called silencer elements and contain ARS consensus sequences. Silencer elements are required for silencing both in budding yeast and fission yeast. Earlier it was shown that swi6 and clr1-4 mutants exhibit a modest effect on silencing alone but this effect was greatly enhanced in combination with deletion of centromere proximal repression element, REII of mat2P (Thon et al., 1994). Therefore, a strain was taken in which centromere proximal silencer of mat2P (1.8kb BglII-BssH1 fragment) was deleted and ura4^+ marker gene inserted distally to mat2P (genotype: mat1M, smt0, REIIΔ mat2P::ura4^+). To check the effect of polα (D984N) mutation on mating type silencing the polα (D984N) mutant plasmid was transformed into this strain. In wild type strain this ura4^+ marker gene remains repressed as assayed by serial dilution experiments. Equal number of cells (10^7 cells/ml) were serially diluted (10-fold) and spotted on complete plates, Leu^− selective plates, lacking uracil and plates containing FOA. These results show almost negligible effect of (D984N) mutation in ura4^+ expression (Figure 18). However, on iodine staining (D984N) transformants show high proportion of speckled colonies in comparison to vector control and nmt polα transformed cells (Figure 19A and 19B). Since the strain used in this study is a nonswitching strain (mat1M, smt0, REIIΔ mat2P::ura4^+, leu1-32, ura4D18, ura4^+, ade6-216) and presence of high proportion of speckled colonies are indicative of silencing defect. Similar effect has been reported in swi6, clr1-clr4 mutants (Thon et al., 1999) and later in polα/swi7H4 (Ahmed et al., 2001). These results indicate an interesting finding that interference with DNA replication can elicit a strong silencing defect.

3.1.10 The Catalytic function of Pola is not required for imprinting at mat1 locus

Restoration of double strand break by the catalytically dead polα was indeed a surprising observation, which encouraged us to check the intactness of mutant form in various transformants.
Fig. 18. Serial dilution assay to check the effect of polα (D984N) mutation on expression of ura4+ inserted at mat2P. Msmt0Δmat2::ura4+ strain was transformed with vector control, wt polα and (D984N) mutant polα. Cells of each transformant were grown overnight, serially diluted, spotted on indicated selective plates and allowed to grow at 30°C for 3-4 days. Results shows that (D984N) mutation has no effect on silencing as shown by expression of ura4+ gene was not altered in transformants.

Fig. 19. Colony phenotype of Msmt0 REII Δmat2::ura4+ strain transformed with (D984N) mutant polα. (A) The Msmt0REIIΔmat2::ura4+ strain was transformed with vector control, wt polα and (D984N) mutant polα. Transformants were stained with iodine. The proportion of speckled colonies were very high in (D984N) transformants. (B) Graph showing the relative proportion of white and speckled colonies in different transformants as indicated.
Chapter 3. Role of DNA polymerase α in setting up Imprint and chromatin Organization.

For that diagnostic PCR was performed with genomic DNA and plasmids recovered from wild type and swi7-1 transformants. The oligos used for PCR are: wtDNF+swiDNR, (diagnosis of wt copy at D984N), mutantDNF+swiDNR, (diagnosis of D984N mutation) and wtswi71F+swi71R, (diagnosis of wt copy at swi7-1), mutantswi71F+swi71R, (diagnosis of swi7-1 mutation). The D984N mutant plasmids were recovered from transformants and diagnostic PCR was performed to check the intactness of mutation on chromosomal copy and on plasmid copy using genomic DNA and recovered plasmids as template. The result shows that the recovered plasmids are intact and still harbor the D984N mutation (Figure 20). Since expression of D984N plasmid has a dominant negative effect on cell growth (Bhaumik and Wang, 1998), the growth of wild type and swi7-1 mutant containing pola (D984N) plasmid was compared. The increase in cell number on induction by removal of thiamine was monitored by taking absorbance at 595 nm. After 15-16 hrs of removal of thiamine from the media (Maundrell, 1993), the over expression of catalytically dead Pola (D984N) caused a significant slowdown of cell growth in swi7-1 mutant (Figure 21). These results further rule out the possibility of plasmid repair and suggest that the catalytic function of pola is not required for setting up imprint at mating type locus and even replication inefficient pola can generate the imprint.

3.1.11 Presence of DX (6-30) [D/E] XK motif in Pol α

Since pola is involved in setting up imprint at matl locus, its physical presence is sufficient for imprinting and only lagging strand is modified during imprinting (which require pola function more frequently then the leading strand), we checked the presence of other signature sequences in Polα protein that have DNA modification activity. Despite the debate over the chemical nature of imprint (Arcangioli and Kaykov, 2005; Holmes et al., 2005; Vengrova and Dalgaard, 2004; Vengrova and Dalgaard, 2005; Vengrova and Dalgaard, 2006), Polα may have some DNA modification activity, which may not be sequence but structure-specific. Restriction enzymes or type II restriction endonucleases are homodimeric enzymes that cleave their substrates within or directly adjacent to their recognition sequence but in monomeric form they can nick only one strand of DNA (Armalyte et al., 2005; Samuelson et al., 2004). The ‘PD-(D/E)-XK’ core fold is found in most type II restriction endonucleases and serves as signature sequence for identification of restriction endonucleases (Nishino and Ishino, 2001). Examples of such identification include BsoBI (VD\(^{212}...E^{240}LK\)), Eco571 (PD\(^{78}...E^{92}AK\)), HindIII (PE\(^{51}...D^{108}AK\)) and EcoRI (PD\(^{91}...E^{111}AK\)). The PD(D/E)XK motif has also been found in λ–exonuclease (PD\(^{119}...E^{129}LK\)), E.coli MutH (QD\(^{70}...E^{77}LK\)) and in some Holliday junction resolvases (Pingoud and Jeltsch, 2001) (Figure 22, lower panel).
Fig. 20. The diagnostic PCR showing intactness of mutant copy of polα (D984N) plasmid in wild type and swi7-1 strain. Diagnostic PCR was performed with genomic DNA and (D984N) plasmids recovered from transformed wild type and swi7-1 strains. Primers pairs used for PCR are (wtDNF + swiDR, diagnosis of wt allele at D984D), (mutantDNF + swiDR, diagnosis of mutant allele at D984N) and (Wt swi7F + swi7R, diagnosis of wt copy of swi7-1 allele at G1116G), (mutantswi7F + swi7R, diagnosis of mutant swi7-1 allele at G1116E). Control PCR was performed with primer pair (pold=nf + pold=nr). PCR product was run on 1% agarose gel. Results shows that D→N mutation was intact in recovered plasmids (Lane 5, 13) and swi7-1 mutation was also unrepaired (lane 11) and the possibility of repair due to recombination was ruled out.
Fig. 21. The Dominant negative effect on growth of swi7-1 strain upon overexpression of Polα (D984N) mutant. The wild type and swi7-1 strains transformed with (D984N) plasmid were grown in selective media in presence of thiamine to keep nmt1 promoter driven (D984N) expression to a minimum level. At OD₅₉₅ = 0.5 cells were washed and inoculated in fresh (+) thiamine and (-) thiamine media at equal density. After every 6 hrs growth was measured by taking OD at 595nm. After 16 hrs of removal of thiamine from the media (Maundrell, 1993), the overexpression of catalytically dead Polα (D984N) caused a significant slowdown of cell growth in swi7-1 strain.
### Enzyme | PD motif | D/EXK motif
--- | --- | ---
EcoRI | PD$^{31}$ | E$^{111}$AK
EcoRV | PD$^{74}$ | D$^{90}$IK
BamHI | ID$^{74}$ | E$^{111}$FE
PvuII | ND$^{58}$ | E$^{68}$LK
Cfr10I$^a$ | PD$^{134}$ | (E$^{204}$)S$^{188}$VK
FokI | PD$^{450}$ | D$^{46}$TK
BglII | PD$^{116}$ | D$^{142}$IK
MnlI | PD$^{83}$ | E$^{98}$IK
NaeI | TD$^{86}$ | D$^{95}$CK
BglIII | ID$^{84}$ | E$^{93}$VQ
NgoMIV$^a$ | PD$^{140}$ | (E$^{201}$)S$^{185}$CK
BsoBI | VD$^{12}$ | E$^{240}$LK
α-exonuclease | PD$^{119}$ | E$^{129}$LK
E. coli MutH | QD$^{70}$ | E$^{77}$LK
E. coli TnsA | D$^{114}$ | (E$^{149}$)Q$^{130}$VK
Pfu Holliday junction resolvase | VD$^{36}$ | E$^{46}$VK
E. coli McrBC | TD$^{243}$ | D$^{256}$AK

---

Fig. 22. Presence of DX$_{(6-30)}$[D/E]XK motif in fission yeast DNA pola. Diagrammatic representation of pola gene showing all the studied mutation with their relative position on C-terminal. The Polα protein contains two overlapping PD-(D/E)XK-like motifs at its C-terminal. The consensus sequence of DX$_{(6-30)}$[D/E]XK and its location on Pol α is indicated. Table showing the presence PD-(D/E)XK motif in restriction nuclease superfamily, in recombination repair proteins like E.coli MutH and in Holliday junction resolvases.
We scanned fission yeast DNA polymerase α for PD (D/E) XK like motif. To our surprise we indeed found two overlapping PD (D/E) XK motifs, located downstream of region-VII and within the region-V (Figure 22, upper panel). The sequence of corresponding region was LDSQGKPNLDVKGLDMK spanning amino acid sequence 1040 to 1056. The two overlapping PD (D/E) XK motifs are D----X(7) ----DVK and D-------- X(12) -------DMK. The Asp (D) residues are involved in metal ion binding and responsible for catalytic activity of restriction endonucleases (Pingoud and Jeltsch, 2001). Within Polα sequence these Asp (D) residues are located at position 1041 and 1049 for the first motif and position 1041 and 1054 for the second PD (D/E) XK motif (Figure 22, upper panel).

3.1.12 Does putative PD (E/X) XK motif in polα play a role in switching?

Out of three Asp (D) in the two overlapping PD (D/E) XK motifs the last two Asp (D) residues at positions 1049 and 1054 fall into the conserved region-V (Figure 23A), which is responsible for dNTP binding (Wang et al., 1989). A single amino acid change of Asn (N) 61 to Lys (K) confers altered sensitivity to aphidicolin and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) in this region. The corresponding residue at this site in human polymerase α is an aspartate (Gibbs et al., 1988; Hall et al., 1989; Wong et al., 1988) (Figure 23B). Mechanism of action of aphidicolin involves a strong competition with dCMP incorporation, a weaker competition with dTMP incorporation and very little, if any, competition with dGMP and dAMP incorporation. The first (D) Asp (1041) of fission yeast DNA Polα falls in a nonconserved region between region-VII and region-V (Figure 23A).

With the help of Overlap Extension PCR all three (D) Asp residues were mutated to (A) Ala in a LEU2 + based vector containing full length pola gene in front of adh promoter (Figure 23C). The mutants were named as pART1 pola41(D1041A), pART1 pola49(D1049A) and pART1 pola54(D1054A). Along with vector control and wt pola all three mutant plasmids were transformed into swi7-1 and wild type strains. Both intact and BglII linearised plasmids were used for transformation to check the effect of integrated (single copy) and episomal (multicopy) form. Complementation of pola defect was monitored by iodine staining. It was observed that both in integrated as well as in episomal form these mutants behave as wt pola and complement the defect of swi7-1 mutation (Figure 24A). To further confirm these results at molecular level the double-strand break was checked in different swi7-1 strains transformed with linearised and episomal plasmids of mutant pola. It has been observed that the level of DSB was normal in transformants whether wt pola gene was introduced in linear as well as episomal form (Figure 24B, lanes 3 and 7) or in mutants both in episomal (Figure 24B, lanes 4 to 6) as well as in
Fig. 23. The $\text{DX}_{6-30}[D/E]XK$ motif in fission yeast DNA polα located in region-V. (A) the sequence of polα gene showing the three Asp (D) residues (red circles) of D(E/E)XK motif. The conserved residues in region-V among different eukaryotic DNA polymerases are marked by red bars. (B) The pair wise alignment of region-V of human DNA polymerase α and Herpes simplex polymerase, the dotted squire shows single 61Asn (N) to Lys (K) mutation confers sensitivity to drugs like aphidicolin (C) ClustalX alignment of different fission yeast DNA polα clones in which the three Asp (D) residues were mutated to Ala (A).
Fig. 24. The \textit{DX(6-30)[D/E]XK motif DNA pola} plays no role in switching. (A & B) The three D(D/E)\textit{XK} mutants namely, \textit{pART1pola41}(D1041\rightarrow A), \textit{pART1pola49}(D1049\rightarrow A) and \textit{pARTpola54}(D1054\rightarrow A) were integrated after \textit{BglII} linearization into \textit{swi7-1} strain and switching was checked by iodine staining. At colony level all mutants were found to complement the switching defect of \textit{swi7-1} strain (B) The double strand break was checked in all \textit{swi7-1} transformants with the help of Southern blotting and it was found that all three mutants \textit{pART1pola41}, 49 and 54 restore the break in both episomal as well as in integrated form.
Chapter 3. Role of DNA polymerase α in setting up Imprint and chromatin Organization.

integrated form (Figure 24B, lanes 8 to 10) in comparison to wild type (Figure 24B, lane 1) and wt pola (Figure 24B, lanes 3 and 7). These result shows complementation of swi7-l defect by PD (D/E) XK mutated pola. Therefore, it is inferred that PD (D/E) XK motif in Pola has no role in switching.

3.1.13 Alteration of donor loci upon expression of (D984N) mutant in swi7-l strain:

Earlier results (Figure 15, left panel) suggested a surprising absence of mat3 HindIII fragment upon expression of the pola (D984N) in swi7-l mutant strain. Since the probe used was mat1M HindIII fragment which gives a very weak signal at mat2P position, the status of mat2P in the above transformants is not clear. However on over exposure, the blots did indicate absence of both mat2 and mat3 bands (Figure 15 A, open arrowheads). To characterize the excised donor loci, the Southern blots of the HindIII digests of the genomic DNA of the pola (D984N) transformants of swi7-l mutant were hybridized with the mat2P probe. Surprisingly, a truncated band of ~5.9 kb, which possibly corresponds to mat2P and another band of 4.2 kb were observed. The later band comigrates (Figure 25, lane 2 to 7) at the position of mat3M (Figure 25, lane1). Because it gives strong hybridization signal with mat2P probe, we infer that all the three cassettes, mat1, mat2 and mat3 in pola (D984N) transformants of swi7-l mutant may harbor the Plus allele, with the mat2P having undergone a truncation event.

3.1.14 Search for DNA pola mutant defective in switching by random mutagenesis approach

Since all the site-directed mutagenesis attempts did not affect the function of Pola in imprinting, we attempted the random mutagenesis approach. For this swi5-swi7 double mutant was employed, which gives a complete nonsporulating phenotype. The swi7 affects initial step of switching and responsible for imprinting but on iodine staining it gives some dark staining and speckled colonies and swi5 affects the last resolution step of switching with normal level of imprinting and produced speckled colonies on iodine staining. However, in double mutant of swi5-swi7 both double-strand break and switching are reduced, such that the colonies give very low iodine staining. The double mutant was transformed with hydroxylamine treated nmt pola plasmid and transformants were plated on thiamine containing selective plates. Switching defective light staining colonies were identified (Figure 26A). Out of 8494 transformants only 8 were found to be light staining (spo⁻). Genomic DNA from wild type and light staining colonies were prepared and checked for double strand break. However, result of Southern blotting shows that some of the transformants had a rearranged mat1 locus, which might be due to the effect of
Fig. 25. Alteration of donor loci upon expression of pola(D984N) mutant in swi7-1 strain. Southern blots of the HindIII digests of the genomic DNA of the pola (D984N) transformants of swi7-1 mutant were hybridized with the mat2P probe. Surprisingly, a truncated band of ~5.9 kb, which possibly corresponds to mat2P and another band of 4.2 kb were observed. The later band comigrates (lane 2-7) at the position of mat3M (lane 1).
Fig. 26. Search for DNA pola mutant defective in switching by random mutagenesis approach. (A) The double mutant swi5-swi7 was transformed with hydroxylamine-treated nmt pola plasmid along with vector control. The transformants were plated on thiamine containing selective plates and after 3-4 days the colonies were stained with iodine to identify switching defective light staining colonies (black arrows). Out of 8494 transformants only 8 were found to be switching-defective (B) Southern blotting was carried out to check double-strand break at mat l in all switching defective derivatives. However, all of them were found to be defective in organization of mating type locus. The mating type locus of some of the light derivatives was found to be rearranged (Light 2, 3 and 8) and in remaining the double strand break was not normal (compare wild type with light 1, 4 and 5 to 7).
Chapter 3. Role of DNA polymerase α in setting up Imprint and chromatin Organization.

swi5 mutation on resolution step (Figure 26B, Light 2, 3 and 8). However, some other transformants have intact mat1 and mat3 loci with an additional band (whose origin is not known) (Figure 26B, Light 1, 4, 5 to 7) but also lacking in double strand break. It is possible that these could be caused by a resident mutant pola gene. Future studies will address this issue further.

3.1.15 In vitro endonuclease activity assay of Polα

Since, we were unsuccessful in isolating any switching-defective mutant of pola by site directed as well as by random mutagenesis approach, so we decided to check the endonuclease activity (if any) of fission yeast DNA polymerase α by in vitro endonuclease activity assay. For this MBP pola clone was taken in which pola was cloned in fusion with MBP (Maltose Binding Protein). Expression of MBP-pola clone in E. coli was standardized and it was found that MBP-Pola fusion protein was well expressed after induction for 8hrs at 25°C (Figure 27), pMALP2 vector was taken as a control. Both MBP and MBP-Pola were purified with the help of amylose resin. Since double-strand break is generated at H1 box of mat1 locus, Bluescript mat1P (BSmat1P) clone was taken as substrate in which Xhol fragment of mat1P was cloned. The substrate plasmid was incubated with MBP and MBP Polα in 1X NEB buffer 2 at 30°C, aliquots were taken at 15, 25, 35, 45 and 55 minutes and subjected to electrophoresis on a 1% agarose gel. In lane 1 supercoiled BSmat1P was incubated without MBP Polα and in lanes 2 to 6 aliquots taken at different time points were run as shown in (Figure 28). In MBP-Pola panel the nicking activity was quite pronounced: within 55 minutes of incubation about 75% supercoiled fraction of BSmat1P was converted into open circular form. In the MBP-treated samples some nicking activity is detectable but the shift of supercoiled form into open circular form was not significant (Figure 28) and this effect may be the result of spontaneous nicking due to incubation with buffer. MBP-Pola was also incubated with PCR amplified linear mat1P DNA, in presence of 1X NEB buffer 2. Aliquots were taken at different time points and subjected to electrophoresis in a 1% agarose gel. Result show that there was no double strand cleavage activity in MBP-Pola (Figure 28, bottom panel). These results suggest that while MBP-Pola has some nicking activity, it does not cut both the strands of DNA and it needs supercoiled DNA as substrate for nicking.

3.1.16 In vitro Nicking activity of Polα is not H1 box specific

In S. pombe switching is initiated by imprinting of a single strand at H1 box of mat1 cassette. The upper strand in mat1M and mat1P DNA is nicked not in a sequence but position-(probably structure) specific manner. There is a difference of three nucleotides in the site of nicking between mat1M and mat1P. To determine the strand specificity of cleavage by MBP
Fig. 27. Expression of fission yeast recombinant DNA polymerase α. The fission yeast DNA polymerase α was cloned in *E. coli* expression vector pMAL-P2 and expression of MBP fused recombinant *polα* was checked by SDS polyacrylamide gel electrophoresis. Expression was standardized at various temperatures as indicated. Uninduced sample was run on the same gel as control. The expression was also confirmed by Western blot analysis with anti *polα* antibody.
**Fig. 28. in vitro endonuclease activity assay of Polα.** DNA nicking activity of purified MBP-Polα was determined by incubating BSmat1P supercoiled plasmid with MBP Polα at 30°C, taking MBP alone as control. Aliquots were withdrawn at indicated time points, and the reaction products were analyzed by agarose gel electrophoresis. The supercoiled (sc) substrate was nicked to give the open circular (oc) DNA without the accumulation of the linear form. The accumulation of OC form was measured by quantitating the band intensity using Scion image software. It was observed that in presence of MBP Polα (top left panel) almost 75% DNA was converted from SC to OC form within 55 minutes. There was about 3 fold increase in OC form, while in case of MBP alone the relative accumulation of OC form was not significant (top right panel), while no cleavage activity was observed on using linear DNA as substrate (bottom panel).
Chapter 3. Role of DNA polymerase α in setting up Imprint and chromatin Organization.

Pola we planned a run-off sequencing (Samuelson et al., 2004), in which both the strands of a nicked DNA are sequenced with the help of convergent oligos approaching the nicked site from both the ends. As a result, the intact strand sequence proceeds smoothly till the end, while in nicked strand sequence soon runs off due to discontinuity of template strand and the nicked site is marked by an additional A residue added by the terminal transferase activity of AmpliTaq DNA polymerase used in sequencing reaction (Magnuson et al., 1996). As predicted, MBP Pola should be making a nick on top strand of mat1P cloned in BS (Figure 29, top panel). The DNA substrate BSmat1P was incubated with purified MBP-Pola for 55 min at 30°C in the presence of 1X NEB buffer 2. The nicked DNA product was resolved by electrophoresis and eluted using Qiagen gel elution kit. Both the strands were sequenced with two primers that converge towards the known nicking site, namely mat1Chip14f: ggattaacgcgtactcaattacg and mat1Chip16r: tcaagatgtatggggagaaagag. The mat1chip16r oligo was diagnostic for top strand nick. Sequencing was conducted using the AmpliTaq dideoxy terminator kit (Applied Biosystems) and an ABI XL3130 sequencing instrument. The sequencing chromatograms indicates that sequence proceeds smoothly beyond the predicted nicking site and does not indicate any run off in sequencing reaction (Figure 29, bottom panel). One might argue that presence of some amount of randomly nicked DNA, which bears nicks at random sites other than the predicted site, might be acting as an intact template for sequencing reaction. Considering this scenario and assuming that only 50-60% of isolated DNA was nicked at the predicated position, and given that the last Adenine (A) residue is added by AmpliTaq (Magnuson et al., 1996), a higher peak height of A residue than the neighboring peaks should be present at the putative nicking site, However, this is not the case. In all sequencing chromatograms, all peaks around the predicted nicking site are nearly of same height (*) sign (Figure 29, bottom panel). These results indicate that Pola definitely has some nicking activity and it requires supercoiled substrate for nicking although we were not successful in linking this nicking activity to the predicted nicking site on mat1P DNA. However, these results do not rule out the possibility of structure-specific nicking activity of fission yeast DNA polymerase α. The AT-rich sequences at homology boxes in fission yeast are capable of adopting various secondary structures (Egel, 2005), which might act as substrate for nicking by DNA Pola.

3.1.17 Evidence of unusual DNA structure at mating type locus

Since the observed in vitro endonuclease activity of Polα seems to be directed towards supercoiled plasmid and theoretical considerations suggest occurrence of specific structure at the H1 box, we were prompted to check the bending propensity of DNA at mat1P and mat1M locus (Figure 30A). Probing the presence of DNA super secondary structures i.e. cruciform, hairpin etc.
Fig. 29. Run-off sequencing to determine the nicking site of MBP Polα. The supercoiled substrate BSmat1P was incubated with purified protein for 55 min at 30°C. The nicked circular DNA product was isolated by agarose gel electrophoresis and sequenced using primers that converge towards the predicted nicking site as shown in mat1P DNA (top panel). The mat1chip 16 rev primer was the diagnostic primer for nicked template. The chromatograms of two independent run off sequencing experiments were shown the predicted nicking point where sequence was expected to run off was marked by (*) sign. However, the nicking activity of MBP Polα could not be mapped to the predicted nicking site as shown by lack of run off in the sequencing chromatograms.
Fig. 30. Presence of unusual sequence at mating type locus. (A) Replication pausing at matl (MPS1) The position of the homology domain H1 is indicated by the gray shade and the imprinted nucleotides are circled. The palindromic bases around the imprint are labeled by arrows, indicating the center of the palindrome (Nielsen and Egel, 1989) (B) The 17 PCR primers spanning the entire matl region were designed at every 163 bp distance, which is approximately equal to a nucleosome repeat length 156 (± 2) in fission yeast (Godde and Widom, 1992). Positions of PCR primers used for detection of bending and circular permutation assay are shown by red bar across the entire matl region.
in *in vivo* condition is not very straightforward. Therefore, we decided to check the bending potential of *mat1* locus by simple high percentage acrylamide gel electrophoresis technique.

The phenomenon of sequence-specific DNA bending was first demonstrated by anomalous electrophoretic migration behavior of 212bp fragment of kinetoplast DNA of the trypanosome *C. fasciculata*. The 212bp DNA, due to its curved structure, migrated as if it were 1000 bp long in 6% polyacrylamide gel (Kitchin *et al.*, 1986; Marini *et al.*, 1982). The sequence of kinetoplast DNA has several adenine tracks, which are responsible for its curved structure and anomalous electrophoretic behavior. The curved structure of kinetoplast DNA was visualized by scanning electron microscopy (Griffith *et al.*, 1986). The sequence directed bending of DNA has been shown to play an important role in gene regulation especially by regulating the activity of promoters (Plaskon and Wartell, 1987).

To check the bending propensity of mating type region DNA from Plus and Minus mating type strains was amplified by PCR. The 17 PCR primers spanning the entire *mat1* region were designed at every 163 bp interval which is approximately equal to a nucleosome repeat length 156 (± 2) in fission yeast (Figure 30B) (Godde and Widom, 1992). The amplified PCR products were subjected to 1% agarose and 6% poly acrylamide gel electrophoresis. The results show significant gel retardation of PCR products corresponding to H1 and H2 box in both *mat1M* and *mat1P* DNA (Figure 31A and 31B) on acrylamide gels. On the other hand, all products migrate at the same distance in agarose gels (Figure 31A and 31B, top panels). The observed gel retardation coincides with the H1 and H2 boxes, which has preponderance of AT tracts.

3.1.18 **Bending propensity of *mat1M* DNA is higher than that of *mat1P* DNA**

The reduced mobility of curved DNA during polyacrylamide gel electrophoresis is more pronounced at higher gel concentration. Therefore, a 16% polyacrylamide gel was used and the overlapping PCR products were amplified by taking forward oligo of one amplicon with the reverse oligo subsequent amplicon. In this manner the size of PCR product becomes double of that of the first experiment and each 163bp sequence was represented twice in an overlapping manner. The 163bp fragment towards the 3' end of one amplicon is located to 5' end of the subsequent amplicon. This strategy was applied to get an idea of sequence determinant responsible for slow migration of a particular DNA fragment. These amplified PCR products were subjected to 1% agarose and 16% polyacrylamide gel electrophoresis.
Fig. 31. Bending propensity of **mat 1 DNA.** (A) **mat1P**  (B) **mat1M.** The amplified PCR products were subjected to 1% agarose and 6% polyacrylamide gel electrophoresis. The results show significant gel retardation in PCR product corresponding to H1 and H2 box in both **mat1M** and **mat1P** DNA on 6% PAGE, while in agarose gels all products migrate at the same distance.
Chapter 3. Role of DNA polymerase α in setting up Imprint and chromatin Organization.

Results show the bending potential of mat1M DNA around H1 box is much higher than that of same sequence in mat1P DNA (Figure 32A and 32B, compare lane 14/15 of lower panel with the lane 14/15 of upper panel). Moreover, the DNA region between H1 and H2 box in mat1M seems to be more curved than that of mat1P DNA (Figure 32A and 32B). This sharp curved nature of mat1M DNA around H1 box might contribute to the internal pause site MPS1 in mat1M, which is yet not reported in mat1P (Dalgaard and Klar, 2000; Vengrova and Dalgaard, 2004).

3.1.19 The bend center coincides with the Imprinting site

The center of curvature within a particular DNA fragment can be determined by the circular permutation assay (Wu and Crothers, 1984). In this assay, the sequence of a target DNA is circularly permuted with respect to its ends. If the target DNA is intrinsically curved or contains a region of anisotropic (hinge-like) flexibility, the relative position of the curved region will vary in different permuted sequence isomers. The sequence isomer with the fastest mobility is the one with its locus of curvature located at or near one end of the fragment and this sequence position will be described as the apparent bend center. For circular permutation analysis of mat1 DNA around the H1 box PCR products were amplified using different combinations of oligos as shown in Figure 33A. The amplified PCR products were subjected to 1% agarose and 16% acrylamide gel electrophoresis. The 100 bp ladder (NEB) was run in each gel as a normal mobility marker. Mobilities of various DNA fragments were calculated as described by (Lu et al., 2005). The results show that bend center is located at amplicon Number 13F15R and 14F116R for both mat1P (Figure 33B, left panel) and mat1M DNA (Figure 33B, right panel), which correspond to the sequences around the imprinted site. This experiment clearly indicates that imprinted DNA around the imprinting site takes an unusual bent structure. Moreover the value of mobility determinant is -5 for mat1M DNA (Figure 33C), which indicates that mat1M DNA is more curved than that of mat1P DNA (Figure 33D) (see Methods Section).

3.2 Role of DNA polymerase α in Chromatin Organization

3.2.1 Mutations in polα/δ affect heterochromatin structure at mating type locus

Since loss of silencing in S. pombe has generally been associated with decrease in localization of H3-K9 di-methylation (H3-K9-me2) and Swi6 binding, we tested the localization of H3-K9-me2 and Swi6 in polα/ swi7H4 and polδ mutants. Earlier work has shown that H3-k9me2, and Swi6 are localized to a 20-kilobase silent heterochromatic interval. In contrast, H3 methylated at lysine 4 (H3-K4-me2) is specific to the surrounding euchromatic regions (Noma et
Fig. 32. Greater bending propensity of *mat1M* DNA than that of *mat1P* DNA. (A) *mat1P* (B) *mat1M*. Amplified PCR products were subjected to 1% agarose and 16% poly acrylamide gel electrophoresis. Results shows that the bending potential of *mat1M* DNA around the Swi1-Swi3 binding site and H1 box is much higher than that of the same sequence in *mat1P* DNA. Moreover the DNA between H1 and H2 box in *mat1M* seems to be more curved than that of the *mat1P* DNA.
Fig. 33. Determination of bending center by circular permutation assay. (A) Schematic representation of circular permutation assay (B) Circularly permuted amplified PCR products were subjected to 1% agarose and 16% poly acrylamide gel electrophoresis for matIP (left panel) and matIM (right panel) DNA. The results show significant gel retardation in PCR product corresponding to the sequence around imprinted site. (C) Determination of mobility determinant [100 (μR) − 1] for matIP (left panel) and matIM (right panel).
Chapter 3. Role of DNA polymerase α in setting up Imprint and chromatin Organization. al., 2001). To directly check the localization of Swi6 and H3-K9-me2, we performed the Chromatin immunoprecipitation assay (ChIP), in strains where a ura4+ marker gene was inserted near mat2P region and the endogenous ura4+ gene was replaced by a truncated version of ura4+, called DS/E. Common primers were used to amplify products of 694 bp (ura4+ reporter) and 426 bp for the ura4-DS/E minigene located at the endogenous ura4+ locus. This minigene serves as internal control for ChIP experiments (Partridge et al., 2002) (Figure 34A). Results showed that localization of Swi6 was drastically reduced at the ura4+ reporter flanking the mat2P in both swi7H4/polα and pol8ts2 mutants (Figure 34B and 34C). The level of H3-K9-me2 was also reduced at both loci in both swi7H4/polα and pol8ts2 mutants (Figure 34B and 34C).

3.2.2 Mutations in polα and polδ affect the specialized chromatin structure at centromeres

The fission yeast centromeres consists of two domains the outer domain (otr) and central domain. Silencing at centromeres also involves additional centromere-specific factors including a chromodomain protein Chp1 (Doe et al., 1998). Chp1 along with Swi6 is mainly restricted to otr region (Partridge et al., 2000) and CENP-A (Cnp1) and some histone H3 containing nucleosomes that are dimethylated on lysine 4 of histone H3 are associated with the central core domain The H3 histone variant Cnp1 imparts an unusual chromatin structure to the central domain (Polizzi and Clarke, 1991; Takahashi et al., 2000; Takahashi et al., 1992). The ChIP on Chip analysis in swi7H4/polα and polδ mutant strains with Swi6 and anti H3-K9-me2 antibody show strong enrichment of Swi6 and H3-K9-me2 at central domain in swi7H4/polα and polδ mutant strains. This analysis suggests that in polα and polδ mutant strains the central domain chromatin has lost its unusual chromatin structure and Swi6 and H3-K9-me2 from outer repeats seems to be redistributed to central domain in replication mutants (Figure 35A, 35B and 35C). This redistribution of Swi6 and H3-K9-me2 in all three centromeres might be a consequence of loss of Cnp1 from central domain or defective recruitment of Cnp1 during replication. The delocalization of CENP A from central domain is yet to be checked in swi7H4/polα and polδ mutants by us. Although, the delocalization of Cnp1 from central domain in swi7 mutant has been reported recently (Natsume et al., 2008). The loss of unusual chromatin structure might be the possible reason for lagging chromosome phenotype in polα and polδ mutant strains (Saini, 2005).
Fig. 34. Mutations in pola and polδ influence the heterochromatin localization of Swi6 and histone di-methyl H3-K9. (A) The diagrammatic representation of mat2P::ura4 strain used for ChIP analysis and strategy for the quantitative PCR assay used in ChIP experiment. Common primers amplify products of 694 bp (ura4 reporter) and 426 bp for the ura4-DS/E minigene located at the endogenous ura4 locus (B) Chromatin was extracted from wild type, swi7H4/pola and polδts2 mutants followed by immunoprecipitation with antibodies against Swi6 and H3-K9-me2. Immunoprecipitated samples were subjected to radiactive PCR followed by 4% polyacrylamide gel electrophoresis and autoradiography. (C) Quantitation results of autoradiography, where ratio of immunoprecipitated and whole cell extract was plotted to show the fold enrichment. Results show that Swi6 and H3-K9-me2 were drastically reduced at ura4 gene inserted in the mat2P locus in swi7H4/pola and polδts2 mutants.
Fig. 35. (A) Mutations in pola and polδ affect the unusual chromatin structure at the centromere. Chromatin was extracted from wt, swi7H4/pola and polδ mutants followed by immunoprecipitation with antibodies against Swi6 and H3-K9-me2. Immunoprecipitated samples were fluorescently labeled and hybridized to S. pombe whole genome microarray slides. Results show the redistribution of Swi6 and H3-K9-me2 at the central core domain (cnt1) at centromere1 in swi7H4/pola and polδts2 mutants.
Fig. 35. (B) Mutations in polα and polδ affect the unusual chromatin structure at the centromere. Results show the redistribution of Swi6 and H3-K9-me2 at the central core domain (cnt2) at centromere2 in swi7H4/pola and polδts2 mutants.
Fig. 35. (C) The mutations in polα and polδ affect the unusual chromatin structure at the centromere. Results show the redistribution of Swi6 and H3-K9-me2 at the central core domain (cnt3) at centromere3 in swi7H4/pola and polΔts2 mutants.
3.3 Discussion

The role of swi7 gene in setting up imprint was reported nearly twenty four years ago (Egel et al., 1984). Later, swi7 gene was found to encode the catalytic subunit of DNA polymerase α and a single mutation at position 1116 Gly to Glu was reported to abolish its activity in setting up imprint at matl locus (Singh and Klar, 1993). However, the exact mechanism of polα in the imprinting in fission yeast is still not known. Although, significant reports have appeared which were mainly focused on understanding the nature of the matl imprint and the exact chemical nature of the imprint still remains the subject of controversy. The imprint was initially reported as a site- and strand-specific nick (Arcangioli, 1998). Later, it was reported to be an alkali-labile or RNase-sensitive modification in single strand of DNA (Dalgaard and Klar, 1999), where either one or two ribonucleotides have been reported to be incorporated into the matl DNA, creating a DNA–RNA–DNA hybrid strand (Vengrova and Dalgaard, 2004). Recently, the chemical nature of the imprint was again reported as nick contains 3'OH and 5'OH unphosphorylated termini resistant to RNase treatments (Kaykov and Arcangioli, 2004). All recent studies were focused more on defining the chemical nature of imprint but none of them have attempted to address how the imprint is generated. This study is an attempt to understand how DNA polymerase α is involved in setting up the imprint at matl locus.

DNA polα is involved in switching and in swi7-1 mutant of DNA polα the level of imprint is reduced (Singh and Klar, 1993). During unidirectional replication of matl cassette, only lagging strand is imprinted in a site-specific manner, which requires polα function more frequently for replication then the leading strand replication (Dalgaard and Klar, 2001b; Egel, 2005). These considerations make Polα a suitable candidate for a role in setting up imprint at the matl locus. Some of the DNA polymerases are known to code for intein homing endonucleases (Matsumura et al., 2006; Nishioka et al., 1998). The homing endonucleases are notable for their long target sites and a tolerance for sequence polymorphisms in their substrates (Kowalski and Derbyshire, 2002). The vast majority of homing endonucleases fall into four families defined by the presence of conserved sequence elements, the LAGLIDADG, the GIY–YIG, the His–Cys Box, and the HNH families (Kowalski and Derbyshire, 2002). Fission yeast DNA polymerase α was also found to contain a putative LAGLIDADG homing endonuclease motif. Science in most of the LAGLIDADG endonucleases active site constitutes Asp (D) or Glu (E) residues, the DADG domain of fission yeast was mutated to check its effect in switching. However, none of the mutations affected the imprinting, ruling out the role of the putative LAGLIDADG motif in imprinting. Further sequence search revealed presence of a restriction endonuclease like domain
Chapter 3. Role of DNA polymerase α in setting up Imprint and chromatin Organization.

in Pola, known as PD(D/E)XK domain. Nearly all structurally characterized REases belong to PD(D/E)XK superfamily restriction endonuclease-like proteins, also called a PD-(D/E)XK nucleases, constitute a large and diverse superfamily of enzymes that are involved in numerous nucleic acid cleavage events important for various cellular processes. These include the restriction endonuclease-like superfamily, including among others 15 different restriction endonucleases (Bujnicki, 2003), Holliday junction resolvases (endonuclease I, Hjc) (Hadden et al., 2001; Nishino et al., 2001), lambda exonuclease (Kovall and Matthews, 1997) and very short patch repair (Vsr) endonuclease (Tsutakawa et al., 1999). However, mutational studies failed to show a role of the PD (D/E) XK motif of Polα in imprinting.

One interesting outcome of this study is the demonstration of the role of catalytically dead polα in mating type switching. We explored the effect of a catalytically dead but structurally intact polα mutant known as polα D984N (Bhaumik and Wang, 1998) in mat1 imprinting. This mutation involves a critical Asp (D) residue in region-1, the most conserved region of the α-like DNA polymerases. Similar mutation of the conserved Asp residue in human Pola Asp (D)1004 to Asn(N) completely abolishes the catalytic activity of Polα. This mutation, however, does not alter either the protein structure of Polα or the ability of the mutant Polα protein to assemble into the Polα-primase complex (Copeland and Wang, 1993a; Copeland and Wang, 1993b). The corresponding Asp(D) residue in fission yeast is located at position Asp(D)984. Surprisingly, we found that catalytically dead polα mutant D984N restores the imprint in swi7-1 mutant. Thus, so merely the physical presence not the catalytic function of Polα may be sufficient for mat1 imprinting in fission yeast. Since the D1004N mutation in human Polα does not affect synthesis of RNA primer by the primase subunit but affects the extension of the RNA primer by the catalytic subunit (Copeland and Wang, 1993b), the catalytic function of Polα is not required for imprinting. It is possible that the RNA primer may be sufficient for the imprinting.

We also checked the in vitro strand nicking activity of Polα and considerable nicking activity was detectable in recombinant Polα. However, we could not map the nicking site to that of the normal nicking in mat1. This may be because of experimental limitations of exactly replicating the template structure required for in vivo imprinting. Since imprinting is a replication coupled process and the nick is generated during S-phase of cell cycle (Holmes et al., 2005) and the homology boxes have the potential to adopt stem-loop like secondary structure (Egel, 2005), it may be possible that under in vivo conditions a unique secondary structure of H1 box might be acted upon by Polα. The AT-rich sequences, which are located at mat1, can adopt some secondary
structures during replication. Such structures are reported to be associated with chromosome fragile site. These secondary structures can perturb replication as the polymerases encounter them. Although the majority of these perturbations are detected by the ATR-dependent DNA damage checkpoint and DNA repair machinery, some escape and present themselves as gaps and breaks on chromosomes (Durkin and Glover, 2007). Sharp DNA bending at H1 box was also detected by 16% acrylamide gel electrophoresis. DNA bend and transient pause of replication fork at nicking site might allow the DNA to take a novel secondary structure which can be acted upon either by Polα or Polα associated endonuclease. All these possibilities will be investigated in future studies.

A series of chromatin immunoprecipitation experiments performed in polα and polδ mutant strains revealed some interesting clue about replication and heterochromatin formation coupling. Loss of Swi6 and H3-K9-me2 from mating type region in polα and polδ mutant strain suggests that replication proteins play an important role in Heterochromatin formation and maintenance. Loss of unusual heterochromatin structure from central domain of centromeres suggests that replication proteins are essential for centromere integrity and kinetochore assembly. The observed redistribution of Swi6 and H3-K9-me2 from outer repeats to central domain in polα and polδ mutants suggests the possibility of CENP A delocalization from central domain in polα and polδ mutants coupled with redistribution of Swi6 and H3-K9-me2 from outer repeat region to central core domain. These possibilities will be investigated in future studies.