SUMMARY

Heterochromatin represents transcriptionally silent domains of the genome wherein large tracts of DNA are assembled into a specialized chromatin structure that is inhibitory to transcription. Proper heterochromatin assembly is crucial for a variety of chromosomal processes, including gene regulation, nuclear organization, chromosome segregation. Transcriptional silencing is a complex process that is awaiting full characterization. The study of the process in simple, genetically tractable organism, *S. pombe*, has served as a model for studying eukaryotic gene regulation.

The present study was focused on studying the following aspects of heterochromatin assembly in fission yeast:

I Role of DNA polymerase δ in structure and function of heterochromatin

II Role of DNA Polα in telomere maintenance

III Role of RNAi machinery in heterochromatin assembly in fission yeast

I Role of DNA polymerase δ in structure and function of heterochromatin

It has been suggested that DNA replication may be coupled to transcriptional silencing and this ensures the proper establishment and propagation of silent chromatin states. Findings in our lab also support the existence of such a coupling. A temperature-sensitive mutant of DNA polymerase α, *swi7H4* (Murakami and Okayama, 1995) was found to be defective in silencing at all the heterochromatic loci (mating-type loci, centomeres and telomeres) (Ahmed, 2000; Ahmed and Singh, 2001; Ahmed *et al.*, 2001) pointing to a role of this polymerase in silencing. It was seen that DNA polymerase α interacts with the chromodomain protein Swi6 (HP1 homolog), the major structural component of heterochromatin. The interaction between DNA polymerase α and Swi6 has indicated the involvement of Polα in epigenetic control of higher order chromatin assembly and propagation of the silenced chromatin during cell division.

In view of the silencing role of DNA Pol α, it was speculated that DNA polymerase δ, another replicative polymerase, might also be involved in the heterochromatin assembly process. Hence, in the present study, it was planned to investigate a possible role of this polymerase in silencing.

In this study, three ts⁻ alleles of *polδ*: *polδts*1, *polδts*2 and *polδts*3 were used, with the major focus on *polδts*2. These three mutant alleles were generated earlier by *in vitro* random mutagenesis (Shortle *et al.* 1984). These alleles have missense transition of G-C to A-T. The *polδts*1 allele has two adjacent point mutations. The *polδts*2 and *polδts*3 alleles have single point mutation. None of three point mutations are located in the 3'-5' exonuclease domains or in the DNA polymerase δ catalytic domains (Francesconi *et al.*, 1993). The major findings of this part of the present study were:

- Upon examining the influence of *polδ* mutations on mating-type silencing, it was observed that *polδ* mutations lead to a derepression of the silent mating-type loci. The silencing defect was observed in a silencer-deletion background (a deletion that removes a cis-acting silencer element at centromere proximal side of *mat2*-P). *polδts2* mutation causes derepression of the *ura4*$^+$ marker gene located at the silent mating type locus *mat2*-P.
- We found that in combination with deleted centromere-proximal silencer, *polδ* ts mutants show two types of silencing states, dark staining and light staining. The result clearly suggests that these two states, dark and light, differ in their extent of derepression of silent loci.
- *polδts2* mutation leads to alleviated centromeric position effects as it was observed that this mutation causes a derepression of *ade6* marker gene inserted within the outer centromeric repeats. However, this effect was restricted to *otr* region only as no derepression was apparent at *cnt* or *imr* regions of the centromere.
- Since the silencing defect of *polδts2* at the *mat* locus is dependent on the silencer, similar to mutations in *swi6* and *clr1-4*, it appears that Polδ act in same pathway as Clr1-Clr4 and Swi6. In agreement, it was observed that Polδ interacts with Swi6 and Clr4 and *polδ ts* mutants are defective in Swi6 localization at mating-type region and centromeres.

Hence, we provide evidence for a direct role of DNA replication in the assembly of the heterochromatin state. The present results indicate that Polδ may act in concert with Clr4/Rik1, wherein a close interaction of Pol*δ* with Swi6/Clr4/Rik1 complex may bring about a concerted chain of events involving recruitment of Clr4 and Swi6 to *mat* and *cen* loci, histone H3-K9 methylation and subsequent binding of Swi6. DNA polymerase δ may perform an imprinting function in establishing a chromatin state by recruiting the

components of heterochromatin assembly. Further studies will help to decipher the order of events involved in heterochromatin assembly with respect to DNA replication. Considering that proteins related to Swi6 and Clr4 are found in higher eukaryotes, similar interactions in other species might be involved in the assembly of specific chromatin structures.

In agreement with a role of Polδ in transcriptional silencing at the centromeres, we found that mutations in this polymerase influences centromeric functions. Centromere is essential for the proper segregation of chromosomes during mitosis and meiosis, mediating chromosome movement along microtubules and normal sister chromatid cohesion and separation. Investigating this aspect, the following findings were made:

- *pol&s2* mutants display cold sensitivity and elevated sensitivity to the microtubule destabilizing drug, TBZ. The sensitivity of *polδ* mutants to the drug indicates that Polδ interact directly or indirectly with some aspects of tubulin function.
- DNA Polδ is required for centromeric cohesion and for accurate mitotic chromosome segregation as mutations in this polymerase leads to centromeric cohesion defects and aberrant mitotic segregation events.
- Mutations in *polδ* influences chromosome dynamics during meiosis as well. The perturbation of the meiotic chromosome segregation in *pol&s* mutants suggests that DNA Polδ is required for the fidelity of meiotic chromosome segregation. Though several aberrant phenotypes were observed in sporulating *pol&s* mutant, the phenotypes resulting from missegregation events during Meiosis II were predominant. In view of this, it is speculated that DNA Polδ may also influence Rec8-mediated centromeric cohesion during meiosis.

II Role of DNA Polα in telomere maintenance

Telomeres are essential for genome stability in all eukaryotes. Changes in the telomere functions and the associated chromosomal abnormalities have been implicated in human aging and cancer. Understanding the mechanisms maintaining telomeres and insights into their action have advanced considerably in recent years. These includes processes acting on the telomeric DNA and on telomere proteins. In the absence of a telomere maintenance system, many eukaryotes (fungi, trypanosomes, flies, mosquitos) lose terminal repeat sequences. Human and mouse telomeres shorten much faster, suggesting that chromosome ends in these organisms might be actively degraded. If telomere erosion is not balanced by elongation, telomeres will progressively shorten, eventually leading to chromosome

instability and cell death. The most versatile and widely used method of telomere maintenance is based on telomerase. However, telomerase is not the only activity that affects the length of telomeres. Other proteins have been shown to affect the telomere length maintenance. The replication machinery components have been shown to effectively influence the telomere length. Recent studies have suggested the close relationship between the replication and telomerase complexes. Thus, mutations of the components of replication machinery can affect the telomere length (Dahlen *et al., 2003)*. In a *pol*α mutant, abnormal telomere lengthening was observed but the expression of wild-type *pol*α^+ gene restored the telomere length to near wild-type length. In this study, we analysed telomeres in fission yeast *pol*α temperature sensitive mutant, *swi7H4*. The following findings were made in the present study:

- The *swi7H4* mutant exhibited a unique telomere pattern showing a combination of chromosomes with longer telomeric repeats and a subtelomere lacking telomeric repeats.
- The telomere elongation seen in *swi7H4* mutant is an extension of the G-strand, not the C-strand.
- The effect of *swi7H4* is observed in senescence and the telomere elongation was found to persist in survivors of senescence in *swi7H4* mutant. The reduced telomere length without the telomere repeats in the survivors may be stably maintained without recombination, possibly by generating a stable, heritable chromatin structure.
- The genetic studies have indicated a dominant effect of *swi7H4* on telomere length and this effect is propagated during meiosis.
- We checked whether proteins required for epigenetic heterochromatin functions, like Clr1-Clr4 and Swi6 (Nimmo *et al.*, 1998; Thon and Hansen, 2000), are also required for the normal telomere pattern. A similar effect of *swi7H4* mutation and (*clr2*, *clr4* and *swi6*) mutations on telomere loss was observed, suggesting that the Polα-mediated recruitment of the heterochromatin proteins Clr2, Clr4 and Swi6 may play a critical role in maintaining telomeric repeats.

The stable persistence of such telomeres and their inheritance during mitosis and meiosis, suggest that the subtelomeres lacking the telomeric repeats may adopt an alternative chromatin structure, which protects the broken ends lacking the repeats from nucleases, and which can be propagated stably during mitosis and meiosis. Thus, the subtelomeric end

lacking the telomeric repeats can be maintained and propagated as a stable epigenetic chromosomal locus during mitosis and meiosis. As the unique pattern may be generated by a recombination event, Polα, Clr2, Clr4 and Swi6 may act together in generating a chromatin structure, which suppresses recombination.

III RNAi machinery in heterochromatin assembly in fission yeast

It has been firmly established that the RNAi pathway influences heterochromatin assembly process. Despite significant advances, the mechanistic details of how the RNAi pathway machinery influences the global transcriptional silencing by heterochromatinization process are not fully known. An important aspect that is unknown is how H3-K9-methylation is initially established by the RNAi pathway. It was hypothesized by Volpe *et al.* (2002) that the siRNAs may form complex directly with Swi6 and/or Clr4 or exist in complex containing the proteins. The complex may be carried to the DNA regions homologous to the siRNA sequences where the protein components Swi6 and/or Clr4 may initiate the histone H3-Lys9 methylation. The following findings were made in the present study:

- To determine whether Clr4 interacts with siRNA directly, we studied the *in vitro* interactions with recombinant protein, GST-Clr4. We demonstrate that siRNA duplex interacts with Clr4.
- Studies to map the domain in Clr4 that is responsible for the interaction, assays were performed. These assays revealed that derivative with single amino-acid substitution (G486D) in SET domain had weaker interaction with siRNA whereas, chomodomain mutations W31G and W41G and SET domain mutation W487stop codon did not affect the nature of ds-siRNA binding significantly. This result suggested the involvement of SET domain in siRNA binding.
- Swi6 also interacts with siRNA duplex, although relatively weakly, as compared to Clr4.
- The three regions of Swi6 namely the chromodomain, chromoshadow domain and hinge domain were tested for binding to siRNA. Surprisingly, all three domains were found to interact with siRNA, suggesting that there is no domain specificity for RNA interaction.
- Interestingly, Clr4 exhibits binding not only to the synthetic siRNA duplex, but also to For ssRNA but not to the Rev ssRNA, indicating a strand and possibly, sequence-

specificity of Clr4 binding. Competitive studies indicate that Clr4 binds to nucleic acids in order of affinity ssDNA ≅ ssRNA> ds-siRNA.

- Most interesting property of Clr4 uncovered by our results is that of its binding to RNA-DNA hybrid. It is worth noting here an additional point revealed by competition studies that Clr4 could also bind to RNA-dsDNA complex, although the nature of interaction between the strands of RNA and DNA is not known at present.

In view of the above results, it is proposed that the helicase component of RISC may unwind the siRNA duplex to generate the ssRNA, which gets complexed with Clr4 and/or Swi6. The complex is carried to the site of the homology (*dg* and *dh* repeats in the *cen* and *K* region) and is incorporated into a "specific triple-strand complex" in the chromatin context. Thus recruited, Clr4 may cause the H3-Lys9 methylation, following which, Swi6 would bind to the H3-Lys9 methylated sites and, subsequently, through multimer formation, to the assembly of heterochromatin. The results that Swi6 also bind to ssRNA and some other work in our lab showing a direct interaction between Clr4p and Swi6p *in vitro*, lead us to suggest that Swi6 may also participate in the above pathway as a complex with Clr4.