CHROMATIN STRUCTURE AND TRANSCRIPTION OF YEAST U6 snRNA [SNR6] GENE

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CERTIFICATE

The research work embodied in this thesis has been carried out at the Centre for Cellular and Molecular Biology, Hyderabad. This work is original and has not been submitted in part, or full, for any other degree or diploma of any other university.

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To

My Amma and Achan

CONTENTS

List of Abbreviations:

Bp	Base pairs
BSA	Bovine serum albumin
^{0}C	Degree Centigrade
dNTP	Deoxy-ribonucleoside Tri-phosphate
DNase	Deoxy-ribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
Fmol	Femtomoles
HAT	Histone acetyl transferase
kDa	Kilo Daltons
mg	Milligram
min	Minutes
MNase	Micrococcal nuclease
Ml	Milliliter
µg	Microgram
µl	Microliter
NFR	Nucleosome free region
ng	Nanogram
nM	Nanomolar
NPS	Nucleosome positioning sequence
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
PIC	Pre initiation complex
PMSF	Phenylmethylsulfonyl-fluoride
PNK	Polynucleotide kinase
Pol I	RNA Polymerase I
Pol II	RNA Polymerase II
Pol III	RNA Polymerase III
rNTP	Ribonucleoside tri-phosphate
SDS	Sodium dodecyl sulphate
TSS	Transcription start site

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SYNOPSIS

Prokaryotes use a single RNA polymerase to transcribe all genes in the organism but in eukaryotes, transcription is carried out by three different RNA polymerases. RNA polymerase I (pol I) is dedicated to transcribe rRNA genes while RNA polymerase II transcribes all protein coding genes. In the budding yeast, RNA Polymerase III (pol III) transcribes ~300 genes coding for the short non-coding RNAs like tRNA, U6snRNA (SNR6), 5S rRNA etc. The basal transcription factor of pol III, TFIIIC, binds to the two intragenic promoter elements, boxes A and B and recruits the initiation factor TFIIIB 30 bp upstream of the transcription start site, which recruits pol III in turn. Generally, A and B boxes are separated by 50-60 base pairs.

Eukaryotic transcription process is much more complicated as compared to prokaryotic transcription owing to the packaging of DNA in to chromatin. Chromatin is not an inert packing material, but an additional means for precise regulation of gene expression as well as other nuclear transactions involving DNA. The major chromatin modifying machineries include ATP dependent chromatin remodelers, protein complexes that can add or remove chemical groups (covalent modifications) to and from histones and the histone chaperones that can deposit or eject nucleosomes as well as exchange canonical histones with histone variants involved in chromatin mediated regulation of transcription.

Active participation of chromatin in the transcription by pol II is well documented. In contrast, little is known about the role of chromatin in regulation of pol III-transcribed genes. This may be partly because of the prevailing notion that pol III-transcribed genes are devoid of nucleosomes. Recently, several genome-wide studies have reported association of chromatin modifying complexes with pol III-transcribed genes. The chromatin remodeling complex of yeast, Isw2 localizes to a large number of tDNAs, while RSC is targeted to virtually all pol III-transcribed genes. Histone chaperone Asf1 also localizes on many pol III genes including SNR6. Global nucleosome depletion activates transcription of some unusual pol III genes that are not transcribed under normal conditions, showing a repressive role of chromatin on pol III transcription. All

these studies suggest that various chromatin remodeling and modification machineries may be required even for the pol III transcription in vivo.

Transcription by pol III is tightly regulated in response to growth stage, growth conditions and stress by a central regulator Maf1. Maf1 localizes to yeast SNR6 in vivo and represses SNR6 transcription in vitro. There are no studies available on the response of chromatin structure of pol III-transcribed genes to repression.

U6 snRNA is conserved from yeast to mammals and is transcribed by RNA Polymerase III. Unlike other pol III transcribed genes, the box B in SNR6 (U6 snRNA) gene of *S. cerevisiae* is found 120 bases downstream of the terminator, positioning it ~200 bp away from the box A, in contrast to the optimal 50-60 bp separation of the two found in most tDNAs. The TATA box at –30 bp position can drive the transcription in vitro on naked DNA templates but box B and TFIIIC are required to relieve the chromatin-mediated repression of SNR6, indicating TFIIIC helps alter the chromatin structure in favor of TFIIIB binding.

The reported in vivo chromatin structure of SNR6, which is lost in the absence of TFIIIC binding, has one nucleosome downstream of B box, an array of nucleosomes upstream of TATA box and a sub-nucleosomal size (~100 bp) protection between the boxes A and B leading to the idea that the DNA between A and B boxes is occupied not by a nucleosome but a non-histone protein like Nhp6. Previous in vitro studies from our lab had shown that binding of TFIIIC and TFIIIB to SNR6 leads to the positioning of a nucleosome between A and B boxes as well as upstream of TATA box. ATP dependent chromatin remodeling after TFIIIC binding is necessary for the high level activation of SNR6 on chromatin templates advocating for the role of chromatin in regulation of SNR6.

This study was taken up with an aim of resolving the actual chromatin structure of SNR6 and establishing any possible involvement of chromatin in regulation of SNR6 in vivo. First chapter of the thesis introduces to the topic of the study while the second chapter gives the methodology used. Chapters that follow constitute the findings of this study which helped to propose the hypothesis in the concluding chapter.

In the first chapter, an overview about pol III transcription machinery, its transcriptome and regulation is given. This is followed by an introduction to ATP dependent chromatin remodelers, covalent histone modifications and histone variants. Chromatin mediated regulation of pol II transcription is described as a background followed by a detailed description of involvement of chromatin in pol III transcription. A survey of research on SNR6 gene, followed by formulation of the questions asked in this study is described in the last.

The second chapter describes the various materials involved in the study. Antibodies, yeast strains and primers used are listed along with the commonly used buffers. Various techniques used in this study are also described with detailed protocols.

The third chapter begins with demonstration that SNR6 can be repressed by nutrient starvation which can be used as a tool to study the involvement of chromatin in regulation of SNR6. Chromatin footprinting coupled to nucleosome depletion and ChIP assays shows that the DNA between A and B boxes is indeed condensed by a nucleosome. ATP dependent chromatin remodeler RSC creates a ~ 100 bp nucleosome free region (NFR) around the TATA box by sliding a nucleosome covering TATA box upwards during active transcription. Repression of SNR6 by nutrient starvation is associated with loss of RSC from the gene and sliding of the upstream nucleosome towards the TATA box.

Fourth chapter follows the dynamics of histone variant H2A.Z in the upstream nucleosome. The variant is present during the active condition but lost during transcription repression. ATP dependent chromatin remodeler SWR1 complex is required to deposit H2A.Z in the upstream nucleosome. H2A.Z has a slightly negative effect on SNR6 transcription and the chromatin structure is unaffected in htz1Δ cells. HAT complex SAS acetylates H4K16 on the upstream nucleosome under active condition. Significantly, H4K16 acetylation by SAS takes place after H2A.Z deposition and shows a negative effect on SNR6 transcription similar to H2A.Z.

The fifth chapter shows the striking relation between histone acetylation and SNR6 transcription: Histone H3 acetylations (H3K9, K14 and K18) that are associated with active transcription by pol II show increased levels under repression. Histone deacetylase protein Rpd3 associates with SNR6 under active conditions and deacetylates H3K9 and H3K18. The histone acetyl transferase protein Gcn5 is required to maintain the chromatin structure of SNR6 and deletion of Gcn5 leads to decreased transcription. Surprisingly, deletion of Gcn5 also led to an increased acetylation. These results suggest that Gcn5 may be playing a role other than histone acetylation on SNR6 transcription.

The sixth chapter summarizes all the work presented in this study and a model for the events associated with chromatin is presented. All the results, when taken together, suggest that high levels of histone acetylations in the repressed state of SNR6 keep the gene in a poised state. RSC is known to require acetylation for its remodeling activity. Thus, high acetylation in repressed state can facilitate RSC action for quick return of the gene to the active state.

Chapter 1

Introduction

1.1 Overview

Transcription is the process of copying of the genetic information in DNA in the form of a complementary RNA molecule. All living organisms make use of specialized molecular machineries called RNA Polymerases for this purpose. Chemically, RNA polymerases are nucleotidyl transferase enzymes which unwind DNA, pass through each base on one of the strands while picking up the complementary bases and joining them by making a phosphodiester bond between them to synthesize an RNA molecule. As the complexity of the organisms increase, complexity of Polymerase also increases.

Many viruses like T7 bacteriophage contain RNA polymerases with a single subunit capable of completing the RNA synthesis (Cheetham and Steitz, 2000). Eukaryotic mitochondrial RNA Polymerase is also a single subunit protein showing similarity to T7 RNA Polymerase (Gaspari et al., 2004). Plant plastids contain a nuclear encoded, T7 like single subunit RNA polymerase (NEP) and a plastid encoded eubacteria-type multisubunit RNA polymerase enzyme (Hess and Borner, 1999; Kanamaru and Tanaka, 2004). Both bacteria and archaea contain a single RNA polymerase complex which transcribes all kind of genes. Eukaryotes, in contrast, have different RNA Polymerases with specialized duties.

Bacterial RNA polymerase is a protein complex with a 5 subunit core (α,α, β, β' and ω) which is conserved in sequence, structure and function in all kingdoms of life. Besides the 5 subunit core enzyme, bacterial polymerase requires an additional protein called σ factor for transcription initiation. σ factor is later released from the elongating polymerase. This single core polymerase transcribes all genes in bacteria with the help of different σ factors (Darst, 2001).

Archaeal RNA polymerase is a 11 protein complex that is evolutionarily more close to eukaryotic polymerases than to the bacterial counterpart (Langer et al., 1995). Homologues for all eukaryotic RNA polymerase II subunits except Rpb8 and Rpb9 are found in archaea (Kusser et al., 2008). Archaeal transcription factors TBP (binds to TATA Box), TFB and TFE, the homologues of eukaryotic proteins TBP, TFIIB and TFIIE

respectively are required for proper initiation of transcription (Geiduschek and Ouhammouch, 2005).

Eukaryotes have three different RNA polymerase complexes, each devoted to transcribe a specific set of genes and this gene specificity is conserved throughout eukaryotes. RNA polymerase I transcribes all rRNA genes except 5S rRNA. 18S, 28S and 5.8S rRNA genes are present as a single transcription unit found in tandem repeats of 100 or more copies. Each transcription unit contains promoter elements such as core promoter (CP), upstream control element (UCE) and enhancer. CP is recognized by core factor (CF) and UCE is recognized by upstream activating factor (UAF). Pol I transcription is coupled to ribosome assembly and takes place in the nucleolus (Grummt, 2003).

RNA polymerase II transcribes all protein coding genes and many non coding RNA genes like snRNAs. Amongst the three polymerases, Pol II has the least number of subunits but highest number of transcription factors (TFIIA, TFIID, TFIIB, TFIIE, TFIIF and TFIIH) indicating the enormity of transcription complex assembly. Pol II utilizes varied promoter elements and there is no universal core promoter sequence. In general, Pol II promoters can be put into two categories: Focused promoters and dispersed promoters. Focused promoters have either a single transcription initiation site or a distinct cluster of initiation sites within a short span of nucleotides. Focused promoters are more common in lower eukaryotes as only one third of vertebrate core promoters are the focused kind. Dispersed promoters are more common in vertebrates wherein the transcription initiation sites may be dispersed in a window of 50-100 nucleotides, in CpG islands. Little is known about the mechanism of start site selection in dispersed core promoters (Muller et al., 2007; Juven-Gershon et al., 2008).

RNA polymerase III is the biggest of the three RNA polymerases. Yeast RNA polymerase III is probably the best characterized eukaryotic transcription system. In vitro transcription system with all recombinant factors is established for Pol III transcription (Ducrot et al., 2006). Pol III transcribes all tRNA genes, 5S rRNA gene, U6 snRNA gene (abbreviated as SNR6 in this thesis), 7SL RNA gene and some other non coding RNA genes. Pol III genes are scattered throughout the genome and accumulating

evidences indicate that the Pol III transcription happens at or near the nucleolus (Haeusler and Engelke, 2006).

Apart from the essential RNA polymerases I, II and III, plants have two additional non-essential RNA polymerases: RNA Pol IV and V (Herr et al., 2005; Onodera et al., 2005). Both Pol IV and Pol V share many subunits between themselves and with RNA polymerase II. While the largest subunit of Pol IV is coded by NRPD1 and that of Pol V is coded by NRPE1, the second largest subunit is shared between Pol IV and pol V (coded by NRPD2/NRPE2) (Ream et al., 2009). Though both Pol IV and Pol V are involved in gene silencing mediated by siRNA, they are functionally distinct. Pol IV functions upstream of siRNA synthesis in the silencing and Pol V generates non-coding transcripts at a target loci, which is the process downstream of siRNA synthesis (Pikaard et al., 2008; Wierzbicki et al., 2008).

All eukaryotic RNA polymerases transcribe the DNA that is packaged into chromatin. Initially chromatinization was considered a tool of packaging the very long eukaryotic DNA. Later extensive research has shown that chromatin is not just a packing mode but it participates actively in regulation of all nuclear processes like transcription, replication, repair and recombination. Chromatin can impede transcription by masking various DNA binding sites/promoters. However, eukaryotes have evolved various mechanisms to relieve the block by chromatin and to use it as an effective regulatory, epigenetic framework. These mechanisms include ATP-dependent chromatin remodeling, histone modifications, histone variants etc. A large number of genome wide studies have shown specific patterns of chromatin organization on genes based on their activity status (Li et al., 2007). Most of these studies and models are for genes transcribed by Pol II and little is known about the rule of chromatin on Pol III transcribed genes.

1.2 RNA polymerase III transcription machinery:

Eukaryotic RNA polymerases are large protein complexes with more than 10 subunits. Many of the subunits are either shared by the three polymerases or have paralogues

indicating they are structurally and functionally conserved with small differences dictating their functional differences. Studying the structural similarities and differences can be helpful to understand the basic mechanisms used by these polymerases for transcription as well as to know how they differ from each other as the precise functional territories of these enzyme molecules are well conserved evolutionarily. There are two general transcription factors for pol III: the multi subunit TFIIIC and the three subunit TFIIIB.

1.2.1 RNA polymerase III: Structure and function

Several biochemical as well as protein-protein interaction studies have been made to map positions of individual subunits in the complex (Flores et al., 1999). In the last one decade, several papers are published on the high resolution crystal structure of 10 subunit core of Pol II, Pol II complexed with template DNA and nascent RNA, 12 subunit structure of Pol II etc (Cramer et al., 2000; Cramer et al., 2001; Bushnell et al., 2004). The structural details have illuminated the role of each subunit of Pol II in the process of transcription as well as the mechanisms of transcription initiation and elongation (Gnatt et al., 2001). Moreover, the Pol II structure has served as a frame work for elucidating the structures of other two polymerases (Cramer et al., 2008).

Among the three Eukaryotic RNA Polymerases, Pol III is the most complex with 17 subunits while Pol I and Pol II are having 14 and 12 subunits each respectively. Out of the 17 subunits of Pol III, the largest subunits RPC160 and RPC128 show remarkable similarities with the two largest subunits of Pol II-RPB1 and RPB2 (Table 1.1). Pol I and Pol III share the subunits AC40 and AC19 which are homologous to Pol II subunits RPB3 and RPB11 (Geiduschek and Kassavetis, 2001; Huang and Maraia, 2001; Schramm and Hernandez, 2002). RPC11 which shows homology to both RPB9 and transcription factor TFIIS, is involved in the intrinsic RNA cleavage activity of Pol III (Chedin et al., 1998). This is interesting because in the crystal structure of Pol II, both Rpb9 and TFIIS are positioned close to each other (Gnatt et al., 2001). Such a high homology between subunits of different polymerases underlines conservation of their function and structure. The Pol III specific subunits may be responsible for the specific properties of Pol III. Various biochemical studies done on both human and budding yeast Pol III

Module	RNAPIII[a]	RNAPII	Associated Structural Elements
Core subunits	C160	Rpb1	Clamp, jaw (below the lobe), foot
	C128	Rpb2	Lobe, protrusion, wall, clamp
	AC40	Rpb3	Back
	AC19	Rpb11	Back
	ABC27 (Rpb5)	ABC27 (Rpb5)	Jaw (below the clamp)
	ABC23 (Rpb6)	ABC23 (Rpb6)	
	ABC14.5 (Rpb8)	ABC14.5 (Rpb8)	
	ABC10α (Rpb12)	ABC10α (Rpb12)	
	ABC10β (Rpb10)	ABC10β (Rpb10)	
Stalk subunits	C25	Rpb7	Stalk
	C17	Rpb4	Stalk
Termination subcomplex	C53	–	
	C37	–	
	C11[b]	Rpb9	Jaw (below the lobe)
Initiation subcomplex	C82	–	
	C34	–	
	C31	–	

Table 1.1: Comparison of pol II and pol III subunits

Subunits ABC27, ABC 23, ABC14.5, ABC10α and ABC10β are common for all the three polymerases while the subunits C53, C37, C82, C34 and C31 are pol III specific . pol III specific subunits form a termination subcomplex and an initiation subcomplex.

(Table from Fernandez-Tornero et al., 2007)

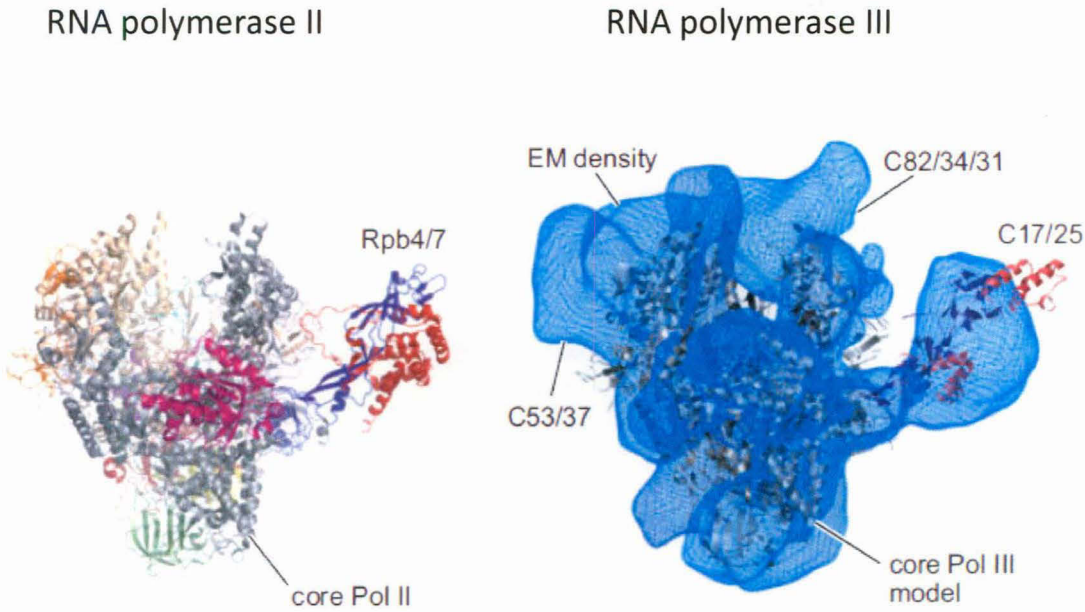


Figure 1.1: Comparison of structures of RNA polymerases II and III.

Pol II structure is the high resolution crystal structure and pol III model is electron microscopic structure superimposed on the homology model (Cramer et al., 2008) .

indicate that they assemble as two distinct sub-complexes on the surface of Pol III. C82/C34/C31, heterotrimer make a sub-complex and a mutation that causes dissociation of this sub-complex, renders Pol III thermo sensitive (Werner et al., 1992). Further studies have shown that this sub-complex is involved in transcription initiation and is required for the recruitment of Pol III to target promoters (Wang and Roeder, 1997; Kenneth et al., 2008). Another such sub-complex is constituted by C37 and C53 along with C11 (Hu et al., 2002). This sub-complex is involved in proper termination of transcription and recycling of Pol III (Landrieux et al., 2006). Facilitated recycling is a mechanism used by Pol III to achieve high levels of transcription, whereby Pol III does not fall off the gene after termination but transferred back to the initiation site by a yet to be understood mechanism. This process keeps Pol III template committed and results in increased transcription efficiency (Dieci and Sentenac, 2003). Thus, all the Pol III specific subunits carry out Pol III specific duties. A recent proteomic study proposed that the core includes C11 because C37/C53 is more easily dissociable from the core; C82/C34/C31 dissociated on treatment with DMSO but C11 was still found associated with the core (Lorenzen et al., 2007). This may indicate that C11 is part of the Core complex and acts as a bridge or docking factor for C53/C37. These observations as well as the protein-protein interaction studies led to a model with the C37/C53/C11 sub complex on the downstream face of the Polymerase and C82/C34/C3 on the upstream face of Pol III, interacting with the recruiting factors (Lorenzen et al., 2007). These positions are compatible with their function in transcription termination and initiation respectively. Finally, it has emerged that Pol III has three Pol III specific sub complexes attached on the periphery of a ten subunit core that is similar to Pol II core (Figure 1.1).

Conservation of core subunits of Pol II and Pol III led Jasiak et al (2006) to predict a homology model for Pol III structure. Their prediction had a RPC17/25 crystal structure attached to the nine subunit core enzyme. RPC11 was not included in the prediction because its similarity to Rpb9 is not good enough to allow the homology modeling. The structure as well as the nucleic acid binding capacity of C17/25 is markedly different from Rpb4/7 (Jasiak et al., 2006). A recent attempt revealed Pol III structure at 17 Å resolution by cryo-electron microscopy (Fernandez-Tornero et al., 2007). Though the overall structure is conserved between Pol II and Pol III, there are

some marked differences. Pol III has a bulkier stalk which is evident from the altered structure of the C17/25 subunit structure (Jasiak et al., 2006). Immunolocalization revealed that the C82/34/31 sub-complex is situated next to the C17/C25 stalk. It agrees with the known fact that C34 and C17 interact with Brf1 (Brun et al., 1997; Ferri et al., 2000). Position of C82/34/31 complex on Pol III is a structural evidence for the role of this sub-complex in transcription initiation. Another sub-complex C53/37 was located to the outer end of the DNA binding cleft. This position enables the sub-complex to sense the incoming DNA and thus explain its role in termination and reinitiation (Fernandez-Tornero et al., 2007).

1.2.2 TFIIIB - the initiation factor proper

TFIIIB is the transcription initiation factor that recruits RNA polymerase III to its target promoters. TFIIIB is a heterotrimeric protein complex composed of TATA binding protein (TBP), TFIIB related factor 1 (BRF1) and BDP1. *S. cerevisiae* TBP and Brf1 make a stable complex termed B' while Bdp1 dissociates from TFIIIB easily, making the B" fraction (hence the name *B double prime* or BDP) (Kassavetis et al., 1991).

TBP is a universal transcription factor required by all three RNA Polymerases. *S. cerevisiae* Pol III promoters generally lack a TATA box except a few genes like SNR6, SCR1 and some tRNA genes (Dieci et al., 2007). In many other organisms including *S. pombe*, TATA box is an essential/ubiquitous Pol III promoter element (Huang and Maraia, 2001). In *S. cerevisiae*, TFIIIB is positioned upstream of the initiation site not through the sequence specific interaction between TATA box and TBP, but by the Box A bound TFIIIC. TBP is known to bend the DNA almost 90 degrees. As part of TFIIIB complex on Pol III transcribed genes, TBP bends the DNA upstream of start site. The bend is enhanced by Bdp1 and is a hall mark of TFIIIB-DNA complex (Geiduschek and Kassavetis, 2001).

Bdp1 is a Pol III specific transcription factor of 594 amino acids with no paralogues in other polymerases (Kassavetis and Geiduschek, 2006). It is loosely bound to the TFIIIB complex indicating that it is recruited after the formation of the stable DNA-TBP-Brf1 complex. Bdp1 determines the physical properties of TFIIIB-DNA complex. Its binding to DNA is not sequence specific and it makes an additional bend between the TBP binding site and transcription initiation site. Bdp1 extends the TFIIIB footprint

upstream of TBP binding site and imparts the extreme stability to TFIIIB-DNA complex. The Brf1 interacting region of Bdp1 has a SANT domain (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). Bdp1 is also known to interact with chromatin remodeler Isw2 in yeast (Bachman et al., 2005). CK2 mediated phosphorylation of Bdp1 is required for cell cycle specific repression of RNA Polymerase III (Hu et al., 2004).

Brf1 is a 596 amino acid protein which has a homology to the Pol II transcription factor TFIIB at its N terminus (hence the name *B related factor* or BRF). The C terminal half is specific to Pol III and provides most of its affinity for TBP and BDP1 binding. N terminal half is essential for its transcription activity. An evolutionarily conserved 70 amino acid homology region II of Brf1 plays a critical role in holding the TFIIIB complex together by acting as a two sided adhesive that binds to both TBP and Bdp1 although other regions are also involved in binding to these factors (Kassavetis and Geiduschek, 2006). TFIIIB is recruited to Pol III genes by TFIIIC mainly via the interaction between Brf1 and the tetratricopeptide (TPR) repeat of Tfc4 subunit of TFIIIC. This interaction involves three separate TPR elements and three sites in Brf1 (two in the N terminal segment and one in the homology segment II in C terminal half) (Geiduschek and kassavetis, 2001; Schramm and Hernandez, 2002). Many of the protein binding sites in Brf1 are overlapping indicating the assembly of TFIIIB is sequential and is associated with structural reorganization of Brf1. The proposed sequence of assembly is: i) TFIIIC binding to A-B Boxes; ii) interaction between Tfc4 subunit of TFIIIC and Brf1; iii) anchoring the Brf1-TFIIIC complex to DNA by the Brf1 bound TBP; iv) reconfiguration of interaction between TFIIIC and Brf1 to accommodate Bdp1 (Kassavetis and Geiduschek, 2006). Brf1 interacts also with C34 and C17 subunits of Pol III (part of Pol III specific initiation sub-complex and stalk respectively). It is believed that through this interaction, TFIIIB recruits Pol III to its promoters (Werner et al., 1992; Khoo et al., 1994; Wang and Roeder, 1997; Ferri et al., 2000).

Brf1 is the target of most of the cellular mechanisms that regulate Pol III transcription. The central regulator of Pol III called Maf1 acts directly on Brf1 as well as Pol III (Upadhya et al., 2002; Desai et al., 2005). Brf1 is the target of Casein Kinase 1 enzyme and this phosphorylation is required for its regulation. Most of the tumor suppressor proteins that regulate Pol III transcription also target Brf1 for their action

(White et al., 1996; Felton-Edkins et al., 2003a; 2003b; Gomez-Roman et al., 2006). Thus Brf1 is an interesting protein that interacts with TBP and Bdp1 to recruit TFIIIB complex, is responsible for the integrity of TFIIIB complex, recruits Pol III by its interaction with C34 and C17 and acts as the main target of Pol III regulatory mechanisms.

1.2.3 TFIIIC – The assembly factor

For in vitro transcription of a gene which can direct TFIIIB binding through its TATA box, no other factors are required for transcription initiation. But for TATA less genes (which constitutes most of the Pol III transcribed genes in budding yeast) and for transcription of chromatin templates of certain TATA containing genes like SNR6, TFIIIC is required to recruit and position TFIIIB. TFIIIC achieves this through the interaction between its TFC4 subunit and Brf1 (Geiduschek and Kassavetis, 2001).

S. cerevisiae TFIIIC is a 6 subunit complex, whose subunits are coded by the genes Tfc1, Tfc3, Tfc4, Tfc6, Tfc7 and Tfc8 making an aggregate molecular weight of 520 kDa. These subunits are arranged in two different domains τA and τB, connected by a flexible and proteolysis sensitive linker (Marzouki et al., 1986). τA is composed of Tfc1, Tfc4 and Tfc7 and binds to A box. Tfc4 subunit plays the key role in recruitment of TFIIIB by interacting with Brf1 and Bdp1. τB is composed of Tfc3, Tfc6 and Tfc8. Tfc8 also acts as the linker between τA and τB. Tfc3 binds to the B box while Tfc6 occupies the most downstream position of TFIIIC complex and binds to the terminator region. Yeast TFIIIC does not show a HAT activity. However, atleast three subunits of Human TFIIIC, TFIIIC220, TFIIIC110 and TFIIIC90 are reported to have histone acetyl transferase (HAT) activity (Kundu et al., 1999). Recently *S. pombe* TFIIIC was found to act as a chromatin organizer independent of Pol III transcription function (Noma et al., 2006). Similar role for TFIIIC in *S. cerevisiae* has also been reported (Simms et al., 2008).

1.2.4 TFIIIA – Assembly factor for 5S

Unlike tRNA genes and other Pol III transcribed genes, TFIIIC does not bind to the 5S rRNA gene in a sequence-directed manner. It has to be recruited by the 5S specific transcription factor TFIIIA. *X. laevis* TFIIIA was the first eukayrotic transcription factor to be purified (Engelke et al., 1980) and the first for which cDNA was isolated (Ginsberg et

al., 1984). Also, it was the founding member of the C_2H_2 zinc finger family of DNA binding proteins (Miller et al,. 1985). TFIIIA binds to the internal control region (ICR) of 5S gene using its 9 zinc fingers and recruits TFIIIC. The mechanism by which it recruits TFIIIC is not well understood.

1.2.5 SNAPc – Vertebrate factor for U6 snRNA

Human and other vertebrate U6 snRNA genes utilize completely upstream, Pol II like promoters, constituting two types of sequence elements. Factor requirement for such promoters are also different from other types of Pol III promoters. The major factor is SNAPc (*sn*RNA *a*ctivator *p*rotein *c*omplex) which binds to proximal sequence element (PSE) (Schramm and Hernandez, 2002). Binding of Oct1 factor to the DSE (distal sequence element) and binding of SNAPc are cooperative events. DSE-Oct1 complex is stabilized by binding of SNAPc to PSE and Oct1 binding is required for SNAPc to bind to PSE. SNAPc and Oct1 are common for Pol II transcribed as well as Pol III transcribed U snRNA genes. The Pol III specificity is achieved by TATA box, which is present only in U6 snRNA gene. SNAPc is a 5 subunit complex with a central 190 k Da subunit that anchors other smaller subunits which later bind to TBP and then recruit Pol III (Hernandez, 2001).

1.3 RNA polymerase III promoters

RNA polymerase III transcribed genes generally have internal promoters, which can be divided in to three types: Type1, 2 and 3 (Figure 1.2). 5S rRNA gene is the only Type 1 promoter known while tRNAs and some other RNAs like VA1 RNA genes have Type 2 promoter structure. Vertebrate U6 promoter and some other non coding RNA genes constitute the Type 3 promoters. Both Type 1 and Type 2 are intragenic while Type 3 is upstream of the transcription initiation site.

1.3.1 Type 1 Promoter elements:

5 S rRNA gene is the only member of this class. This is an intragenic promoter element with an A box, an intermediate element (IE) and a C box in *X. laevis*. These three elements constitute the internal control region (ICR). In *S. cerevisiae*, only the C box is

required for 5S transcription (Bogenhagen, 1985; Pieler et al., 1985; Challice and Segall, 1989).

1.3.2 Type 2 promoter elements:

All tRNA genes have a promoter structure of this type. This type of promoters are composed of an A box, typically present at approx. 20 bp downstream (+20 position) of transcription start site (TSS) and a B box present 50-60 bp downstream of the A box. These two elements are well conserved between species probably because they form the D and T loops of mature tRNA. The distance between A and B box is variable among different tRNA genes depending on presence of introns (Sharp et al., 1983).

Both A and B boxes are bound by TFIIIC. TFC4 subunit of τA domain binds A box and TFC3 subunit of τB domain binds B box. TFIIIC can bind to both these elements simultaneously and covers the whole transcribed region of the gene. However, read through by Pol III only partially dissociates TFIIIC from the gene (Gieduschek and Kassavetis, 2001).

Some of the tRNA genes in yeast as well as most of the tRNA genes in *S. pombe* have a TATA box upstream of the gene (Dieci et al., 2006). Though most of tRNA genes in budding yeast do not have a TATA box or any other recognizable sequence elements upstream, there are evidences of the sequences just upstream of the gene having an effect on their transcription (Braglia et al., 2007).

1.3.3 Type 3 promoter elements

Vertebrate U6 RNA promoter comes under this category. This is an entirely different class of promoter which resembles Pol II promoter rather than a Pol III promoter. In vertebrates, all U spliceosomal snRNAs (U6 as well as other Pol II transcribed U snRNAs) have similar promoter structure with DSE and PSE and utilize special transcription factors while the TATA box gives the polymerase specificity (Schramm and Hernandez, 2002).

Distal Sequence Element (DSE) can be considered as an enhancer element for type 3 promoter. It is composed of at least two different protein binding sites. First one

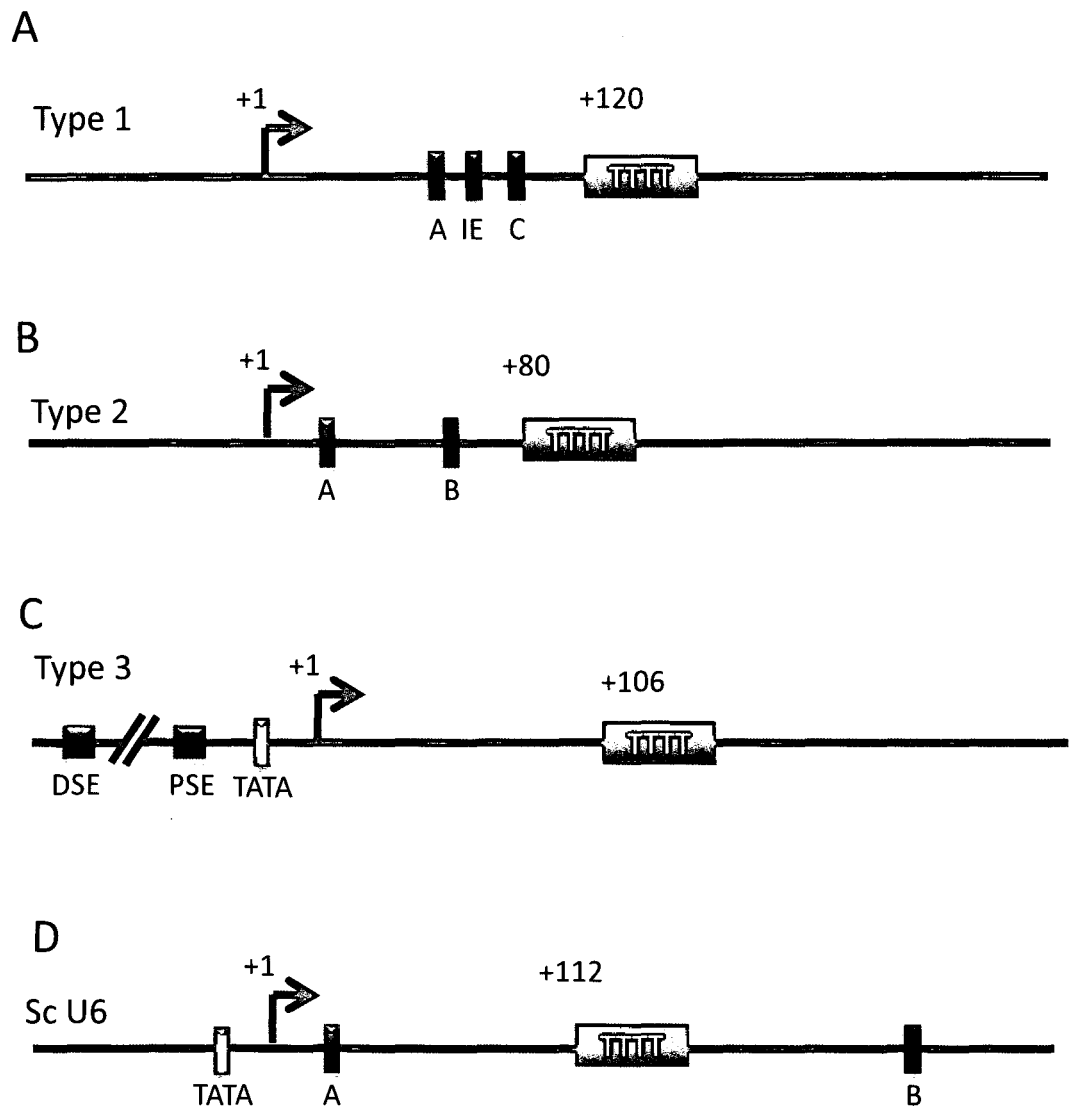


Figure 1.2: Different types of RNA polymerase III promoters

Numbers denote transcription start site at +1 and terminator at given location

A. Type 1 promoter of the *X. laevis* 5S RNA gene consists of an internal control region (ICR), which can be subdivided into A box, intermediate element (IE)and C box
B. Type 2 promoter of the *X. laevis* $tRNA^{leu}$ gene consists of an A box and B box, separated by a distance of 43 base pairs.
C. Type 3 promoter of the human U6 snRNA gene consists of a distal sequence element that enhances transcription and a core promoter composed of a TATA box at -30 position and a proximal sequence element (PSE) upstream of it
D. The *S. cerevisiae* U6 promoter is a hybrid promoter consisting of a TATA box (-30 to -23), and A box (+21 to +31) and a B box located downstream of U6 coding region (+234 to +244).

(Adapted from Schramm and Hernandez, 2002)

is the Oct1 binding site which is an octamer DNA sequence and the second one is a Staf1 binding site.

Proximal sequence element (PSE) is the binding site for a 5 subunit factor called SNAPc. Binding of SNAPc to PSE and binding of Oct1 to DSE are cooperative events. On its own, Oct1 makes a weak complex with target DNA but with a PSE bound SNAPc in the vicinity, this complex becomes stronger. Similarly SNAPc alone cannot bind to PSE efficiently because the PSE binding N terminal domain of the SNAPc-190 subunit is masked by its own C terminal region. Interaction with DNA bound Oct1 is required to remove this mask (Gieduschek and Kassavetis, 2001; Schramm and Hernandez, 2002).

Presence of a TATA box makes the promoter committed to Pol III. Interaction of SNAPc with TBP leads to the assembly of a slightly different TFIIIB complex. TFIIIB on type III promoter utilizes a variant of Brf1 called Brf2 (Kassavetis and Geiduschek, 2006).

1.3.4 Mixed type of promoters:

Though Pol III promoters are classified broadly as 3 types, there are promoter structures seen in various species that do not fall in to any of these groups or their known variables. Some examples of such promoters include Trypanosomal snRNA promoters, which utilize the A and B box elements of an upstream tRNA gene (Nakaar et al., 1994). Budding yeast RNase P RNA and SNR52 snoRNA genes utilize A and B boxes in the transcribed leader region which will be later cleaved to make the mature RNase P RNA (Lee et al., 1991). This strategy is used in many other noncoding RNA genes transcribed Pol III in a variety of organisms (Dieci et al., 2007).

1.4 RNA polymerase III transcriptome

It is a common notion found in most of the articles dealing with transcription that Pol III transcribes housekeeping non-coding RNAs like tRNA genes, 5S rRNA gene and U6 snRNA gene. This in fact gives an impression that Pol III transcription is monotonous. Various attempts to study the genome wide localization of Pol III machinery as well as computational studies revealed that Pol III transcriptome has much more variety than

RNA	Function	Species	Promoter
tRNA		All	A and B boxes (internal)
5S rRNA	Part of large subunit of ribosome	All	A and C Boxes(internal)
U6 snRNA	Splicing	All	Internal or Upstream
RNase P RNA	Component of RNase P RNA (tRNA maturation)	All	Internal or Upstream
RNase MRP RNA	rRNA maturation	All except yeast	Upstream
7 SL RNA	Signal recognition particle	All	A-B boxes or Upstream
7 SK RNA	Binds to P-TEFb and repress Pol II transcription elongation	Vertebrates	Upstream
Vault RNA	Macromolecular assembly and / or transport		Internal and external
Y RNA	RNA quality control and DNA replication		Upstream
BC1 RNA	Neural specific. Involved in dendritic translation	Rodents	Internal and Upstream
BC200 RNA	Neural specific. Involved in dendritic translation	Primates	Internal and Upstream
Virus encoded RNAs	Viral replication		Internal and Upstream
SINE Encoded RNAs			Internal
snR52 (snoRNA)		S. cerevisiae	A and B boxes (upstream tRNA like leader)
ZOD1 RNA		S. cerevisiae	A and B Boxes with intervening transcriptional terminator
tRNA-like SRP RNA		Trypanosomatid	A and B Boxes
snoRNAs	rRNA processing		A-B boxes
Micro RNAs	Gene regulation		A-B Boxes
Unclassified non-coding RNAs	Gene Regulation		Upstream
Stem Bulge (sb) RNAs		C. elegans	

Table 1.2: RNA Polymerase III transcriptome

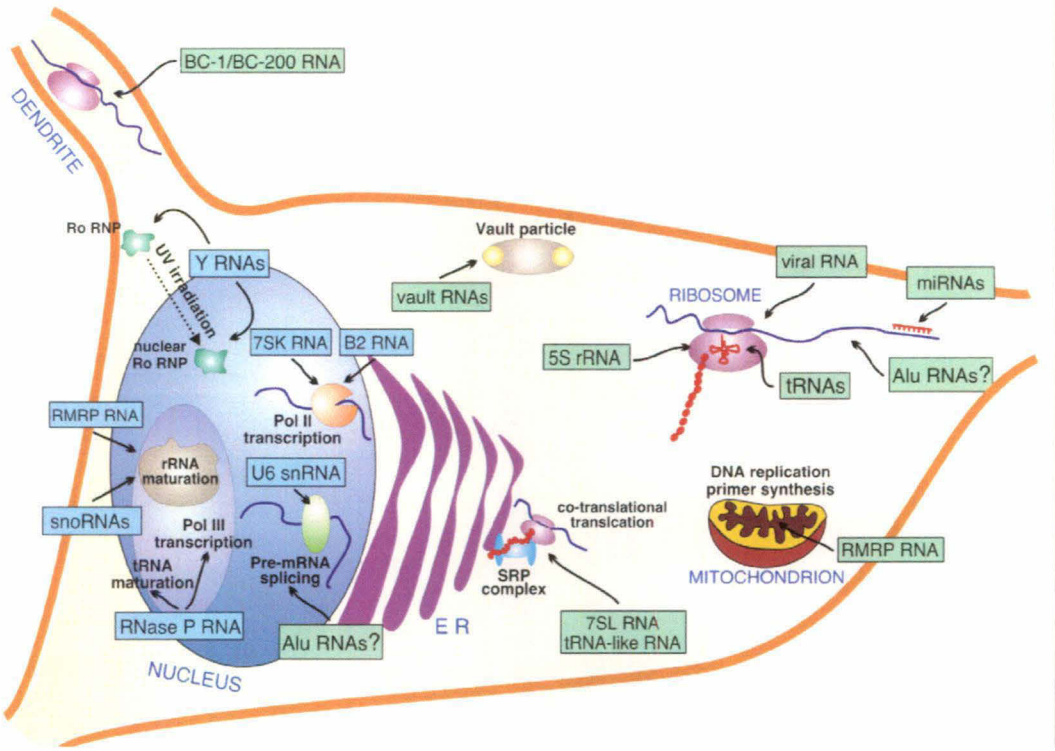


Figure 1.3: Cellular functions of pol III transcribed genes

Pol III transcripts acting in the nucleus and in the cytoplasm are shown in blue and green, respectively. An arrow indicates the involvement of a Pol III–synthesized RNA in a cellular process or its physical association to a ribonucleoprotein particle. The question mark in the case of Alu RNAs is to point out that their involvement in the indicated processes remains poorly defined. (Dieci et al., 2007)

previously thought (Harismendy et al., 2003; Roberts et al., 2003; Moqtaderi and Struhl, 2004; Pagano et al., 2007). Dieci et al (2007) reviewed these recent additions to Pol III transcriptome which is summarized in Table 1.2. This expanding RNA Polymerase III transcriptome reveals a set of RNAs involved in a variety of cellular processes, ranging from transcription regulation (7SK RNA), splicing and RNA processing (U6, RNAse P, RNAse MRP), translation (tRNAs, 5S rRNA, microRNAs), RNA quality control (Y RNAs), co-translational translocation (7SL RNA), replication (Y RNA),just to name a few (Dieci et al., 2007). (A schematic of various cellular roles for Pol III transcribed genes is given in Figure 1.3.)

1.5 Regulation of Pol III transcription

1.5.1 Cell cycle and growth rate dependent regulation

The rate of protein synthesis is regulated to meet the growth rate but cells do not allow it to exceed the requirement. This is achieved by reducing the pol I and pol III transcription. It is known that RNA polymerase III transcription is coupled to growth rate. In budding yeast, pol III transcription as well as pol I transcription increase with increased growth rate and decrease with decreased growth rate. Starvation of various nutrients and many other stress conditions also lead to decreased Pol III transcription. This is of particular interest because in their natural habitats, yeast cells may have to often face lack of nutrients and to survive in such situations, they need to have a reduced growth rate (Clarke et al., 1996). There are also evidences that pol I and pol III transcriptions are controlled separately with an auto regulatory control of tRNA synthesis (Oliver and McLaughlin, 1977).

Pol III transcription is regulated in a cell cycle dependent manner also. Studies on *Xenopus* and HeLa cells show that Pol III transcription is repressed during mitosis due to phosphorylation of TFIIIB by a Cdc2-cyclin B kinase (Gottesfeld et al., 1994; Leresche et al., 1996). After the exit from mitosis, Pol III transcription increases slowly during G1 and reaches the maximum in S and G2 phases (White et al., 1995).

1.5.2 Maf1-the central regulator of Pol III

Different signaling pathways that respond to various stresses like TOR pathway or RAS/cAMP/PKA pathway (both can detect the nutrient availability), secretory signaling pathway and DNA damage pathway are known to regulate Pol III transcription (Willis et al., 2004). All these pathways converge to a single protein Maf1 which down regulates Pol III transcription (Upadhya et al., 2002). Maf1 is required for pol II repression under endoplasmic reticulum stress or oxidative stress (Desai et al., 2005) and is known to couple carbon metabolism to Pol III transcription (Ciesla et al., 2007). Deletion of Maf1 protein, which is well conserved from yeast to humans leads to increased transcription of tRNA genes (Pluta et al., 2001). Recent studies have revealed that human Maf1 protein also functions as a negative regulator of Pol III transcription (Goodfellow et al., 2008; Reina et al., 2006). It can repress the transcription from type I, type II and type III promoters both in vitro and in vivo. It is a phosphoprotein that gets dephosphorylated and localizes to nucleus under stress conditions. hMaf1 represses Pol II transcription in both transformed as well as non transformed cells (Reina et al., 2006).

A synthetic genetic array screening to identify genetic interactors of Maf1 revealed that Maf1 mediated repression of Pol III genes and Med20 mediated repression of ribosomal protein genes act in parallel indicating that Pol III transcription and ribosomal protein transcription are coordinately regulated to tackle the adverse growth conditions (Willis et al., 2008).

In actively growing cells, Maf1 is phosphorylated by Sch9 -a kinase in the TOR pathway or Protein kinase A (PKA) -a kinase in the RAS/cAMP pathway and remains in the cytoplasm as a phosphoprotein (Lee et al., 2009). A stress condition that can activate or alter any of these pathways leads to dephosphorylation of Maf1 by a phosphatase PP2A and subsequent nuclear import of Maf1. When the cells are again in an actively growing condition, phosphorylation of nuclear Maf1 leads to its export to cytoplasm (Moir et al., 2006; Oficjalska-Pham et al., 2006; Roberts et al., 2006). This nuclear export requires a carrier molecule called Msn5 as deletion of Msn5 led to a constitutive nuclear localization of Maf1. But the phosphorylation-dephosphorylation cycle and the proper regulation of Pol III transcription was unaffected indicating that

phosphorylation status of Maf1 is the key determinant in pol III repression; not the cellular localization (Towpik et al., 2008). Protein kinase C1, a central player in the cell integrity pathway is also shown to be required for Maf1 activation but the target of PKC1 that activates Maf1 is not known (Roberts et al., 2006).

Inside the nucleus, Maf1 is known to interact with Pol III and TFIIIB subunit Brf1. No interaction is seen between TFIIIC and Maf1. Co-immunoprecipitation studies have shown that Maf1 can interact with the N terminus of RPC160, the largest subunit of Pol III (Oficjalska-Pham et al., 2006). Evidences show that target of action of Maf1 is Brf, part of TFIIIB (Desai et al., 2005; Upadhya et al., 2002). Like its yeast counterpart, human Maf1 also targets Brf1 and Brf2, which are part of TFIIIB complex (Rollins et al., 2007).

According to a model based on the observations made in budding yeast, Maf1 binds to Pol III and Brf1 and prevents their loading on to the genes. Other evidences like increased occupancy of Maf1 on Pol III transcribed genes under repression (Oficjalska-Pham et al., 2006; Roberts et al., 2006) and the fact that all polymerase III subunits do not show a decrease in occupancy even under repressed conditions (Roberts et al., 2006) led to an alternative working model for Maf1 mediated repression. In this model, Pol III is not dissociated from the gene under repression. It is bound by the activated Maf1 making it transcriptionally incompetent (Geiduschek and Kassavetis, 2006).

A study on human Maf1 revealed another aspect of Maf1 mediated repression. Recombinant human Maf1 could inhibit the de novo assembly of TFIIIB-DNA complex as well as Pol III recruitment in vitro, but failed to inhibit the facilitated recycling of the pol III already present on the gene. Facilitated recycling of Pol III is an important regulatory mechanism which helps to maintain a higher level of Pol III transcript. This observation suggests requirement of additional steps for the down regulation of facilitated recycling under adverse conditions (Cabart et al., 2008). Deletion of Maf1 can affect not only the transcription of RNA polymerase III but also the tRNA mediated gene silencing (TGM) (Moir et al., 2006), wherein a transcriptionally active tRNA gene inhibits the transcription of a nearby Pol II gene.

Though the picture looks simple with a single master repressor for Pol III on which all the signaling pathways converge in yeast, the reality is much more complicated. All pol III transcribed genes are not repressed equally by Maf1. There are differences in the extent of repression for individual tRNA classes by Maf1 while some genes are unaffected by it (Ciesla et al., 2007). It has yet to be determined whether individual tRNAs within a particular class are also regulated differently. This suggests involvement of additional regulators for Pol III transcription in yeast, probably the chromatin and its modifiers.

1.5.3 Regulation of Pol III by other activities

In mammals, many other regulators of pol III are known. They include tumor suppressors and oncogenes like p53, Retinoblastoma protein (Rb) and cMyc, indicating role of Pol III transcription in transformation. In many cancers, Pol III transcription is known to be deregulated (White, 2004, 2005). Some evidences show a role for pol II in pol III transcription. Extragenic accumulation of pol II activates transcription by Pol III on neighboring genes (Listerman et al., 2007) and the genome wide observation detected the Pol II elongation factor TFIIS targeting to Pol III genes as well (Ghavi-Helm et al., 2008). The mechanism by which these machineries regulate Pol III transcription is not yet known. Recently ribosomal proteins are also shown to activate Pol III transcription indicating a networking of different machineries to maintain co-regulation of Pol III transcription with ribosomal biogenesis and functionality (Dieci et al., 2009).

Pol III transcription is known to be regulated in a tissue specific manner in higher organisms. Xenopus 5S rRNA gene (Stutz et al., 1989) and *Bombyx mori* $tRNA^{ala\ SG}$ gene (Taneja et al., 1992) are two well known examples. A recent analysis of tissue specific expression of tRNA genes in humans has shown that expression of individual tRNAs varies in a tissue specific manner and suggests an additional level of translational control in a tRNA abundance-codon usage manner (Dittmar et al., 2006).

1.6 U6 snRNA gene

1.6.1 The U snRNAs

Spliceosome is a ribonucleoprotein complex containing five Uridine rich small nuclear RNAs (U1, U2, U4, U5 and U6 snRNAs). During the process of splicing, U1 and U2 snRNAs specify the splicing site and branch point. U1 snRNA defines the intron sequence by direct interaction between single stranded 5'end of U1 and the 5'splice site while the 5'end of U2 base pairs with the branch site directly. Once the splice site is committed by binding of U1 and U2 snRNPs, the tri-snRNP complex containing U6, U4 and U5 snRNA join to form the complete spliceosome and the splicing reaction takes place. U6 snRNA plays the main catalytic role in the splicing reaction (Guthrie and Patterson, 1988; Brow, 2002).

U6 snRNA varies from other spliceosomal RNAs in several ways. It is synthesized by RNA Polymerase III rather than RNA Polymerase II; it contains a γ-methyltriphosphate 5'end instead of a trimethylated guanosine 5'cap structure and it lacks a Sm-binding site (Guthrie and Patterson, 1988). U6 snRNA is also unique in that uridylate (UMP) residues are both added to and removed from its 3'end post transcriptionally and mature forms of this snRNA contain a 2', 3'-cyclic phosphate terminal group. The maturation process leads to a change in length of the mammalian U6 RNA (Lund and Dahlberg, 1992). Furthermore, generation of U1 to U5 snRNPs requires transit through cytoplasm whereas maturation and functioning of U6 snRNA appears to be accomplished solely in the nucleus at least in some organisms like *Xenopus,* while it travels to cytoplasm in mouse fibroblasts (Zieve et al., 1988; Vankan et al., 1990). Other than these, some other modifications are also present on U6. They include methylation of adenosine residue at position 43 and 2'-O-ribose methylations of human U6 (Epstein et al., 1980; Shimba et al., 1995).

In case of *Arabidopsis thaliana*, both Pol II and Pol III transcribed snRNA gene promoters contain an upstream sequence element (USE) and the TATA box. Both these elements are interchangeable between Pol III and Pol II transcribed genes without affecting the polymerase specificity. The polymerase selecting factor is the spacing

between USE and TATA box. It is 33-34 base pairs for Pol II transcribed genes and 23-24 for Pol III transcribed snRNA (Waibel and Filipowicz, 1990).

1.6.2 The U6 snRNA gene

These features evoked a lot of interest in snRNA genes from transcription point of view. Despite having a similar promoter structure with minor changes, U6 snRNA gene is transcribed by RNA polymerase III while all others are transcribed by Pol II (Reddy et al., 1987). Among all spliceosomal RNAs, U6 snRNA shows highest conservation from yeast to human (60% identical) making it one of the most conserved RNAs (Brow and Guthrie, 1988). Though U6 RNA sequence is highly conserved, U6 gene number varies widely between species, from single copy in yeast (Brow and Guthrie, 1988) to 10 copies in *C. elegans* (Thomas et al., 1990). Similarly, the promoter structure of U6 gene is also highly divergent between species and in most of the species other than fungi, it does not resemble the promoters of tRNA genes or other Pol III transcribed genes (Hernandez, 2001). However, even with such lack of similarity in the promoters, the U6 gene is still transcribed by RNA Polymerase III. It is interesting that evolution has changed the promoter architecture significantly while the Polymerase specificity is untouched

Human and other vertebrate U6 snRNA core promoters including mouse, *Xenopus*, zebra fish, Fugu (Takifugurubripes) etc consist of two elements, the PSE and a TATA Box located at a fixed position downstream of PSE. There is also a Distal Sequence Element (DSE) which acts as an enhancer for both Pol II and Pol III transcribed snRNA genes. PSE and DSE are interchangeable between the Pol II and Pol III transcribed snRNA genes without affecting the specificity of the transcribing Polymerase which is determined by the TATA box (Schramm and Hernandez, 2002; Halbig et al., 2008; Zenke and Kim, 2008).

U6 promoter in *Drosophila melanogaster* has a PSEA and TATA box spaced by 12 bps. Here, PSEA is conserved between Pol II and Pol III transcribed snRNA genes. The Polymerase specificity is determined by 19th and 20th bases of the 21bp PSEA element; g/aG commits the promoter for Pol II and TC commits for Pol III (Jensen et al., 1998).

Trypanosoma brucei U6 gene has an interesting promoter structure. It utilizes the A and B boxes of a divergently transcribed tRNA gene upstream of the start site. An intragenic sequence element close to the 5'end is required to position the RNA polymerase III on the gene (Nakaar et al., 1997).

Sea urchin U6 promoter has a proximal sequence element at -55, a TATA box like element at -25 and a novel, essential E Box at -80 bp positions. A sea urchin specific factor called USF binds to the E Box (Li et al., 1994). Though a number of sea urchin snRNA promoters have been characterized in various sea urchins, the determinant of the RNA polymerase specificity is not yet known. Although all of them have PSE and some have TATA box, the TATA Box is not responsible for polymerase specificity. The PSEs of different snRNA promoters do not show any sequence similarity but they are interchangeable without affecting the polymerase specificity (Li et al., 1996).

1.6.3 U6 snRNA gene in *S. cerevisiae* (SNR6)

S. cerevisiae U6 gene known as SNR6 is an essential, single copy gene (Brow and Guthrie, 1988). SNR6 gene has mixed type promoter with upstream, intragenic and downstream promoter elements in an unusual arrangement. Unlike its mammalian counterparts, SNR6 has a tRNA like A and B Box promoter structure (Figure 1.2D). A suboptimal box A is in its canonical position of +21 (+1 as the transcription initiation site) while B box is 120 bp downstream of the terminator at +112 bp position in the gene. Similar to the vertebrate U6 gene, SNR6 also has a TATA box at -30 bp position. Box B is an essential element for SNR6 transcription in vivo as well as chromatin templates in vitro (Brow and Guthrie, 1990; Shivaswamy et al., 2004). Even a 2 bp deletion in the SNR6 B box, which is known to abolish the binding of TFIIIC, is lethal. Box A binds to the tau A domain of TFIIIC and plays a role in determining the transcription initiation site. Changing the box A sequence to consensus could activate a gene lacking box B, both in vivo and in vitro. TATA box plays a role in determining the transcription initiation site (Burnol et al., 1993b; Eschenlauer et al., 1993).

On naked DNA templates, TATA box can direct the assembly of TFIIIB by sequence specific contacts between TATA box and TBP. This TFIIIB-SNR6 complex shares many important features like the extend of footprint, stability etc with tDNA-TFIIIB

complex and 5S rDNA-TFIIIB complex indicating that TATA box containing U6 promoter also uses the same TFIIIB components (Joazeiro et al., 1994). This is remarkable because in vertebrates, U6 transcription needs a Brf variant Brf2 while tRNA genes use Brf1 (Schramm and Hernandez, 2002; Kassavetis and Geiduschek, 2006). Nevertheless, in vivo, TFIIIB is placed on SNR6 by TFIIIC and not by the direct interaction between TATA box and TBP (Gerlach et al., 1995).

HMG box proteins Nhp6A and Nhp6B are also implicated in SNR6 transcription. These are very similar non-histone chromatin structural proteins which act redundantly with each other. Deletion of both the proteins makes yeast cells temperature sensitive and defective in SNR6 transcription. The temperature sensitive phenotype of nhp6ΔΔ can be overcome by over expression of SNR6 or Brf1 (Kruppa et al., 2001; Lopez et al., 2001). Recent studies on tRNA genes speculate that requirement of Nhp6 for Pol III transcription depends on the bendability of the upstream sequences and Nhp6 function may be to stabilize the TFIIIB-DNA complex (Braglia et al., 2007). The exact mechanism by which Nhp6 facilitates SNR6 transcription is not yet known.

Another DNA element required for transcription of SNR6 was recognized from studies on mutants with a reduced distance between A and B box. Though the SNR6 gene is resistant to mutations in TATA box, a deletion of 42 bp between the terminator and B box made the gene sensitive to TATA box mutations (Gerlach et al., 1995). Further studies revealed that transcription of these Δ42 mutants are more dependent on a $(dT\text{-}dA)_7$ tract immediately downstream of TATA box. A single base pair inversion in the middle of the T7 stretch can nearly abolish the U6 transcription in Δ42 background while the inversion of the whole T7 stretch has no effect (Martin et al., 2001). Interestingly, mutations in T7 stretch are synthetically lethal with nhp6ΔΔ condition even though the A-B box distance is wild type, indicating they act in a coordinate manner to activate SNR6 transcription (Martin et al., 2001).

The importance of distance between A and B boxes of SNR6 became evident from the experiment where the downstream B box of *S. cerevisiae* was deleted and *S. pombe* B box present in an intronic sequence was inserted at a canonical distance from A box, as found in tRNAs. Since the splice sites of *S. cerevesiae* and *S. pombe* are similar,

the *S. cerevisiae* splicing machinery could splice out the intronic B box efficiently and gave the mature U6 RNA. However,SNR6 gene thus created with an intronic B box, that is closer to A box failed to achieve the full level of transcription as compared to the wild type gene (Kaiser et al., 2004).

The upstream region of SNR6 is characterized by a solo δ element spanning from -425 to -91 with its own TATA box (-252 to -259) and UAS (-185 to -222) oriented away from SNR6 (Brow and Guthrie., 1990). δ elements are long terminal repeats flanking Ty1 or Ty2 retrotransposons and are responsible for transcription initiation (5′ LTR) as well as polyadenylation (3′ LTR) of Ty1 and Ty3 RNAs. But due to the absence of internal ε region, solo δ elements will be transcribed only at a basal level (Boeke and Sandmeyer., 1991). The downstream region of the SNR6 has an uncharacterized gene that is transcribed in the opposite direction of SNR6.

1.7 Chromatin and Transcription

Chromatin is known to be involved in regulation of gene expression for quite a long time. Extensive research had been carried out on the β-globin and ovalbumin loci in metazoans and on inducible genes like Pho5, His3 and Gal genes in budding yeast (Straka and Horz, 1991; Lohr and Lopez, 1995; Martinez-Campa et al., 2004) to elucidate the basic mechanisms of chromatin mediated transcription regulation. These studies as well as others revealed many of the basic concepts in chromatin biology and mechanisms that the cells utilize to alter the chromatin structure to regulate the gene expression as well as other nuclear processes. These include ATP dependent chromatin remodelers, covalent modification of histones, histone variants and histone chaperone mediated histone dynamics.

1.7.1 Covalent modifications of histones

Evidence for involvement of covalent modification of histones and their involvement in transcription is at least four decades old (Allfrey et al., 1964; Allfrey, 1966; Pogo et al., 1966). Further studies have cemented the relation between active transcription and histone acetylation. Till date, 8 different histone modifications have been found and all

of them are known to affect transcription in one way or the other (Kouzarides, 2007). The known histone modifications are listed in the Table 1.3. The most important and well characterized modifications are: Lysine acetylation, Lysine and arginine methylation and serine phosphorylation.

Identification of wide variety of histone modifications and their involvement in various chromosomal processes led to the proposal of a histone code hypothesis. According to this hypothesis, covalent modifications on histones, alone or in combination, function to direct specific and distinct DNA template programs.

1.7.1.1 Acetylation and deacetylation

Histone lysine acetylations, one of the first discovered post translational modifications of histones are known to be associated with active transcription (Pogo et al., 1966; Hebbes et al., 1988; Turner, 1991). Acetylation is involved in almost all chromosomal processes including the assembly of newly synthesized histones in to the chromatin; transcription, replication, repair and recombination (Shahbazian and Grunstein, 2007). Several residues in all four histones can be acetylated and a class of enzymes called Histone lysine acetyl transferases (HATs)/Lysine acetyl transferases (KATs) does this acetylation. Many of the HATs/KATs are associated with transcriptional co-activator complexes and can be recruited to the genes by specific activators through protein-protein interactions (Kouzarides, 2007).

First purification of a HAT was from the larvae of Brine shrimp in 1979 by Cano and Pestana (Cano and Pestana, 1979). It took several years to isolate and clone gene for a HAT. HAT1a and Gcn5 were cloned in 1995 and 1996 respectively (Kleff et al., 1995; Brownell, 1996) while the first HAT complex (SAGA) was purified in 1997 (Grant, 1997). Various biochemical studies were made on HATs and the substrate specificity of many of these were identified both in vitro and in vivo (Kimura et al., 2005).

Depending on the Histone acetylase subunit, HATs can be classified in to different groups. GNAT (Gcn5-N acetyl transferases) family of HATs includes Gcn5, PCAF, Elp3, Hat1a, Hpa2 and Nut1a while the MYST (*M*orf, *Y*bf2 (sas3), *S*as2 and *T*ip60) family of HATs is composed of Morf, Ybf2 (sas3), Sas2 and Tip60, Esa1, HBO1 and MOF.

There are other HATs that do not fall in any of these categories. They are called orphan class and include P300/CBP, RTT109, TAF1, human TFIIIC etc (Lee and Workman, 2007). A single subunit with histone acetyl transferase activity can be part of many complexes. For example, yeast Gcn5 makes different complexes like SAGA, ADA, SLIK, and HAT-A2 (Lee and Workman, 2007) revealing complexity of the regulation by acetylation.

Histone deacetylases (HDACs) are the enzymes that can remove acetyl group from the modified lysines. HDACs are classified in to four classes based on their catalytic domain, Class I, II, III and IV (Yang and Seto, 2008). Among these, the zinc utilizing classes I, II and IV are considered as classical family or Rpd3/Hda1 family (de Ruijter et al., 2003). Class III includes an entirely different NAD+ dependent class with proteins similar to yeast Sir2/Sirtuins (Haigis and Guarente, 2006). Class I enzymes are the ones similar to yeast Rpd3 protein. They are in the range of 400-500 amino acids in length and localize to nucleus. The mammalian members of this family include HDAC1, -2, -3 and -8 (Thiagalingam et al., 2003). They have an N terminal deacetylase domain and a C terminal tail, which has sites for many modifications like phosphorylation and sumoylation (Sengupta and Seto, 2004). Class II enzymes are similar to yeast Hda1 and the human members of this family are HDAC4, -5, -6, -7 -9 and -10. They act as signal transducers and often shuttle between cytoplasm and nucleus (Yang and Seto, 2008). Most of the class II HDACs are large enzymes compared to Class I and share homology only in the catalytic domain. Hda1 and its *S.pombe* homologue have an N terminal deacetylase domain while the mammalian ones have a C terminal catalytic domain with an N terminal extension that acts as protein binding site and regulates the HDAC activity (Yang and Gregoire, 2005). Class III HDACs are entirely different from the first two classes. They require NAD+ for their activity. Being similar to Sir2 in yeast, they are also known as sirtuins (SIRT1-7 in mammals). They are mainly implicated in heterochromatin formation, gene silencing, ageing etc (Liu et al., 2009). The lone member of class IV is the HDAC11, which is highly conserved from *C. elegans* to humans. Though this shows similarity to Class I, the Phylogenetic analysis argues to consider this as a separate class (Gregoretti et al., 2004). The functions of this class of deacetylases are not clear.

Histone deacetylation is generally considered as associated with gene repression and HDACs are recruited to genes either by various repressor proteins or as part of co-

repressor complexes. An exception to this rule is the activation of PU.1 gene, a regulator of hematopoiesis. PU.1 gene is repressed by HDAC inhibitors and an H4 HDAC activity is required for its proper activation (Laribee and Klemsz, 2001; Laribee and Klemsz, 2005). Rpd3 is recruited to osmostress and heatshock genes leading to deacetylation of H4 when activated. Activation of these genes is impaired in rpd3Δ cells (de Nadal et al., 2004).

HATs and HDACs can be recruited to specific genes by activators/repressors. They can also function in a global manner. That means, they can acetylate/deacetylate a broad region in a continuous manner without being recruited by a specific factor. All throughout the genome (Shahbazian and Grunstein, 2007), a dynamic interplay of acetylases and deacetylases maintains the chromatin acetylation status at a time. The equilibrium can change in to one or the other direction depending on the type of factor binding to the region.

1.7.1.2 Methylation and demethylation

Methylation of histones on their free NH2 -end is known for some time (Clarke, 1993). Apart from the amino terminus, Lysines and arginines on histone can be methylated. Lysines can be methylated with one (mono), two (di) or three (tri) methyl groups while arginines can be methylated with either one or two methyl groups. The dimethylation on arginine can be either symmetric or asymmetric. One of the best studied histone modifications is lysine methylation. A set of enzymes called histone methyl transferases (HMT) or lysine methyl transferase (KMT) methylate histones (Shilatifard, 2006).

Out of the 4 histones, H3 and H4 are generally methylated. Major methylation sites in these histones are: H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20. Only H3K4, K36 and K79 methylations are discovered in budding yeast. All these are known to be associated with active transcription. H3K9, K27 and H4K20 methylations are observed in other organisms and are associated with transcription repression (Cheung and Lau, 2005).

There are two different classes of KMTs; SET (Su (VAR) 3-9, Enhancer of Zeste and Trithorax) domain containing and non-SET domain containing. SET domain containing

KMTs methylate H3K4, K9, K27, K36 and H4K20 while non-SET domain containing KMT (Dot1 in yeast and its homologs) methylate H3K79 (Shilatifard, 2006).

In budding yeast, -mono, -di and –tri methylation of H3K4 is done by Set1, which shows similarity with mammalian MLL proteins. Set1 is part of the complex called COMPASS (Complex of proteins associated with Set1). COMPASS associates with initiating Pol II phosphorylated at serine 5 with the help of Paf1 complex and is required for the methylation of H3K4 on active genes. Ubiquitination of H2B is required for the methylation of H3K4 (Krogan et al., 2003a; Ng et al., 2003; Wood et al., 2003a; Wood et al., 2003b Morillon et al., 2005; Schneider et al., 2005).

H3K36 is methylated by Set2 in budding yeast (Strahl et al., 2002). Set2 associates with Pol II transcription elongation through the phosphorylated CTD (serine 2) of Pol II with the help of *S*et2 *R*pb1 *i*nteracting (SRI) domain (Li et al., 2002; Krogan et al., 2003c; Li et al., 2003; Schaft et al., 2003; Xiao et al., 2003; Kizer et al., 2005; Morris et al., 2005).

Dot1 is a well conserved, non-SET domain containing KMT. Identified as a chromatin silencing protein and a meiotic recombination checkpoint regulator; Dot1 methylates H3K79 (Dlakic, 2001; Feng et al., 2002; Lacoste et al., 2002; Ng et al., 2002b; van Leeuwen et al., 2002). Dot1 also associates with Pol II with the help of Paf1, and requires H2B ubiquitination for methylation of H3K79 (Ng et al., 2002b; Krogan et al., 2003a; Wood et al., 2003b). Recent Mass spec studies identified mono methylation (no di or tri) of H3K9 and di methylation of H4K20 in yeast but their significance or methylating enzymes are not known (Garcia et al., 2007). It is interesting that other than Set1 and Set2, yeast has 4 more SET domain containing proteins: Set3, 4, 5 and 6. Among these Set3 and 4 also have a PHD domain that can bind to a methyl lysine. Set3 is also part of a complex that has Hos2-an Rpd3 like HDAC and Hst1-a Sir2 like HDAC (Pijnappel et al., 2001). Some of these Set proteins may have a methyl transferase activity that may be specific for other residues, but not yet established.

There are two different classes of lysine demethylases. First class includes LSD1 (*l*ysine specific *d*emethylase *1*), which is a nuclear, amine oxidase homolog. This is an

FAD dependent demethylase that is specific for mono and di methyl H3K4 and part of a corepressor complex with deacetylase activity (Lee et al., 2005).

The second type of demethylases is JHDM (*J*umonji *h*istone *dem*ethylase) class. First examples for this class are JHDM1 and JHDM2A (Tsukada et al., 2006; Yamane et al., 2006). They require Fe(ii) and alpha-ketoglutarate for activity and release succinate and formaldehyde as byproducts. Unlike LSD1, JHDM class of demethylases can demethylate tri methyl lysines also. There are several members of this class with different substrate specificities known by now (Anand and Marmorstein, 2007)

1.7.2 Function of chromatin modifications

There are two implications proposed for the action of chromatin modifications: i) modifications can alter DNA-histone contacts and thus open up the chromatin structure in favor of transcription. Histone tail acetylation, with the negative charge of acetyl group is thought to have this effect; ii) Modifications can act as binding sites for other proteins and these proteins may be responsible for the downstream effects. Discovery of various modified lysine binding domains was the reason behind this model. It is possible that both the effects are seen at different places (Kouzarides, 2007).

There are many specialized domains that can recognize the histone modification marks. They are considered as the readers of the histone code and the mediators of the functions of chromatin modification. These domains generally show high specificity to their substrates. Bromodomains are the acetyl lysine binding domains, found in many chromatin remodelers and transcription activators. In many cases, acetyl lysine binding by a bromodomain can alter the protein function allosterically while in some cases it can help recruit another factor to the chromatin (Ferreira et al., 2007). Chromodomains, PHD (*P*lant *h*omeo *domain*), Tudor domain and WD40 repeats are known to bind methyl lysine while 14-3-3 proteins bind to phospho serines (Seet et al., 2006). Chromodomains and PHD domains are also shown to be site specific and more importantly, specific to the extent of (mono, di or tri) methylation (Lee and Workman, 2007).

An important aspect of these domains is that different chromatin associated complexes have different combinations of domains that recognize different

modifications and their combinatorial mode of action may serve as a regulatory mechanism. This can lead to cross talk between histone modification marks. The classic example of cross talk between modifications is the one between H2B ubiquitylation and H3 methylation. Set1 and Dot1 mediated methylation of H3K4 and K79 on active genes is dependent on ubiquitylation of H2B by Rad6 (Ng et al., 2002b). Interestingly, ubiquitylation is required only for di and tri methylation, not for mono methylation (Shahbazian et al., 2005). Cross talks between modifications may take place at different levels. If a residue can be modified in two different ways, they are mutually exclusive. For example, acetylation of H3K9 will prevent methylation of the same residue. Sometimes, presence of a modification on the adjacent residue can affect the affinity of a protein to its binding site. For example, H3S10 phosphorylation affects binding of HP1 to methyl H3K9 (Fischle et al., 2003; Fischle et al., 2005). Presence of a modification on one residue can also prevent modification of a nearby residue. An example for this kind of cross talk is the prevention of Set2 mediated H3K36 methylation by H3P38 isomerization (Nelson et al., 2006). Yet another way of cross talk is that one modification can increase the affinity of another modification enzyme to its substrate nucleosome. Examples for such a cross talk include the increased affinity of Gcn5 for H3 phosphorylated at S10 (Clements, 2003) and the tethering of NuA3 HAT complex to a H3K4me3 via the PHD domain of its subunit Yng1 is required for the H3K14 acetylation (Taverna et al., 2006).

1.7.3 Chromatin Remodeling Complexes

ATP dependent chromatin remodelers use the energy derived from ATP hydrolysis to restructure the chromatin structure by sliding, evicting or restructuring the nucleosomes. Chromosomal processes like chromatin assembly, transcription, DNA replication, repair and recombination are benefitted from chromatin remodelers. In general, all remodelers have an affinity for nucleosome (Clapier and cairns, 2009) and possess a subunit having similar kind of DNA-dependent ATPase domain (Figure 1.4). This domain that is split in to two parts: DExx and HELICc, acts as a DNA motor which breaks the DNA-histone contacts. These domains are separated by a short insertion in all chromatin remodelers except the INO80 family. In INO80 family, it is a long insertion that separates the DExx and HELICc domains. Apart from the ATPase domain, chromatin

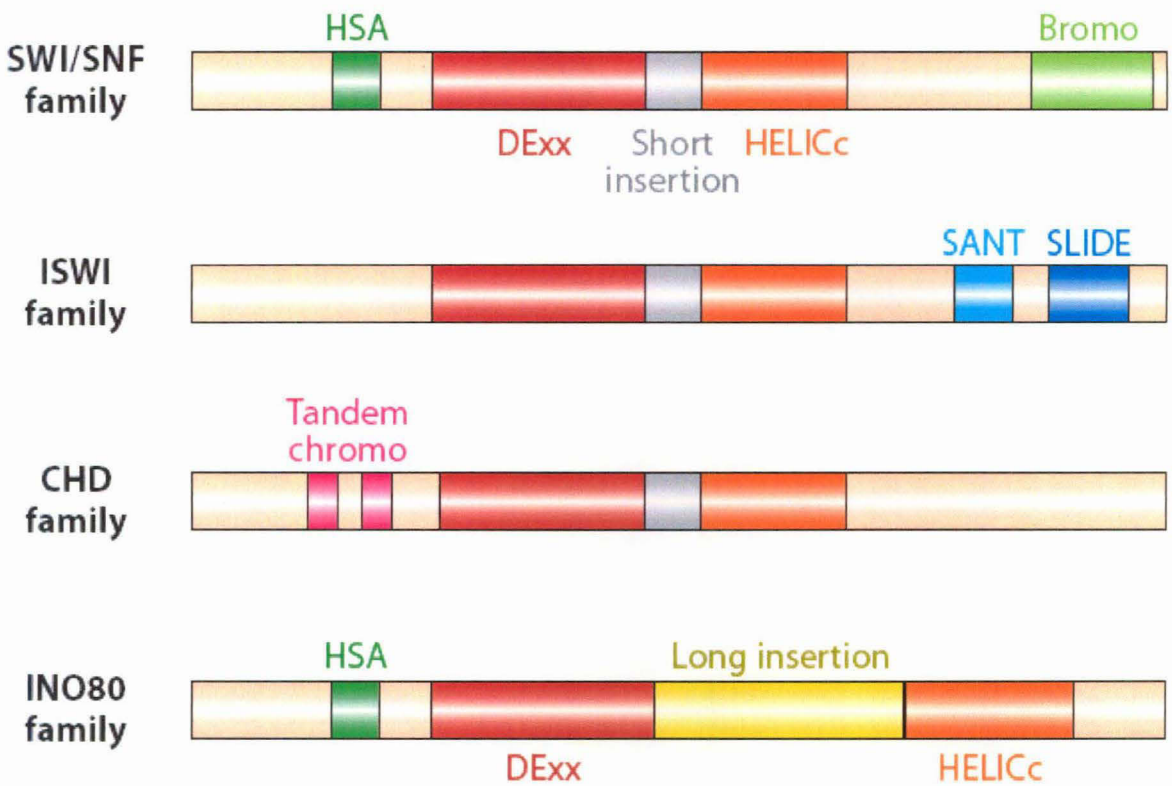


Figure 1.4: Chromatin Remodeler families based on their ATPase domain

Short insertion within the ATPase domain of SWI/SNF, ISWI and CHD family is represented as grey color while the long insertion in case of INO80 family is shown in yellow. (Figure from Clapier and Cairns, 2009)

remodelers possess domains that can identify histone modification marks; have domains and/or subunits that regulate the ATPase domain and domains required for protein-protein interaction.

Chromatin remodelers can be broadly classified in to four classes depending on the additional domains present in the ATPase subunit (Figure 1.4).

1.7.3.1 SWI/SNF Family:

Founding member of Switch family of remodelers is yeast SWI/SNF complex. Various remodeler complexes of this class vary in having 8 to 14 subunits. The ATPase subunit of this family contains an HSA (helicase-SANT) domain and a C terminal bromodomain (Mohrmann and Verrijzer, 2005). Yeast has two complexes in this family: SWI/SNF and RSC. ATPase domain of SWI/SNF is the Swi2/Snf2 protein while that of RSC is Sth1. RSC is an abundant, 14 subunit complex (Cairns et al., 1996) and has 7 out of the total 15 bromodomains present in yeast. This indicates that acetyl lysine binding by RSC is important for its function.

SWI/SNF family of remodelers can slide or eject nucleosomes at many loci and are involved in most of the chromosomal functions except chromatin assembly.

1.7.3.2 ISWI family:

Imitation switch (ISWI) family of remodelers contains 2 to 4 subunits. The catalytic ATPase subunit contains a SANT (Swi3, ADA2, NCoR and TFIIIB) domain and a SLIDE (SANT like ISWI domain) at the C terminus. The SANT and SLIDE domains together bind to unmodified histone tails and DNA (Dang and Bartholomew, 2007; Pinskaya et al., 2009).

ISWI family complexes are involved in many functions like chromatin assembly (nucleosome spacing by dACF, yIsw1 and CHRAC), repression and activation of transcription etc. Yeast has two different ISWI family ATPases: Isw1 and Isw2. Isw1 forms two different complexes: isw1a and Isw1b while Isw2 forms only one complex (Vary et al., 2003).

1.7.3.3 CHD Family:

Members of CHD (Chromodomain, Helicase and DNA binding) family of remodelers can be either monomers or multi subunit complexes. The domain architecture of the ATPase subunit includes two tandem chromodomains at the N terminus (Marfella and Imbalzano, 2007). It is generally found as monomer in lower eukaryotes. Some of the CHD family remodelers are involved in nucleosome sliding and ejection to promote transcription while some others are required for transcription repression.

Yeast Chd1 is part of histone acetylase complexes SAGA and SLIK and plays a role in transcription elongation while Chd1 containing Mi2/NURD complex of vertebrates, involved in transcription repression constitutes histone deacetylase (HDAC1/2)(Denslow and Wade, 2007).

1.7.3.4 INO80 Family

INO80 (Inositol requiring 80) family of remodelers generally have more than 10 subunits. The catalytic subunit is characterized by an ATPase domain with a longer insertion. This insertion serves as binding site for other proteins like Rvb1/2 (a helicase related AAA-ATPase). Yeast has two complexes of this class: INO80 complex and the SWR1 complex. INO80 complex is involved in transcription activation, DNA repair etc while SWR1 complex is required for deposition of histone variant H2A.Z to nucleosomes (Bao and Shen, 2007).

The possible mechanism for a remodeler action is that it acts as DNA translocator anchored on to a nucleosome. The remodeler anchors on the octamer and pulls the DNA from the linker region with the help of ATP hydrolysis, pumping it towards the dyad with the formation of a DNA loop. The similarity in the structure of the ATPase domain and the diversity of the remodeling outcome indicates that other domains/subunits other than ATPase regulate the remodeling action depending on the cues from chromatin.

Chromatin remodelers act as repressors or activators of transcription. In general, the remodelers that organize nucleosomes in to arrays act as repressors while those that disorganize the nucleosomes are activators. The dynamic antagonism of these two kinds

of remodelers may be responsible for setting up equilibrium of nucleosome assembly/disassembly and the presence of activators or repressors probably shifts the equilibrium in one or the other direction. Remodelers are recruited to promoters by repressors or activators depending on the property of the remodeler. For example, Ume6 repressor or Ssn6-Tup1 recruits Isw2 to promoters (Goldmark et al., 2000). The array forming property of Isw2 is also utilized to prevent the antisense transcription (Whitehouse et al., 2007). Many remodeling complexes cooperate with repressive HDACs or contain HDACs as a subunit (Clapier and Cairns, 2009).

1.7.4 Histone Variants

Histone variants are the non allelic variants of histones that are incorporated in a replication independent manner in to nucleosomes. Unlike their canonical counterparts, they are synthesized outside the S phase of cell cycle. There are many variant forms identified for core histones with the variation ranging from a few amino acids to large additions. These variations generally alter the structure in such a way that the property of the nucleosome changes (Pusarla and Bhargava, 2005).

H1 has a large number of variants that are expressed in different cell types, developmental stages etc. Most of the sequence variations are present in the nonglobular N- and C-terminal tail domains (Cole, 1987; Brown, 2001).

Major variant forms of histone H3 are the centromeric H3 (cenH3) and H3.3. Centromeric H3 is conserved from yeast to humans and required for kinetochore assembly. H3.3 varies from the canonical histones by four amino acids. In budding yeast, apart from centromeric H3, only one form of H3 is there and that is equivalent to the mammalian H3.3. H3.3 is associated with active transcription. Vertebrates also have a testes specific H3.4 variant.

H2A has maximum variants among core histones. They include H2AX that plays a role in DNA repair, macroH2A-present on inactive X chromosomes and silent regions, H2A.Bbd-associated with active transcription and H2A.Z (Pusarla and Bhargava, 2005). Compared to canonical H2A, H2AX has a four amino acid extension on the C terminus

and the serine of that extension can be phosphorylated. Budding yeast bulk histone H2A is equivalent to H2AX.

H2A.Z is one of the most thoroughly studied histone variant. Budding yeast version of H2A.Z is a non essential gene (Dhillon and Kamakaka, 2000; Jackson and Gorovsky, 2000) while in mammals, H2A.Z is essential (Faast et al., 2001). The crystal structure of H2A.Z containing nucleosome, highlights its structural differences with the nucleosome containing the canonical H2A (Suto et al., 2000). Differences in the docking region of H2A.Z alter the H3-DNA contacts and can destabilize the nucleosomes. Differences in the structure also indicate that hybrid nucleosomes with H2A.Z-H2B dimer and H2A-H2B dimer are not preferred (Suto et al., 2000). But recent evidences show that H2A.Z can form hybrid nucleosomes in vitro though their presence or significance in vivo is uncertain (Chakravarthy et al., 2004). Studies on biophysical properties of H2A.Z containing nucleosomes also have given conflicting reports on the stability of nucleosomes containing H2A.Z. One set of evidence indicates that H2A.Z destabilizes nucleosomes (Abbott et al., 2001) while the other set argues for a stabilizing role for H2A.Z (Fan et al., 2002; Park et al., 2004).

The INO80 class remodeler SWR1 deposits H2A.Z on nucleosomes in an ATP dependent manner (Krogan et al., 2003b; Kobor et al., 2004; Mizuguchi et al., 2004). H2A.Z is involved in many cellular functions. It is known to be present in upstream regions of active genes-flanking the nucleosome free region (Raisner et al., 2005; Albert et al., 2007) and it prevents the spreading of silent heterochromatin (Meneghini et al., 2003). H2A.Z is involved in marking repressed gene for rapid activation as well as for the localization of genes to nuclear periphery (Adam et al., 2001; Brickner et al., 2007). H2A.Z is also known to be associated with Pol III transcribed genes (Albert et al., 2007).

1.8 Chromatin context of RNA Polymerases:

Each RNA polymerase transcribes a unique set of genes and recent evidences indicate that they transcribe in different chromatin environment and make their own transcription factories in the nucleus.

1.8.1 RNA polymerase I and chromatin

Ribosomal RNA genes transcribed by Pol I are highly repetitive in nature and localize to the nucleolus. Pol I transcription is required for the integrity of nucleolus. Humans have ~400 copies of the rDNA while yeast has 150-200 copies. But only half of these are transcribed at a given time and others are silenced with a heterochromatin like structure forming on the DNA (Conconi et al., 1989). These active and inactive genes are interspersed in yeast. Chromatin remodelers Chd1, Isw1 and Isw2 are also associated with active rDNA (Jones et al., 2007). Active and silent rDNA promoters in humans can be distinguished by the differential nucleosome positioning (Langst et al., 1998; Li et al., 2006). Though regular nucleosome array is absent on the active genes, unphased and dynamic nucleosomes are present on them (Thiriet and Hayes, 2005; Jones et al., 2007; Merz et al., 2008). An HMG box protein Hmo1 binds throughout the active rDNA indicating the chromatin structure of active rDNA is entirely different with very less nucleosomes (Merz et al., 2008). In short, Pol I transcribes rDNA that has less number of nucleosomes (not absent) compared to the silent rDNA. Pol I takes help from histone chaperones nucleolin, nucleophosmin and FACT to transcribe chromatin template (Rickards et al., 2007; Murano et al., 2008). Interestingly, the transcription termination factor TTF-I recruits NoRC1 remodeler that silences the rDNA, thus coupling transcription and silencing (Nemeth et al., 2004).

Chromatin modifications also reveal the functional status of rDNA chromatin. Active rDNA chromatin is hypo methylated and hyper acetylated while the opposite is true for silenced rDNA. Silent chromatin is hyper methylated at H3K9 and is bound by HP1 (Nemeth et al., 2008; Santoro et al., 2002)

1.8.2 RNA polymerase II and chromatin

Most of the concepts of chromatin transcription were revealed by the research on RNA polymerase II transcribed genes. Though many of the basic mechanisms of chromatin mediated regulation of gene expression were revealed by studying individual pol II transcribed genes, these studies were not sufficient to make a general conclusion about the chromatin structure of genes. The advent of techniques like Chromatin immunoprecipitation, microarray and high throughput sequencing enabled researchers

to look at the positions of nucleosomes on a genome wide scale rather than at a single gene level (Rando and Chang, 2009).

Studies on global nucleosome positioning were most fruitful in yeast owing to the simplicity of the organism as well as the fact that most of yeast genome is transcribed euchromatin. ChIP on chip (Microarray hybridization after chromatin immunoprecipitation) studies with low resolution were done initially to reveal that promoter regions are generally depleted of nucleosomes (Bernstein et al., 2004; Lee et al., 2004). This was followed by high resolution tiling oligonucleotide micro arrays and high throughput parallel sequencing (Yuan et al., 2005; Albert et al., 2007; Lee et al., 2007; Whitehouse et al., 2007; Mavrich et al., 2008; Shivaswamy et al., 2008). Enormous amount of this data helped to make a high resolution atlas of the nucleosome positions in the whole budding yeast genome. One striking observation from these studies was the presence of a large volume of strictly positioned nucleosomes throughout the genome. These nucleosome positions showed a great correlation with the computationally predicted nucleosome positioning sequences (NPS) in yeast (Ioshikhes et al., 2006; Segal et al., 2006; Peckham et al., 2007; Yuan and Liu, 2008; Kaplan et al., 2009). NPSs are stretches of DNA that can encode positioned nucleosomes. These DNA have an internal curvature and therefore thermodynamically favor the formation of nucleosomes (Gottesfeld and Bloomer, 1980; Young and Carroll, 1983). These studies as well as the detailed study of yeast U6 snRNA gene (Vinayachandran et al., 2009) put forward a theory that yeast genome has an intrinsic property to encode the nucleosome positions and thereby form a blueprint of the chromatin structure on which various transcription factors and other chromatin regulatory machineries work to generate the particular chromatin structure of a locus in particular growth state. It may be noted however, that most of these genome wide studies were done in actively growing wild type cultures with a few exceptions (Whitehouse et al., 2007; Shivaswamy et al., 2008).

These studies also revealed certain common patterns that are widespread throughout the yeast genome. These patterns or motifs provide insight in to the general rules of establishment of chromatin architecture and help define the chromatin architecture of a typical yeast promoter/gene (Figure 1.5). However, deviations from

this general model are also observed and the atypical genes shed light on different gene regulatory mechanisms at play (Rando and chang, 2009). For a typical yeast ORF, the upstream region is characterized by a nucleosome free region (NFR) encompassing the transcription start site (TSS) flanked by two well positioned nucleosomes (the nucleosome near the TSS is designated as +1 and the one at the opposite end is designated as -1; Figure 1.5) containing histone H2A variant H2A.Z (Albert et al., 2007). NFR is the region where majority of the transcription factor binding sites are present though some are found in the -1 nucleosome as well. Most of the -1 nucleosomes are rotationally phased in such a way that the major groove of protein binding sites face away from the histone octamer. This enables the factors to access the target sites in nucleosomal DNA (Albert et al., 2007). In many cases, the NFR are coded either by a nucleosome excluding sequence like poly dA/dT (Yuan and Liu, 2008) or by binding of transcription factors like Abf1 and Reb1 (Kaplan et al., 2009). Nucleosome at the +1 position is also encoded by a strong NPS while the strength of NPS reduces going further downstream (Ioshikhes et al., 2006). Micro array and sequencing studies also report that though positioned nucleosomes are seen downstream of the +1 nucleosome (+2, +3 etc), they become fuzzier or delocalized gradually, arguing for a barrier model of nucleosome positioning (Mavrich et al., 2008). This model is based on the argument of Kornberg and Stryer that many times nucleosomes are positioned due to the constraints of packing many nucleosomes in to a small region (Kornberg and Stryer, 1988). Here, the +1 nucleosome acts as a barrier to the downstream array of nucleosomes restricting the positions these nucleosomes can take. The restriction is the highest for the +2 nucleosome and gradually reduces as it goes further downstream. Such a barrier dependent positioning of nucleosomes is experimentally proved. Binding of lac operator binding protein R3 to its binding site can position an array of nucleosomes on either side when a chromatin is assembled using a Drosophila crude extract and the positioning is lost when the barrier R3 is removed (Pazin et al., 1997). Similar study with chromatin assembled by salt dilution method also has shown array of positioned nucleosomes in an R3 dependent manner and interestingly, removal of R3 after assembly did not affect the positioning because of the absence of chromatin remodeling machinery in salt diluted chromatin (Pusarla et al., 2007). Like the 5' NFR, a good proportion of 3' gene ends are also characterized by NFR (Mavrich et al., 2008; Shivaswamy et al., 2008). It may be of

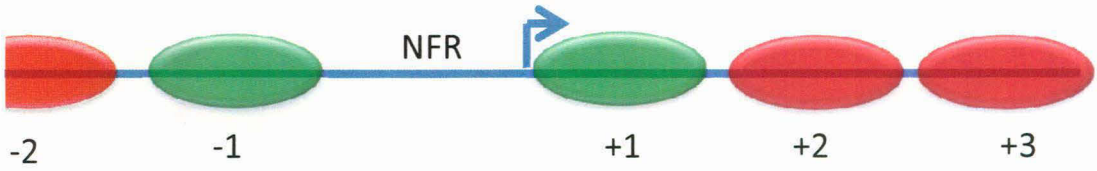


Figure 1.5: Chromatin structure of a typical pol II transcribed gene

Green ovals are H2A.Z containing nucleosomes while red ovals are nucleosomes with canonical H2A. NFR is nucleosome free region where most of the transcription factor binding sites are present. Bent arrow denotes the TSS.

physiological significance because many of the antisense transcripts initiate in this region (Mavrich et al., 2008).

Results of many genome wide studies on chromatin modifications can be overlaid on this picture. Histone acetylations are the highest on the 5' end of the ORF where both H2A.Z and trimethyl H3K4 show highest occupancy. H3K79 methylation, H3K4 dimethylation and H3K36 dimethylation are enriched on the middle region of an average ORF while H3K4 me1 and H3K36 me3 peak over the 3' end of the ORF (Li et al., 2007). The role of H3K36 methylation in transcription is well studied. H3K36 methylation by Set2 brings the deacetylase Rpd3s complex via the interaction between Eaf3 and di methyl H3K36. This deacetylation mediated by Rpd3s is a pathway to prevent spurious transcription initiation from within the ORF by keeping the chromatin structure intact (Carrozza, 2005; Joshi and Struhl, 2005).

The gene structure of budding yeast described above is only an averaged one and there are notable deviations from this structure. In yeast ~80% of the genes are TATA less and are TFIID dependent for transcription. These genes generally show the characteristics of a typical gene while the minority of TATA box containing genes shows a wide variety of chromatin structures. These TATA containing genes are generally stress responsive, their transcription is characterized as noisy or bursty and the nucleosome positions on these genes are more fuzzy (Ioshikhes et al., 2006; Alberts et al., 2007). Another genome wide study on the nucleosome dynamics has shown that nucleosomes on the promoters are rapidly replaced compared to the ORF nucleosomes. The conclusion was made by looking at the replacement of normal H3 by tagged histone H3 (Dion et al., 2007; Jamai et al., 2007; Rufiange et al., 2007).

The principles established with yeast studies have relevance in the mammalian system also. Though the complexity of mammals and the very large size of the genome make mammalian chromatin structure more complex, the basic principles hold true for mammals. Ubiquitously expressed genes in human genome are enriched for nucleosome excluding sequence upstream of the TSS (Radwan et al., 2008). DNaseI hypersensitivity studies as well as chromatin immunoprecipitation studies revealed ~200 bp NFR centered around the position -85 flanked by nucleosomes on each side. The +1

nucleosome position differs depending on whether the gene is actively transcribed or Pol II is in a paused state. There is no NFR seen on nontranscribed genes (Heintzman et al., 2007; Ozsolak et al., 2007; Schones et al., 2008).

1.8.3 RNA Polymerase III and chromatin

One of the initial attempts to correlate the chromatin structure and transcription status of Pol III transcribed genes was made by Coveney and Woodland in 1982. They checked the DNaseI hypersensitivity of a 5S rRNA gene in its repressed state and a tRNA gene in both active and repressed states. They could find that, unlike the Pol II transcribed genes like Globin gene, these genes were DNaseI hypersensitive in active as well as repressed conditions (Coveney and Woodland, 1982) indicating that tRNA genes are devoid of nucleosomes (Morse et al., 1992).

Most of the initial studies on the chromatin mediated regulation of Pol III transcription was on 5S rDNA transcription. Studies on *Xenopus* 5S rRNA genes revealed that the oocyte specific gene is repressed by a chromatin structure that involves linker histone H1 (Schlissel and Brown, 1984). A detailed in vitro DNaseI footprinting study of a ternary complex of 5S rRNA gene, TFIIIA and a histone octamer revealed that a nucleosome gets positioned on the gene and this position overlaps with TFIIIA binding site. But TFIIIA can still bind its site in the nucleosome by disrupting the histone-DNA bonds at the overlapping region. This provided a possible explanation of H1 mediated repression of 5S gene (Rhodes, 1985). Wolffe and Brown hypothesized that it is the stability of the transcription factors that plays the major role in developmental regulation of 5S transcription. In somatic cells, the oocyte specific 5S gene loses its transcription factors and allows chromatin to repress the transcription (Wolffe and Brown, 1988). In vitro transcription studies of 5S gene on reconstituted chromatin showed the need of a topoisomerase activity for proper binding of TFIIIA and TFIIIC and chromatin transcription by Pol III (Glikin and Blangy, 1986; Kmiec et al., 1986). While depletion of histone H4 led to changes in the transcription by Pol II, it did not make much difference in global transcription level of Pol I and Pol III (Kim et al., 1988). A recent study with the same yeast strain which looked at the Pol III transcription at individual genes revealed that many unusual Pol III genes that are repressed under

normal conditions are expressed when nucleosomes are depleted (Guffanti et al., 2006). It has been shown long back that nucleosomes as well as chromatin condensation can block Pol III transcription if the pre initiation complex (PIC) is not formed before chromatin assembly or the nucleosome is complete with H2A-H2B dimer. Absence of H2A-H2B dimer allows TFIIIA to bind the nucleosomal DNA (Felts et al., 1990; Tremethick et al., 1990; Hansen and Wolffe, 1992). Most of the nucleosome-mediated repression must have prevailed at the initiation stage because Pol III is known to transcribe over a nucleosome on its own (Studitsky et al., 1997) while RNA polymerase II requires involvement of other factors to transcribe nucleosomal DNA.

Studies on the role of histone acetylation in transcriptional activation of 5S rDNA transcription showed that acetylation has a positive effect on Pol III transcription in vitro and in vivo (Ura et al., 1997; Howe et al., 1998; Tse et al., 1998). In humans, the HAT complex P300 is recruited by TFIIIC to tRNA genes and U6 snRNA gene for activation of transcription. P300 is required for the stabilization of TFIIIC-DNA complex (Mertens and Roeder, 2008). Human TFIIIC itself is known to possess a HAT activity though this activity does not seem to affect the transcription (Kundu et al., 1999).

Relation between RNA Polymerase III and chromatin also plays a unique role in the maintenance of chromatin structure. Actively transcribed Pol III genes act as chromatin boundary that can stop spreading of heterochromatin (Donze and Kamakaka, 2001). Also, in *S. pombe*, solitary B boxes present in genome are bound by TFIIIC and act as chromatin organizer (Noma et al., 2006).

Pol III genes are involved in cohesion of sister chromatids (Dubey and Gartenberg, 2007). Pol III transcribed genes also act as condensin binding sites. Presence of a B box where TFIIIC binds can make a minimal condensin loading site (D'Ambrosio et al., 2008). It is the interaction between the Pol III transcription machinery and condensin that brings all tRNAs together near the nucleolus (Haeusler and Engelke, 2008). The mechanism and regulatory role for nucleolar localization of tRNA genes is not yet known.

1.9 SNR6 and chromatin

Yeast U6 snRNA gene was isolated in 1988 by Brow and Guthrie (Brow and Guthrie, 1988). SNR6 gene has a novel promoter structure (Brow and Guthrie, 1990). Involvement of chromatin in regulation of SNR6 had been studied in detail (Marsolier et al., 1995; Shivaswamy et al., 2004; Shivaswamy and Bhargava, 2006). TFIIIB alone can drive transcription of SNR6 gene in vitro on naked DNA templates due to its interaction with the TATA box. When the gene is assembled in to chromatin or in vivo, TFIIIB is not sufficient for transcription to happen. Binding of TFIIIC is required to relieve the repression by chromatin assembled in vitro as well as in vivo (Burnol et al., 1993a; Burnol et al., 1993b; Eschenlauer et al., 1993). It is TFIIIC bound to A and B boxes that positions TFIIIB at the TATA box region and mutations in B block that can inhibit TFIIIC binding are lethal (Gerlach et al., 1995; Kaiser and Brow, 1995).

Studies on chromatin structure of SNR6 in vivo by MNase footprinting method revealed an array of nucleosomes upstream of TATA box and a nucleosome downstream of B box. A mutation in the B box which abolishes TFIIIC binding disrupted the chromatin structure over whole locus indicating it is the TFIIIC binding that dictates the chromatin structure of the region (Marsolier et al., 1995). The region between A box and B box was protected from MNase digestion, but the protection was of sub-nucleosomal size. A high resolution structure analysis by DNaseI also showed that the protection between A box and B box is subnucleosomal in size. These mappings measured the protection size as starting from the terminator and ending before the B box. Deletion of stretches of DNA between A box and B box did not extend the protection towards either side. This intriguing result along with the observation of subnucleosomal protection was explained by suggesting that the region between A and B boxes is protected by some non histone protein and not by a nucleosome. Deletion of 42 bases of DNA between terminator and B box resulted in loss of the TFIIIB footprint, nuclease hypersensitivity of the upstream region and simultaneous reduction of transcription (Gerlach et al., 1995).

Later, requirement of Nhp6, a non histone chromatin structural protein was demonstrated for SNR6 transcription (Kruppa et al., 2001). Yeast Nhp6 is coded by two

different genes NHP6A and NHP6B which are very similar to each other and redundant in function. A double deletion mutant of these genes compromised SNR6 transcription making growth temperature sensitive. This phenotype could be reversed by over expression of U6 in multi copy plasmids or by over expression of Brf1 (Kruppa et al., 2001; Lopez et al., 2001). There have been conflicting reports on whether Nhp6 acts in a TFIIIC-dependent or TFIIIB-dependent way (Lopez et al., 2001). Absence of Nhp6 made TATA box region hypersensitive indicating that Nhp6 stabilizes TFIIIB-DNA complex (Lopez et al., 2001). In the absence of Nhp6, the T7 stretch between the start site and TATA box also becomes essential indicating that they cooperate to enhance SNR6 transcription. Interestingly, transcription is almost abolished by reducing the distance between A and B boxes by 42 base pairs in T7 mutant background (Martin et al., 2001). Reduction in the spacing between A and B boxes to the optimal tRNA distance (<60 bp) by introducing an intronic box B led to a decreased transcription revealing the importance of 200 bp distance between them (Kaiser et al., 2004). At the same time, an increased distance between A box and terminator also decrease the transcription in vitro. These results show that the increased distance between A and B boxes, Nhp6 and the T7 stretch work together to form the stable pre initiation complex.

Previous studies from our lab had shown that the in vitro transcription of SNR6 gene assembled in to chromatin shows a high level activation in TFIIIC dependent manner. Detailed study of the chromatin structure had shown that TFIIIC binding causes positioning of a nucleosome between the A and B boxes (Shivaswamy et al., 2004;). This positioning depends on the involvement of an ATP dependent remodeler and this remodeling is necessary for transcription activation. TFIIIC binding recruits TFIIIB to the TATA box. This binding of TFIIIB leads to further chromatin remodeling which positions a nucleosome upstream of TATA box. Thus the sequential chromatin remodeling following TFIIIC binding is required for high level activation of SNR6 (Shivaswamy and Bhargava, 2006). An in vivo study had earlier reported that mutation of acetylatable lysines of Histone H3 (K9, K14, K18 and K23) leads to an increase in the transcription of some promoter mutants while these deletions did not have any effect on wild type gene. Similarly, nucleosome depletion also caused an increase in the transcription of some promoter mutants (Marsolier et al., 1995). This may be because nucleosome

depletion led to exposure of TATA box and thus TFIIIB could direct the transcription efficiently. These experiments explain that in vivo, TATA box is not accessible for TFIIIB binding and the chromatin remodeler, probably recruited by TFIIIC, makes room for TFIIIB binding by clearing nucleosomes from TATA box.

1.10 Aims of the thesis work

The present study aims to understand the role of chromatin in transcription regulation of yeast U6 snRNA gene. One of the most important aspects of this study was also to confirm the in vitro observation that a nucleosome is present between A and B boxes. The specific questions addressed in this study are as follows:

1. What is the active state chromatin structure of SNR6 and how does it change when transcription is repressed in vivo?

2. Whether any ATP-dependent chromatin remodeling activity is associated with SNR6 transcription in vivo?

3. Whether any covalent histone modification is associated with SNR6 and how does the modification pattern change when the transcription is repressed in vivo?

4. Whether any histone variants are involved in regulation of SNR6 in vivo?

To address these questions, structural analysis of chromatin in vivo using low resolution nucleosomal footprinting under different conditions as well as in mutant strains was carried out. To understand the response of chromatin to the regulation of transcription, chromatin immunoprecipitation assays under different conditions or mutations were used. This study reconfirms the presence of a nucleosome between A and B boxes and reveals that key regulatory mechanisms of U6 transcription mediated by chromatin are entirely different from Pol II transcribed genes as well as any other part of the genome.

Chapter 2

Materials and Methods

This chapter describes the materials and methods used in this study. Major techniques used are described in detail.

2.1. Materials

Most of the fine biochemicals were from Sigma. The restriction enzymes and other DNA modifying enzymes were from NEB. Micrococcal nuclease (MNase) was from Sigma or USB. Zymolyase was from US Biologicals. Radio isotopes were from BRIT (India), GE Healthcare or Perkin Elmer, USA. Antibodies were purchased from Millipore (Upstate) or Abcam. FLAG M2 agarose was from Sigma while all other resins used were from GE Healthcare. Real-Time qPCR master mixes (2X) were purchased from ABI or Invitrogen.

2.1.1. Commonly used buffers

10X TBE (per litre)

108g Tris Base
55g Boric acid
40 ml 0.5M EDTA

20X SSC(per litre)

175.3g NaCl
88.2g Sodium citrate
Adjust pH to 8 with 10N NaOH

20X SSPE (per litre)

175.3g NaCl
27.6g NaH2PO4
7.4g EDTA
Adjust pH to 7.4

Denaturing solution for southern blotting (per 400 ml)

8g NaOH
35.06g NaCl

Neutralizing buffer for southern blotting (per 400 ml)

200 ml Tris pH 7.5
35.06g NaCl

Sequencing gel loading buffer

98% deionized formamide
10mM EDTA
0.025% xylene cyanol FF
0.025% bromophenol blue

Buffer Mo(For ChIP washing)

20mM Tris pH 8
2 mM EDTA
1% Triton X 100
0.1% SDS
200 mM NaCl

Buffer M1(For ChIP washing)

20mM Tris pH 8
2 mM EDTA
1% Triton X 100
0.1% SDS
500 mM NaCl

Buffer M2(For ChIP washing)

10mM Tris pH8
2 mM EDTA
1% Na deoxycholate
1% NP40
250mM LiCl

SD buffer 1 M sorbitol 50 mM NaCl 10 mM Tris-HCl (pH 7.4) 5 mM MgCl2 1 mM CaCl2 1mM β-mercaptoethanol 0.5 mM spermidine 0.075% NP-40) **Zymo buffer** 1M sorbitol 10mM β-mercaptoethanol 10 mg/ml Zymolyase 100T from US Biologicals) **Buffer Z** 1M sorbitol 40 mM tris Cl pH7.5 10mM β-mercaptoethanol **ChIP elution buffer** 10 mM Tris pH8 2mM EDTA 200mM NaCl 1% SDS	**TE** 10mM Tris Cl pH 8 1mM EDTA **NPS buffer** 0.5 mM spermidine 1mM β-mercaptoethanol 0.075% NP40 100 mM NaCl 10 mM Tris Cl pH 7.5 5 mM $MgCl_2$ 1 mM $CaCl_2$ **Buffer L** 50 mM Hepes KOH pH 7.6 1% Triton X 100 0.1% Na deoxycholate 2mM EDTA 150 mM NaCl **FA-Lysis buffer** 50 mM Hepes KOH pH 7.6 1% Triton X 100 0.1% Na deoxycholate 0.1% SDS 2mM EDTA 150 mM NaCl

2.1.2. Primers used in this study

All the oligonucleotides used in this study were custom synthesized by Bioserve, Hyderabad. Short oligos were obtained as HPLC purified and longer oligos were PAGE purified. List of oligos used in this study are listed in Table 2.1

2.1.3. Yeast strains

List of yeast strains used in this study are given in Table 2.2.

2.1.4. Media and growth conditions

Yeast extract and peptone were purchased from Difco. Galactose was from Sigma and Glucose used was from Qualigens. Cells were grown in an incubator shaker at 30^0C in

Primer Name	Sequence
ARS504Rev	CACCCCGAAGCTGCTTTCA
ARS504For	GGCTGTCAGAATATGGGGC
Gcn5 S1	CCGTGAGCCGCCCAAAAGTCTTCAGTTAACTCAGGTTCGTATTCTACAT TAGATG CGTACGCTGCAGGTCGAC
Gcn5 S3	GCTAGAGAAATTCTTCAATAATAAAGTAAAAGAAATACCTGAATATTCT CACCTTATTGATCGTACGCTGCAGGTCGAC
GCN5 screen rev	CAATTGATCACATCGTCTCGCC
GCN5 scren for 1	CTGCGTAAATGTTTGATTAAGC
Gcn5S2	CTAAACATTTATTTCTTCTTCGAAAGGAATAGTAGCGGAAAAGCTTCTT CTACGCATTAATCGATGAATTCGAGCTCG
Gcn5screen for	CGAATGTACAATGGCGAGAATACG
Tel VIR For	GCGTAACAAAGCCATAATGCCTCC
Tel VIR Rev	CTCGTTAGGATCACGTTCGAATCC
Pst1 for	GGTGCAATCCAAGACCCTCCC
Pst1 rev	GTGTTGTTTGAAAACAAGTTC
RPS11B for	CCCGCTTTGTTTTTATTCCAC
RPS11B rev	GCCCGCTGAAAGGTTGGCG
RT Both	TCTCTTTGTAAAACGGTTCATCCT
snr14	GCGAACACCGAATTGACCATG
A-B Box For	GTTCCCCTGCATAAGGATGAACCG
A-B Box Rev	GGAAGATAAAGATACACTGCTG
TAG common rev	GCTAAATGTACGGGCGACAGTCACATC
YLRC Delta BOTTOM	CTAAAAGAGGAATAATCTCAATAC
YLRC Delta Top	GAAGATACTAAATGTTCTCTCCG
U6down for	GTACTTATGTGCTTTATGAATGTG
U6down rev	CCTTTCTCTTCTGTTTGACA
U6maxiRT	GGCTGCAGGAATTCGATATCAAGC
Upstream for	GTCATCTTCCTGGACCTCATG
Upstream rev	GCAATGAAACTCTAAAGTATCATCGATTCAG
TATA Box for	CGATGATACTTTAGAGTTTCATTGC
TATA Box rev	CTTCGCGAACACATAGTTGC
Ylrcdelta5 RT	GTTGGGATTCCATTGTTCGTAAACGC
LTR down	CAGAATTTATATTGCTACCATGACTG

Table 2.1: List of primers used in this study

Sl. No.	Yeast Strain	Genotype	Reference
1.	UKY403	MATa, ade2-101, his3-Δ200, leu2-3,-112, lys2-801, trp1-Δ901, ura3-52, GAL$^+$, thr$^-$, tyr$^-$, arg4-1, Δh4-1, [HIS3$^+$], Δh4-2 [LEU2$^+$]/pUK421(TRP$^+$, GAL1-H4-2$^+$)	Han et al., 1988
2.	MHY308	MATa, ade2-101, his3-Δ200, leu2-3,-112, lys2-801, trp1-Δ901, ura3-52, GAL$^+$, thr$^-$, tyr$^-$, arg4-1, Δh4-1, [HIS3$^+$], Δh4-2 [LEU2$^+$]/pUK499(URA3$^+$, H4-2$^+$)	Han et al., 1988
3.	FLAG-H2B	(MAT a hta1-htb1Δ::LEU2, hta2-htb2Δ::TRP1, leu2-Δ1, ura3-52, trp1-Δ63, his3-Δ200/pFB1251 (HIS3 CEN ARS HTA1, FLAG-HTB1)	Ng et al., 2002a
4.	RSC2-Myc	MATa, ura3-52, trp1-Δ63, his3Δ200, leu2::PET56,RSC2-9Myc:TRP1	Ng et al., 2002b
5.	MW671-Myc	MATα ade2-101, his3Δ-200, leu2-Δ1, lys2-801, trp1-Δ63, ura3-52, rpc160::HIS3, MAF1-13Myc:KanMX6, pC160-240(TRP1, 3HA-RPC160)	Oficjalska-Pham et al., 2006
6.	MW4034	MATa, ade2-101, his3- Δ200, leu2-•Δ1, lys2-801, trp1-Δ63, ura3-52, BRF1-3HA :kan	Oficjalska-Pham et al., 2006
7.	YM1730	MATα his3Δ0 leu2Δ0 ura3Δ0 lys2Δ0 htz1::Kan	Meneghini et al., 2003
8.	SWR1-TAP	MATa, his3-1, leu2-0, met15-0, ura3-0, SWR1-TAP:HIS	Durant et al., 2007
9.	MW3993	MATα ura3-52 his3-Δ200 ade2-101 trp1-Δ63 lys2-801 leu2-Δ1 rsc4-Δ4::HIS3	Soutourina et al., 2006
10.	MW4019	MATα ura3-52 his3-Δ200 ade2-101 trp1-Δ63 lys2-801 leu2-Δ1 rsc4-Δ4::HIS3, STH1-13Myc:KanMX6	Soutourina et al., 2006
11.	YBL467	MATa, ura3-1, lys2Δ::hisG, trp1-1, his3-11, -15, leu2-3, -112, can1-100, Hta1-Flag:LoxP/Hta2-2FLAG:Kan	Li et al., 2005
12.	YBL325	MATa, ura3-1, lys2Δ::hisG, trp1-1, his3-11, -15, leu2-3, -112, can1-100,Htz1-3xFlagP:LoxP	Li et al., 2005
13.	YBL556	MATa, his3-1, leu2-0, met15-0, ura3-0, Htz1-TAP:HIS	Li et al., 2005
14.	YBL557	MATa, his3-1, leu2-0, met15-0, ura3-0, swr1Δ::KanMX6, Htz1-TAP:HIS	Li et al., 2005
15.	YJW253	MATa, ade2-1, his3-11, -15, leu2-3, -112, trp1-1, ura3-1, can1-100, sas2Δ::TRP1	Shia et al., 2006
16.	YJW458	MATa, ade2-1, his3-11, -15, leu2-3, -112, trp1-1, ura3-1, can1-100, SAS4-13Myc:KanMX6, SAS2-TAP:TRP1	Shia et al., 2006
18	YWJS069	MATa, his3-Δ1, leu2-Δ0, met15-Δ0, ura3-Δ0, HTZ1-TAP:HIS3MX6, sas2Δ::KanMX	Shia et al., 2006
19	W3031a	MATa, ade2-1, his3-11, -15, leu2-3, -112, trp1-1, ura3-1, can1-100	Lab stock
20	gcn5Δ	MATa, ade2-1, his3-11, -15, leu2-3, -112, trp1-1, ura3-1, can1-100, gcn5Δ::HIS3	Lab stock
21	rtt109Δ	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, rtt109Δ::Kan Mx6	Lab stock
22	rmd6Δ	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, rmd6Δ::Kan Mx6	Lab stock
23	yir042cΔ	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, yir042cΔ::Kan Mx6	Lab stock
24	spt10Δ	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, spt10Δ::Kan Mx6	Lab stock
25	hat1Δ	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, hat1Δ::Kan Mx6	Lab stock

Table 2.2: List of yeast strains used in this study

different media. Rich media used were YEPD (1% Yeast extract, 2% peptone and 2% glucose) or YEPGal (1% Yeast extract, 2% peptone and 2% galactose). MaxiU6 containing cells were grown in synthetic dropout (SD) Ura- medium with 2% glucose as carbon source. Cells were exposed to nutrient starvation by shifting to 0.15X YEP without any carbon source after the A_{600} reached 0.7 (for IEL) or 1 (ChIP and RNA isolation)

2.2. Indirect End Labeling (IEL)

Indirect end labeling (IEL) is a low resolution chromatin foot printing method in which large regions of chromatin (2-3 Kb) can be examined at a time. This is one of the most powerful techniques used to find the presence of translationally positioned nucleosomes. A schematic of IEL method is given in the Figure 2.1. In short, the chromatin is digested with MNase with a single hit kinetics. At this low level of digestion, MNase preferentially cuts the linker DNA, leaving the nucleosomal DNA uncut. In other words, the nucleosomal DNA is "protected" from MNase digestion. Later, the digested chromatin sample is deproteinized and subjected to a secondary digestion with restriction endonuclease to generate DNA fragments with a uniform end. These fragments are then separated by electrophoresis and visualized with a probe that hybridizes at the uniform end. A sample of purified DNA (naked DNA) is also processed along with the chromatin sample. A "protection" is the absence of one or more bands from the chromatin sample in comparison with naked DNA and a protection of more than 145 bp is considered as nucleosomal (Thoma et al., 1984). One of the most important facts about IEL is that it can detect only translationally positioned nucleosomes. i.e. a nucleosome present between two fixed boundaries, but not the rotationally positioned or non-positioned nucleosomes.

We used a modified version of indirect-end-labeling described by Kent and Mellor (1995). Advantage of this method is that this is a rapid protocol which gets over in ~8 minutes time, thus reducing the chance for any change in chromatin structure due to prolonged sample processing stress. This is important because pol III transcription

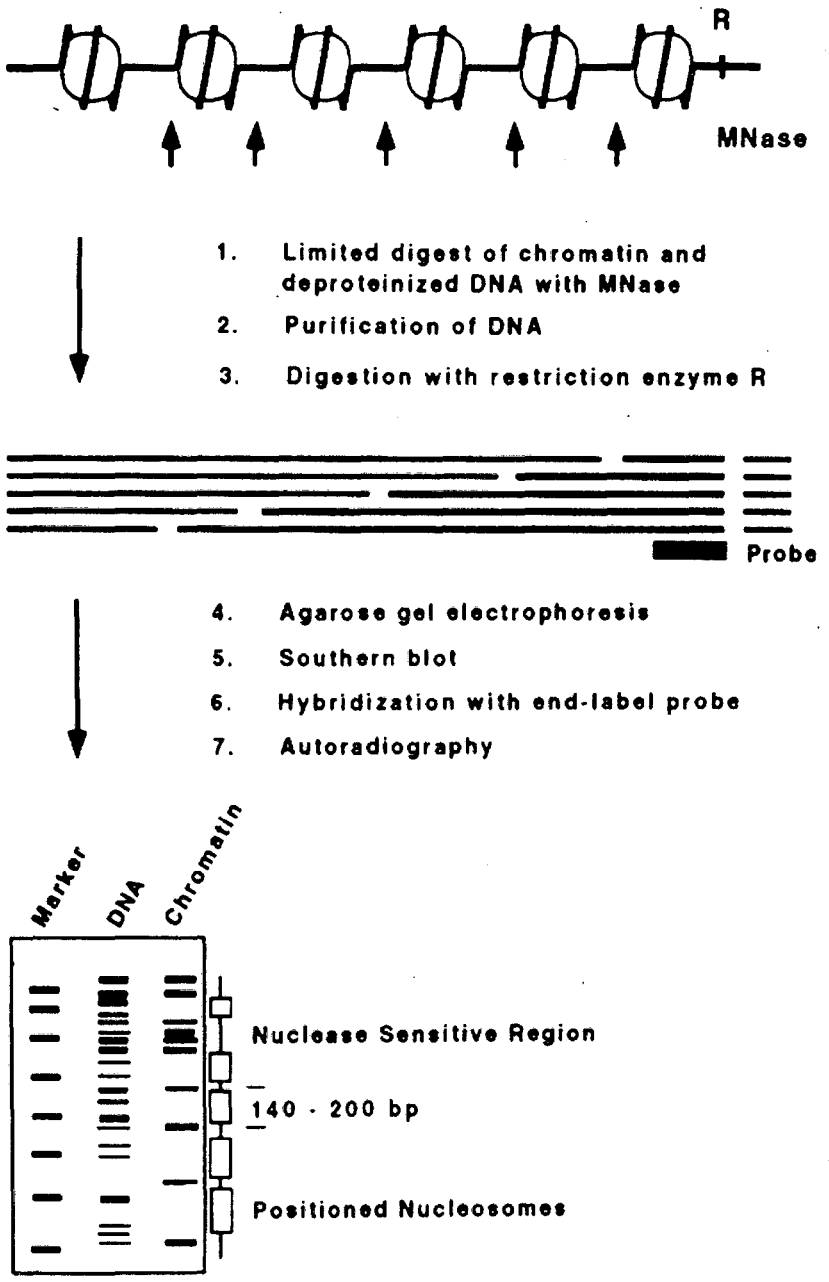


Figure 2.1: Mapping Nucleosome Positions by Indirect End Labeling.

This figure shows a schematic representation of the procedure used for IEL analysis.

responds to cell wall stress and the transcription is repressed within a short time (Upadhya et al., 2002; Willis and Moir., 2007).

Cells from 100ml yeast culture were harvested after growing in appropriate media and growth conditions to a specified OD and period of time. The cell pellet was resuspended for spheroplasting in 950µl of Zymo buffer and mixed well with gentle inversion for 40 seconds (for cells grown in glucose) or for 1 minute (for cells grown in galactose). Spheroplasts were harvested by centrifuging for 10 seconds and washed twice with 1M sorbitol. The pellet was then resuspended in 1ml of SD buffer, divided into 6 equal aliquots and digested with 15, 30 and 45 units of MNase from USB or 0.5, 1 and 2 units of MNase from Sigma for 4 minutes at 37^0C. Digestion was stopped by adding 20 µl of the stop solution (250 mM EDTA and 5% SDS) followed by thorough vortexing. The lysate was then incubated for 1 hour with 10 µl of 10 mg/ml DNase free RNase A (From Roche) at 37^0C followed by overnight digestion with 20µl of 20mg/ml pronase (Roche). Samples were extracted with equal volumes of Tris equilibrated Phenol (pH 8) and chloroform mixture (1:1) till the inter phase became clear. DNA was precipitated by adding Ammonium acetate (1.5M final concentration) and 300 µl isopropanol.

Precipitated DNA was digested overnight with 2.5 Units of *Pst*1 (cuts at +822 bp position) and *Nde*1 (cuts at -1458 bp position) per µg of DNA at 37^0C. DNA was again precipitated and resolved in a 1.5% agarose gel. 1KB or 100 bp DNA (NEB, USA or Fermentas) ladder was loaded to mark the sizes. After the run, gel was transferred on to a nitro cellulose membrane with a pore size of 0.2 micron (Protran, Whatman) by capillary method with 20XSSC. After 24 hours transfer, the blot was baked in vacuum oven for 1 hour at 80^0C and probed with a radio actively labeled 231 bp PCR product generated with "*Pst*1 for" and "*Pst*1 rev" primers (Table 2.1). The probe was designed to hybridize with the region abutting the *Pst*1 end. Blot was visualized on Fuji phosphor imager. The marker lane was probed separately and used to identify the size of bands.

2.3. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) is a powerful technique for studying association of a particular protein to a particular genomic region in vivo. A schematic of ChIP assay is given in Figure 2.2. In short, the live cells are treated with formaldehyde to create DNA-protein, as well as protein-protein crosslinks between molecules in the close proximity before lysing and the chromatin is sheared in to fragments of desired size. This chromatin extract is now incubated with antibody against a protein or a modified peptide (acetyl, methyl etc) to form immune complexes containing antibody, protein and the DNA associated with the protein if any. Epitope tags on proteins are particularly useful for ChIP assays because the cost of producing antibody against each and every protein can be avoided by using them. These immune complexes are purified over Protein A/G agarose. Heating can reverse these cross links and allow the purification of protein bound DNA to analyze it further by PCR or microarray.

2.3.1 Preparation of whole cell extract

100 ml of yeast culture was cross linked by shaking with 1% formaldehyde at 30^0C either after growing to an A_{600} of 1 or after growth in 0.15X YEP for a specified time period. Crosslinking time varied between experiments depending upon the protein under study. For histones and pol III machinery, a 15 minute crosslink was enough while some HAT components required overnight crosslinking. Crosslinking was stopped by adding glycine to a final concentration of 125mM. After incubating for 5 minutes at 30^0C, cells were harvested and washed twice with water.

Depending upon the epitope used, two different methods were used to lyse the cells. The first method is lysis of spheroplasts. The harvested cells were resuspended in 10 ml Buffer Z containing 2mM PMSF and spheroplasted by adding 250 µl Zymo buffer with 10 mg/ml zymolyase 100T to this at room temperature for 20 minutes. Spheroplasts were harvested and the pellet was resuspended in 1 ml NPS buffer containing 2 mM PMSF. Spheroplasts were lysed at this step and the chromatin fraction was pelletted by centrifugation.

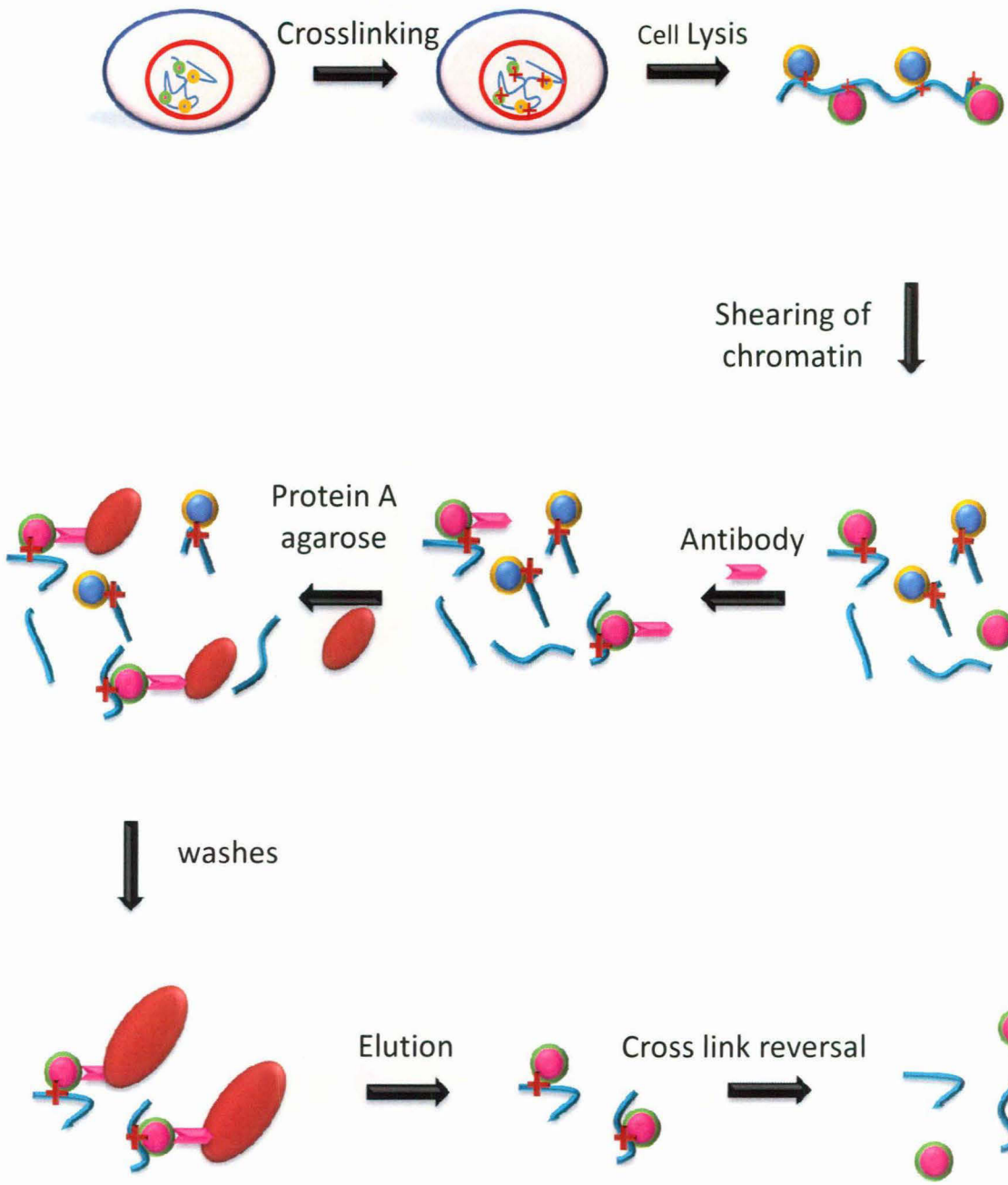


Figure 2.2: Schematic diagram of chromatin immunoprecipitation assay.

Blue lines represent DNA while pink and blue rounds with green rim are DNA bound proteins, small red crosses are crosslinks generated by formaldehyde, large maroon ovals are protein A agarose beads, and pink solid arrows represent antibody molecules

For TAP tagged strains as well as for experiments with overnight crosslinking, zymolyase mediated lysis was not possible (Zymolyase treatment degraded the TAP tag and zymolyase couldn't spheroplast overnight cross linked cells). In these cases, the cells were harvested after crosslinking, washed twice and resuspended in 1 ml FA-Lysis buffer. To the cell suspension, equal volume of 500 micron glass beads (From Sigma or Biospec) was added and vortexed at maximum speed for 1 hour at 4^0C. The lysate was recovered and centrifuged to pellet the chromatin fraction. This pellet was washed once with NPS buffer.

The chromatin pellet was resuspended in 1 ml NPS buffer and digested with either 250 units of USB MNase or with 1 unit of Sigma MNase for 2 hours at 37^oC. Digeston was stopped by shifting the tubes to ice and by addition of EDTA to 10 mM final concentraton. This lysate was then supplemented with "buffer L" components. Extract was then centrifuged and the supernatant was saved as chromatin solution.

MNase digestion was preferred over sonication to achieve high resolution for the assay. But for a particular antibody against Myc tag (9E10) which did not work in this method, sonication method was used to shear the DNA. Spheroplasts were resuspended in FA-Lysis buffer with 2mM PMSF instead of NPS buffer and centrifuged. The pellet was resuspended in 1 ml FA-Lysis buffer with 2 mM PMSF and subjected to sonication in a sonifier. 6 cycles of 30 seconds pulses with 20% amplitude were used with 2 minutes cooling time in between. The lysate was centrifuged and the supernatant was saved as the chromatin solution.

2.3.2. Formation and purification of immune complexes

To the chromatin solution, 75µl of 50% slurry of Protein A/G agarose equilibrated in Buffer L was added and incubated for 1 hour at 4^0C for preclearing. This step was intended to remove all non-specifically interacting proteins and DNA. The chromatin solution prepared as described was from 100 OD units of cells (100 ml x 1 OD/ml). Only 20 OD units of cells were used per immunoprecipitation reaction. 20% of the extract used for IP was kept aside as input material. In case of purified antibody, 2 µg of antibody was used per IP and in case of crude anti serum, 5µl was used. Always one or two samples were kept without adding antibody to be treated as mock

immunoprecipitation. In case of FLAG tag and TAP tag ChIP, the affinity resin (FLAG M2 Agarose from Sigma and Ig G sepharose from GE Healthcare) was added.

The extracts were incubated with antibody at 4^0C overnight followed by incubation for 2 hours with 25µl of 50% protein A/G agarose equilibrated in Buffer L at 4^0C. After the incubation, the beads were washed 8 times (twice each with Buffer M0, M1, M2 and TE). Each wash was for 5 minutes at room temperature and the centrifugation was for 2 minutes at 1000g. After the last wash, samples were eluted twice with 250 µl of elution buffer at 65^0C for 15 minutes and both eluates were pooled. The input sample was taken and 450 µl of elution buffer was added to it. 20 µl of 20 mg/ml pronase was added to all samples and incubated at 42^0C for 1hour followed by an overnight incubation at 65^0C to reverse the cross links. Samples were twice extracted using equal volume of Tris Cl (pH8) saturated phenol and chloroform and precipitated with sodium acetate and ethanol. DNA was pelletted and dissolved in 500 µl of water. Input samples were further diluted ($1/10^{th}$ of the IP sample).

2.4. Real-Time quantitative PCR

DNA samples obtained from ChIP assay were analysed using Real-Time qPCR with SYBR green chemistry. Though SNR6 gene is very short, the MNase cleavage method provided the enough resolution to look at different regions of the gene. Major primer sets used in this study as well as their position in the locus is given in the Figure 2.3 with the sequences in Table 2.1. Three amplicons of the major primer sets for SNR6 are: Upstream (-174 to -96), TATA box (-120 to +10) and A-B Box (+61 to +187). There is a significant overlap between the Upstream and TATA box amplicons. As control primer set, a region approx 500 bp downstream of the right telomere of 6^{th} chromosome was used (TelVIR). This region was selected because it is a region where pol III genes are found. Unless stated otherwise, all the occupancies measured in this study are normalized to TelVIR. In other cases, a nucleosome free mitochondrial gene Cox3 was also used as a control.

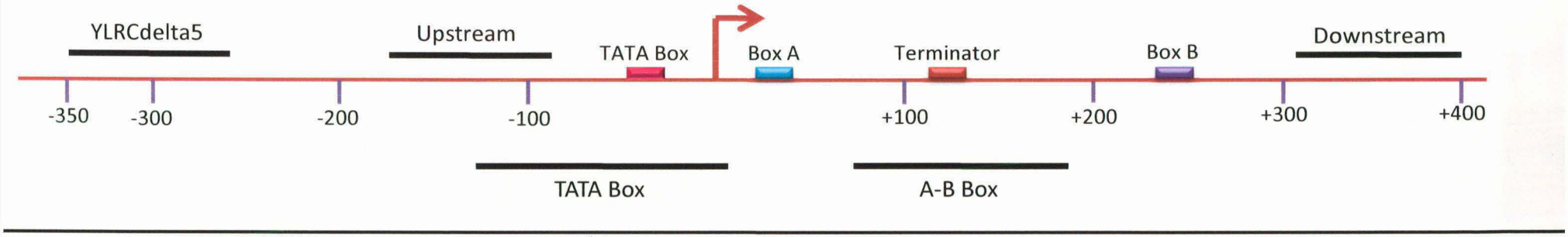


Figure 2.3: Positions of the primer pairs used for Real-Time qPCR quantification of ChIP DNA.

Five amplicons covering different positions on SNR6 are shown. Primer pair Upstream amplified a region from bp position -174 to -96, TATA box amplified the region -120 to +10 while A-B Box set amplified from +61 to +187. YLCdelta5 primer pair amplified the region from -259 to -352 and downstream primer pair amplified from +311 to +415. There is a short overlap of ~ 24 bp between the amplicon "upstream" and "TATA Box". The figure is roughly to the scale

For each Real-Time reaction, 2 µl of the ChIP DNA and 2.5 picomoles of primers were used along with2X qPCR master mix. Reactions were done in triplicates and the Ct values which fell within 0.3 cycles were taken for calculation. The data after the reaction was exported to Microsoft Excel and analysed essentially as described by Aparicio et al. (2004). In short, reaction was done for IP samples and input samples with both test and control primer sets. Average Ct for each sample was calculated and ΔCt was calculated for IP and input samples by subtracting average Ct value of control primer set from that of test primer set. ΔΔCt was calculated by subtracting the ΔCt of input from ΔCt of IP. The degree of occupancy was calculated by the equation "degree of occupancy= $2^{-\Delta\Delta Ct}$". Values obtained for Mock precipitation were also calculated in the same way and considered as background. The actual occupancy (fold enrichment) was expressed against the mock precipitation (Occupancy = degree of occupancy of IP/degree of occupancy of Mock). A value of 1 or more was taken as presence and a value less than 1 was considered absence. The average values from three experiments with standard deviation were plotted in graphs given as final result of measurements.

2.5. RNA isolation

Isolation of total RNA was carried out as described by Schmitt et al. (1990). 10 ml of yeast culture was harvested by centrifugation and washed twice with water. Cells were transferred to a 1.5 ml microfuge tube during the second wash and resuspended in 400 µl AE buffer (50mM Na acetate, pH 5.3, 10mM EDTA) and mixed with 40µl 10% SDS. To this suspension, 400 µl of water saturated phenol pre-warmed to 65°C was added and vortexed at maximum speed. The tube was incubated at 65°C for 7 minutes with intermittent vortexing. Samples were dipped in liquid nitrogen and again incubated at 65°C for 1 minute. Samples were centrifuged at maximum speed for 5 minutes at room temperature and supernatant was transferred to fresh tube. RNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) followed by an extraction with chloroform:isoamyl alcohol (24:1) and precipitated with sodium acetate and ethanol. RNA pellet was dissolved in 50 µl water and stored at -70°C.

2.6. In vitro transcription with purified proteins

In vitro transcription was done on pCS6 plasmid that contains SNR6 locus from -123 to +312 bp position using lab stocks of pure TFIIIB, TFIIIC and pol III according to Shivaswamy et al (2004). Briefly, 100 ng (50 fmols) of pCS6 plasmid was transcribed with 100 fmols of TBP, 64 fmols of Brf1, 150 fmols of Bdp1, 125 fmols of TFIIIC, 5 fmols of RNA Polymerase III and 500 μM each of rNTPs at 21^0C for 30 minutes in a buffer containing 10 mM Tris pH 8, 7 mM MgCl2, 45 mM NaCl, 3mM DTT and 100 μg/ml BSA. The reaction was stopped by adding transcription stop buffer (20 mM EDTA, 200 mM NaCl and 1% SDS) containing a radioactively labeled short DNA of known size as recovery marker. The samples were de-proteinized and extracted with phenol:chloroform:isoamyl alcohol (50:24:1) and precipitated. The product was visualized by reverse transcription reaction with a radio labeled primer.

2.7. Reverse transcription reaction

RT Both primer was used to detect native U6 level while U6Maxi primer was used to detect transcript from MaxiU6 construct. SNR14 primer against U4 was used as a control for RNA levels. One advantage of U4 is that it is not transcribed by pol III. Another and the major advantage is that U4 RNA is more stable than even U6 (Fury and Zieve, 1996) and under the reaction conditions, it is very unlikely that U4 level changes.

For reverse transcription, the primers were labeled at the 5' end by using γ-[^{32}P]-ATP and T4 polynucleotide kinase. The labeled primer was allowed to anneal to the transcript in presence of 10mM tris pH 7.8. 1mM EDTA and 250 mM KCl at 55^0C followed by incubation at 42°C for reverse transcriptase reaction in presence of 400μM dNTPs and 5 units of AMV reverse transcriptase. The reaction was allowed to happen for 1 hour and later processed and resolved on a 10% polyacrylamide 8M urea denaturing gel in 1X TBE. The gels were dried and subjected to phosphorimaging analysis. Bands were quantified using the Image Gauge program (Fuji).

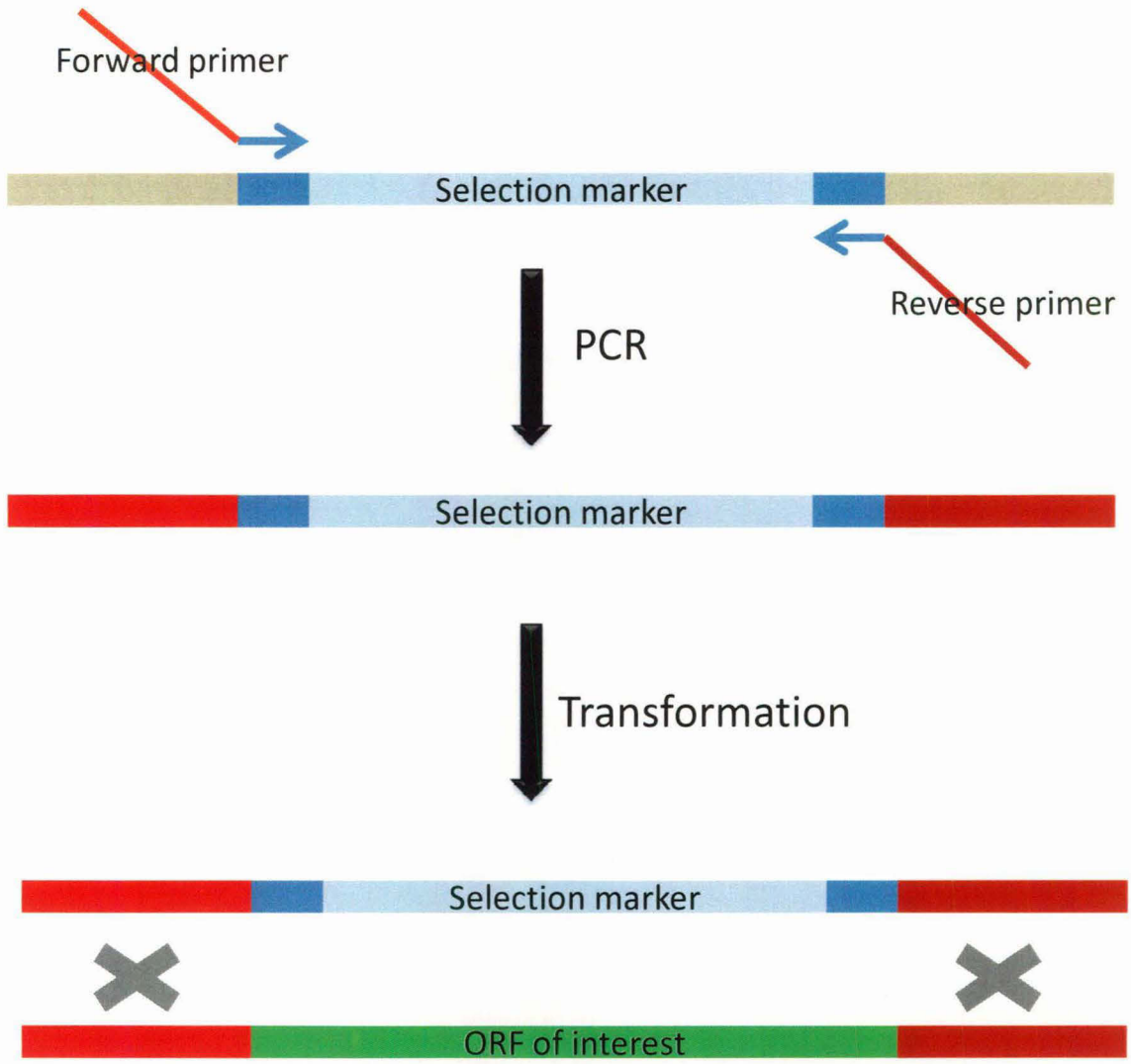


Fig 2.4: PCR based gene disruption in yeast

The figure shows a schematic representation of procedure for PCR based gene disruption in yeast. The forward primer used to amplify the cassette has an overhang of 55 bps identical to the 5' flanking region of the gene (shown in red color) while the reverse primer has an overhang of 55 bps identical to the 3' flanking region of the gene (Shown in maroon color)

2.8. Genetic manipulation of yeast

Genetic modification of yeast had been carried out using the PCR tool box from Euroscarf (Janke etal., 2004). To synthesize a null mutant, a cassette containing a marker gene was amplified using a pair of primers with a 5' overhang of 55 bp that is identical with the flanking region of the gene to be deleted. This PCR product was then transformed in to the yeast so that the fragment gets integrated in to the genome by homologous recombination and leads to the replacement of the gene with the selection cassette (See Figure 2.4).

Yeast transformation was done by heat shock method. Cells from 25 ml log phase culture were harvested and washed twice with water. The pellet was resuspended in 1 ml water and 50 µl was transferred to a fresh tube. Water was removed from the remaining suspension after spinning again. The cells were resuspended in 100 µl Lithium acetate solution and incubated at 30^0C for 30 minutes. Cells were recovered after spinning, mixed with 10µl of PCR product plus 50 µg of single strand denatured salmon sperm DNA and incubated at 30^0C for 30 minutes. 100 µl of PEG solution was added and incubated at 30^0C for 30 minutes followed by addition of DMSO to 10% before giving heat shock at 42°C for 30 minutes. After the heat shock, cells were pelletted, resuspended in 200 µl YPD and allowed to grow for 4 hours before plating on to the antibiotic selection YPD agar plates.

Colonies formed were screened by colony PCR assay with one primer specific for the chromosomal locus, while the other specific for the cassette. The positive colonies were confirmed by sequencing.

Chapter 3

Chromatin Structure and Remodeler of SNR6

3.1. Overview

Various studies have shown that Pol III transcription in *S. cerevisiae* can be repressed in response to a large number of stimuli like general nutrient starvation, DNA damage, cell wall integrity, stationary phase growth etc by a central regulator called Maf1 via targeting Brf1 and Pol III (Geiduschek and Kassavetis, 2006; Willis and Moir, 2007). Repression of transcription by starvation or during late growth phase leads to an increased occupancy of TFIIIC and a little or no change in Brf1 occupancy. But Pol III occupancy shows an interesting feature as some of the subunits show a decrease in occupancy; some others do not show any difference under repression (Harismendy et al., 2003; Roberts et al., 2003; Roberts et al., 2006). These observations led to two different models for the action of Maf1. First one is that Maf1 can bind to Brf1 and Pol III in solution and prevent de novo assembly of PIC while the second model states that Maf1 binds to the PIC that is already present on the genes and renders it incompetent for transcription (Geiduschek and Kassavetis, 2006; Willis and Moir, 2007). Repression of Pol III transcription through Maf1 seems to exert a general effect on Pol III transcription, but it is not the case. All tRNA genes do not respond to Maf1 in the same way (Ciesla et al., 2007). One explanation for this observation may be the involvement of other proteins or the involvement of epigenetic factors.

Previous high resolution footprinting studies on the chromatin structure of SNR6 in vivo had shown a sub-nucleosomal size protection between A and B boxes leading to speculation that the DNA between A and B boxes is condensed not by a nucleosome but by a non histone protein like Nhp6 (Gerlach et al., 1995; Marsolier et al., 1995). A nucleosome-free TATA box followed by an upstream array of nucleosomes covering the solo δ element was also evident from these studies. A nucleosome was found downstream of B box also. The observed chromatin structure of the whole locus was dependent on TFIIIC binding to B box (Marsolier et al., 1995).

In vitro studies on SNR6 had shown that TFIIIC is required to relieve the repression by chromatin (Burnol et al., 1993a) suggesting role of a TFIIIC dependent chromatin remodeling on the locus. Previous studies from our lab had shown a two step ATP-dependent chromatin remodeling after TFIIIC binding which was required for the

high level activation of the gene when assembled in to chromatin in vitro (Shivaswamy et al., 2004; Shivaswamy and Bhargava, 2006). The chromatin remodeler RSC is known to associate with Pol III transcribed genes and is involved in maintenance of low nucleosome density over Pol III genes (Ng et al., 2002a; Parnell et al., 2008). RSC4 subunit of RSC is known to interact with Pol III. A mutation of this subunit can abolish this interaction and negatively affect transcription of many Pol III transcribed genes including SNR6 (Soutourina et al., 2006).

In order to understand the chromatin mediated mechanisms involved in regulation of SNR6, as a first step, the Pol III transcription was repressed by general starvation (Roberts et al., 2003; Roberts et al., 2006) to check whether SNR6 is repressed under this condition. The repression was tracked by the transcription of maxi-U6 construct as well as by the occupancy of Pol III components on SNR6. In vivo indirect end labeling (IEL) technique was used to follow the changes in chromatin structure under different conditions like nucleosome depletion, starvation and mutation of the chromatin remodeler RSC. Chromatin immunoprecipitation technique was used to understand the activity-occupancy relationship of histones as well as other factors.

Finally, a model for the nucleosome dynamics over the SNR6 gene undergoing repression is given along with the identification of the remodeler responsible for the remodeling.

3.2. Repression of SNR6 by nutrient deprivation

As starvation stress was chosen as tool to study structure-function correlates of chromatin in SNR6 expression, the first requirement of the study was to check whether the general nutrient deprivation can repress SNR6 transcription. Nutrient deprivation was achieved by shifting actively growing cells in to 0.15X YEP medium, which is known to repress the Pol III transcription.

3.2.1. SNR6 is repressed under starvation conditions by Maf1.

U6 RNA is known to be very stable with a half life of over 24 hours (Burnol et al., 1993b; Soutourina et al., 2006). This makes it difficult to follow the repression by looking at the related loss of the native RNA level. As shown in the Figure 3.1A, even after 4 hours of starvation, the native RNA level did not show much difference. To track the exact transcription rate, yeast cells were transformed with a plasmid carrying Maxi U6 gene, which has a 59 bp insert in the coding region. This insert makes the RNA less stable and provides a means to differentiate it from the native RNA, while the promoter structure is intact (Marsolier et al., 1995). Quantification of the Maxi U6 RNA level by primer extension method shows that its transcription is reduced to half by one hour of starvation (Figure 3.1B).

In order to further confirm the starvation led repression, the occupancy of RNA polymerase III was examined over the gene using chromatin immunoprecipitation. As described in the materials and methods, due to the higher resolution achievable by MNase cleavage of chromatin after cross linking, the gene could be divided in to three regions using three different primer sets (Figure 2.3). A yeast strain carrying a 3XHA tag on the N terminus of the RPC160 subunit of Pol III was used to follow its occupancy on the gene. RPC160 occupancy is reported to reduce under repression on Pol III genes (Harismendy et al., 2003; Oficjalska-Pham et al., 2006). The ChIP assays showed that RPC160 occupancy drops drastically after starvation for 40 minutes over both TATA box and A-B box region (Figure 3.1C), indicating severe transcriptional repression of the gene.

Involvement of Maf1 protein in the repression of SNR6 was confirmed by checking the occupancy of 3XHA RPC160 occupancy in Maf1 deletion background (maf1Δ). The results in Figure 3.1D show that RPC160 levels did not change in maf1Δ cells even after one hour of repression. In contrast, maf1Δ cells show higher Pol III occupancy even under repressed conditions as compared to wild type cells.

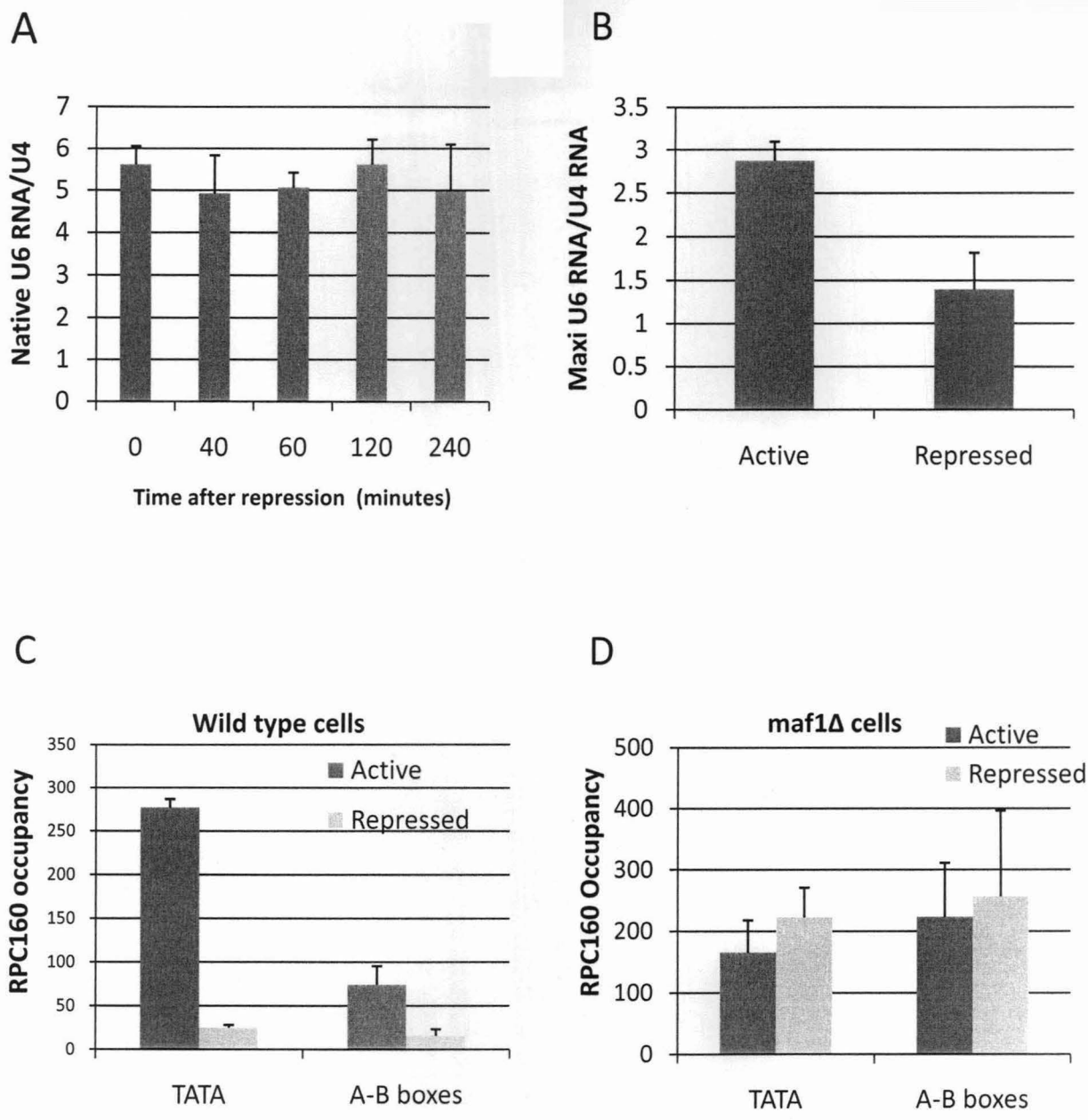


Figure 3.1: SNR6 is repressed under starvation conditions by Maf1.

A. Time course analysis of the effect of repression on the U6 RNA level. Total RNA was isolated at different time points of repression and reverse transcribed with [^{32}P]-end-labeled primers specific for U6 snRNA and pol II transcribed U4 snRNA.
B. Maxi U6 RNA level under nutrient rich condition and after starvation for 1 hour. Expressed as ratio of Maxi U6/U4
C. Effect of starvation on the occupancy of RPC160. ChIP assay was done with a strain carrying N terminal 3XHA tag under nutrient rich condition and after shifting to 0.15X YEP for 40 minutes. Occupancy was expressed as ratio of degree of occupancy of IP/Mock.
D. Effect of Maf1 on occupancy of RPC160. RPC160 was tagged with 3XHA on the N terminus in maf1Δ background and the occupancy was measured by ChIP assay. Occupancy was expressed as ratio of degree of occupancy of IP/Mock.

3.2.2. Pol III machinery is bound to SNR6 under repressed conditions

RPC160 is the largest subunit of Pol III. Loss of Pol III from the gene should be reflected by the occupancy levels of all Pol III subunits. However, a ChIP assay performed against an N terminal FLAG tagged RPC128 at different time points of nutrient deprivation gave an altogether different result (Figure 3.2A). Maf1 mediated repression can establish within 25 minutes of nutrient deprivation (Pluta et al., 2001; Upadhya et al., 2002) and RPC160 has shown a marked decrease in its occupancy by 40 minutes. But RPC128, the second largest subunit of Pol III did not show any difference in its occupancy over the TATA box even after 40 minutes of repression.

Interestingly, though there is no difference in the occupancy of RPC128 over TATA box; it decreased significantly within 40 minutes of starvation on the A-B box region. TATA box region is the site of the PIC formation while A-B Box region contains the transcribed part and terminator of the gene. This result indicates that repression does not dislodge PIC from the TATA box but inhibits transcription initiation and elongation.

To confirm this, the effect of starvation on the occupancy of Brf1 was checked by a ChIP assay with 3XHA tagged Brf1. The results are shown in Figure 3.2C. As expected, Brf1 shows occupancy only on the TATA box region and its occupancy does not change significantly even after repression for 40 minutes. Based on the above observations and previous reports, a time of 1 hour period was chosen for establishing repression in all the experiments performed.

3.2.3. Conclusion

The results described above show that SNR6 gene is regulated by nutrient availability. It is repressed by Maf1 when the cells are starved. Maf1 does not disassemble the PIC formed on the TATA box region of SNR6 but prevents Pol III from moving down to the transcribed region of the gene (Figure 3.3). These results indicate that Maf1 binds to the PIC formed on the gene and renders it incompetent for transcription as depicted in Figure 3.3 and suggested even elsewhere (Roberts et al., 2006).

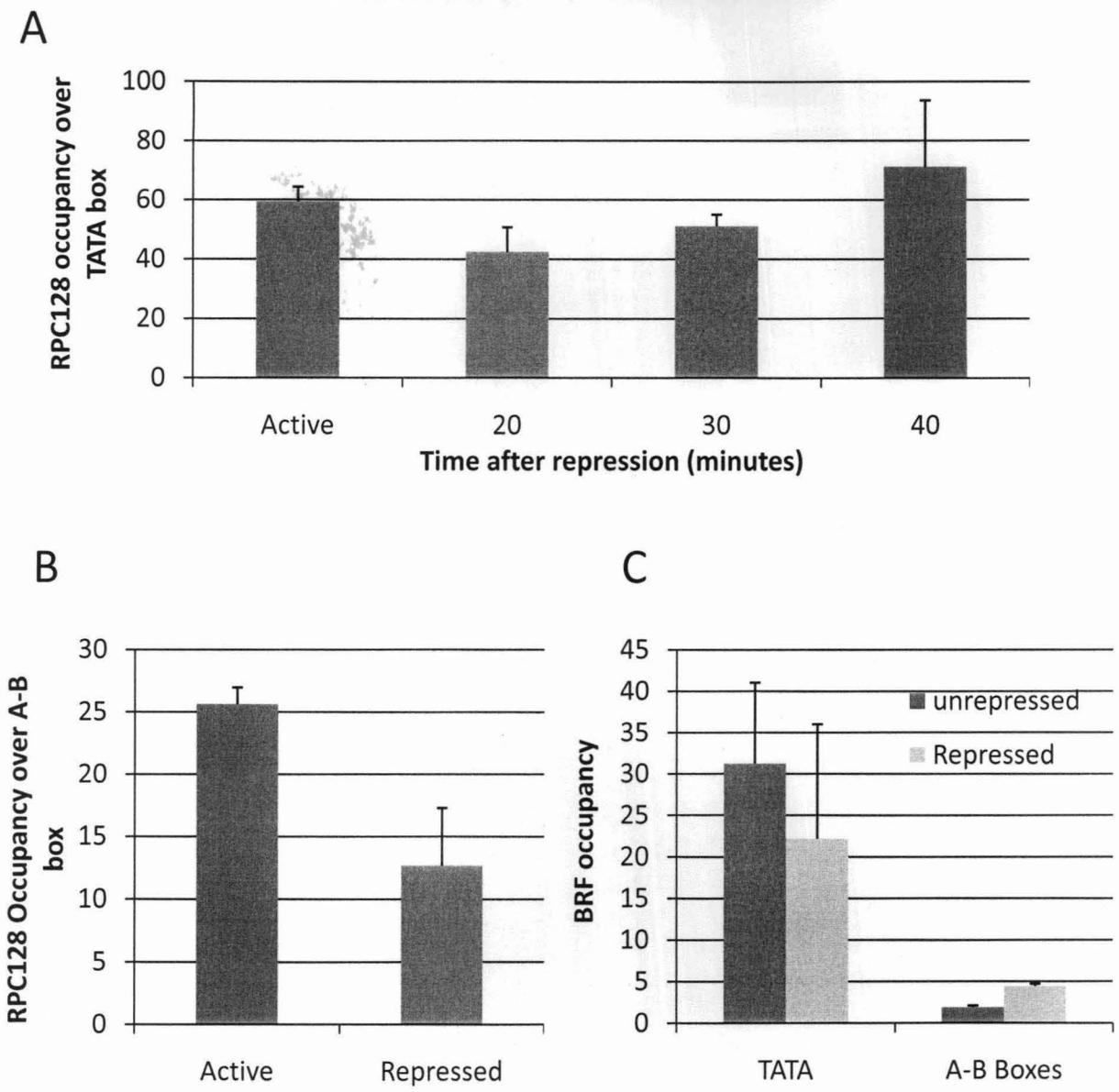


Figure 3.2: Pol III machinery is bound to SNR6 even under repressed conditions.

A. Time course analysis of effect of repression on the occupancy of RPC128. ChIP assay was done using a strain carrying an N terminal 4XFLAG tag on RPC128 at different time points of repression. The graph shows occupancies measured over TATA box region.

B. 4XFLAG-RPC128 occupancy over A-B Box region after 40 minutes of repression.

C. 3XHA-Brf occupancy over TATA box region as well as A-B Box region after 40 minutes of repression.

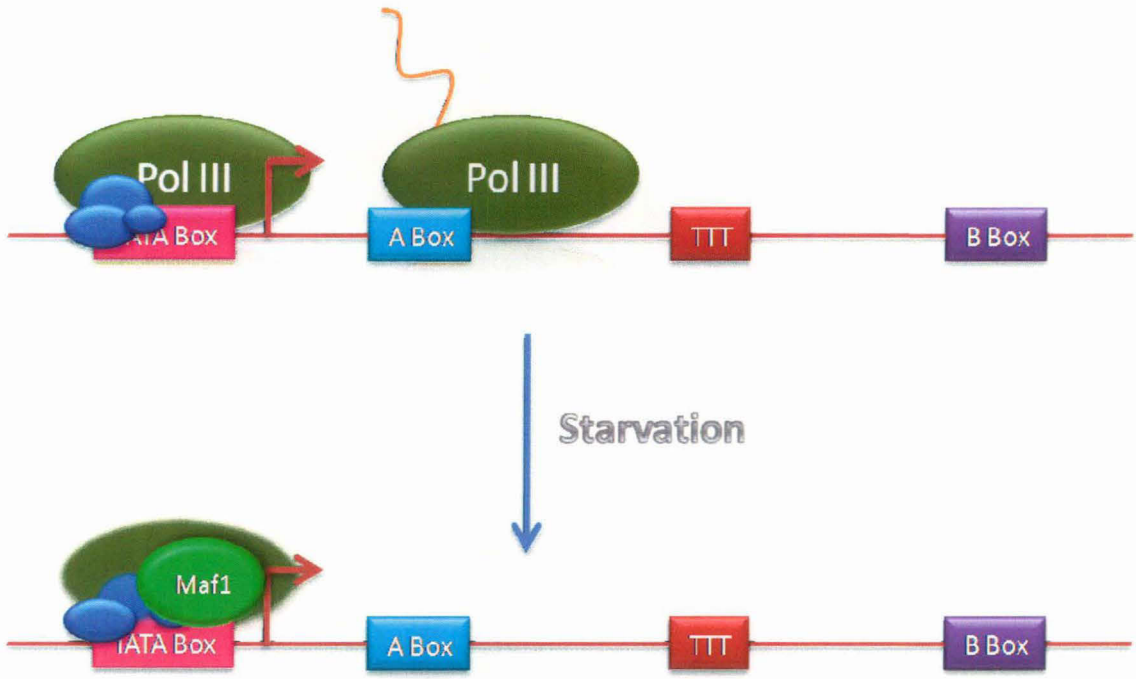


Figure 3.3: Pol III remains bound to the promoter under repression.

TFIIIC is avoided from the model for clarity but its occupancy is known to increase under repression indicating it remains bound to A and B boxes even under repression. The blue ovals are TFIIIB components. Maf1 binding to Pol III may bring in some conformational changes in the transcription complex, under starvation.

3.3. Chromatin remodeling by RSC enables active transcription of SNR6

Confirmation that SNR6 transcription is repressed by starvation made easier to address the question whether chromatin plays a role in this regulation. To find an answer, the in vivo chromatin structure of SNR6 under active as well as repressed conditions was resolved by indirect end labeling (IEL) as well as Chromatin immunoprecipitation methods.

3.3.1. Yeast SNR6 gene is nucleosomal in vivo

Though the in vitro studies show a nucleosome between A and B boxes (Shivaswamy et al., 2004), previous in vivo chromatin structure studies on SNR6 reported a subnucleosomal protection between A and B boxes (Gerlach et al., 1995; Marsolier et al., 1995). To resolve the controversy over the nature of the reported sub-nucleosomal size protection between the boxes A and B of SNR6 in vivo (section 1.9), the yeast strain UKY403 was used, in which histone H4 gene is under the control of Gal promoter such that H4 can be depleted when the cells are shifted from galactose to glucose for growth (Han and Grunstein., 1998). The indirect end labeling (IEL) technique was used to study the chromatin structure of SNR6 in normal and histone depletion conditions (Figure 3.4A). The advantage of coupling nucleosome depletion and IEL is that nucleosomal and non nucleosomal protections can be differentiated. Since IEL is a low-resolution technique, mappings can give an error of ~20 bps and the protections due to TFIIIB or TFIIIC binding to the boxes cannot be seen. Nevertheless, as compared to the naked DNA (lanes 1 and 2), we could see a protection of 190-200 bps size between the boxes A and B (the dark gray oval, lanes 3-4 and 7-8), flanked by two hyper sensitive sites, one close to A box (mapped to ~+28 bp) and one just upstream of B box (mapped to ~+224 bp). This protection persists in the control strain MHY308 which has H4 gene under control of its own promoter, when shifted to glucose (lanes 5-6). But in UKY403, the MNase cut between the boxes A and B on the naked DNA (black dot) reappears in chromatin lanes (lanes 9-11) under the histone depletion conditions, proving that the observed protection is nucleosomal. The region upstream of the A box, constituting start site and TATA box remains exposed to MNase digestion under all conditions,

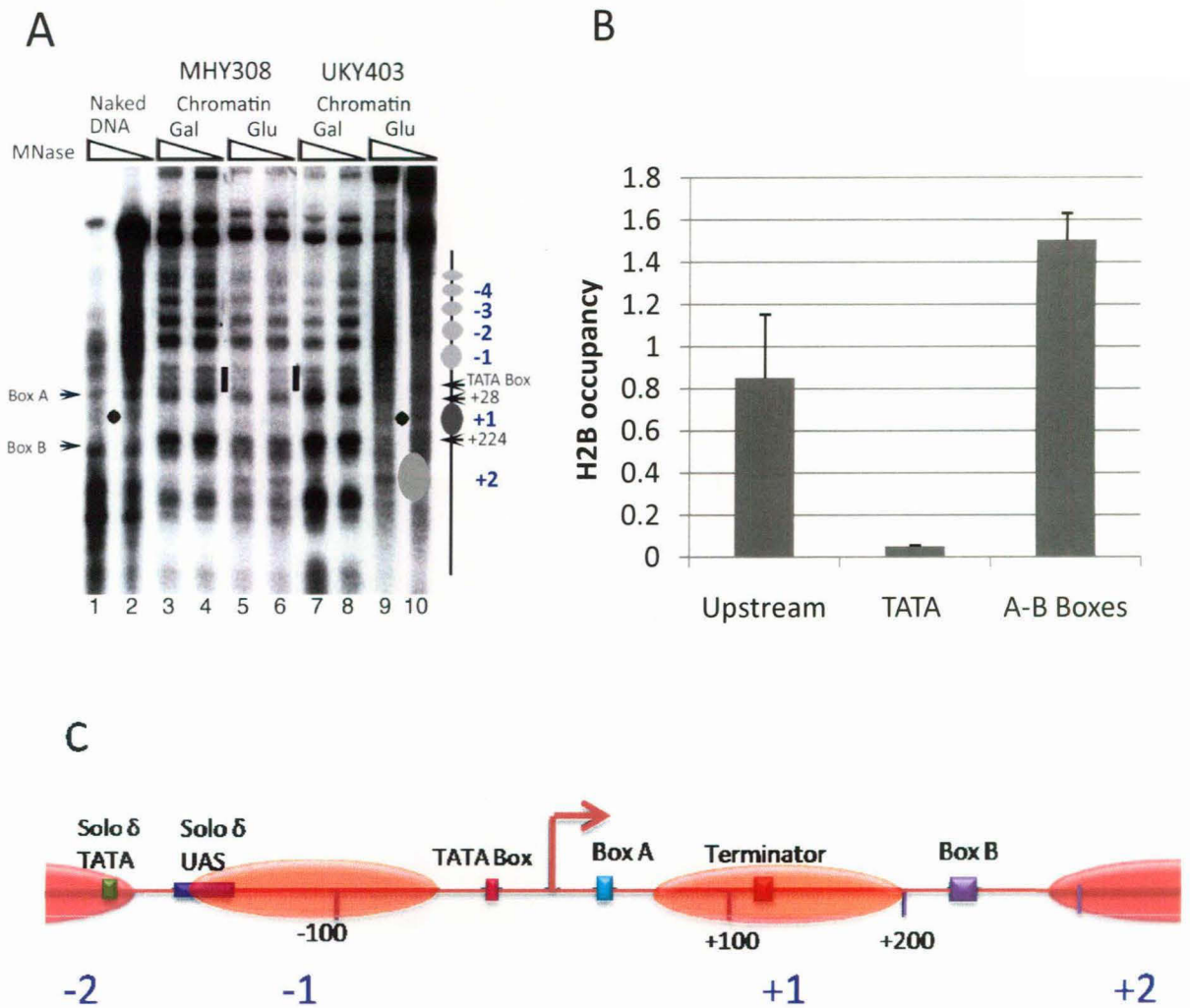


Figure 3.4: Yeast SNR6 is nucleosomal in vivo.

A. IEL analysis of the chromatin structure in strain UKY403 and its isogenic strain MHY308. The cells grown in YEP-galactose medium to an A600 of 0.7 were shifted to YEP medium containing glucose and harvested after 3 h. Gray ovals denote the nucleosomal size protections. The dot denotes the single MNase cut between boxes A and B in the naked DNA. The short bar marks the exposed region around the TATA box in the chromatin. Short arrows mark the Mnase cuts or promoter elements on the SNR6 gene. Nucleosomes are numbered in blue.

B. ChIP assay for FLAG-H2B occupancy on three different regions of SNR6. Upstream, TATA box and A-B Box regions represent the amplicons described in Figure 2.3.

C. Schematic representation of chromatin structure of SNR6 locus as found in this study.

indicating the whole stretch is devoid of nucleosomes (short bars). As compared to naked DNA digestion pattern in the lanes 1 and 2, the region upstream of the TATA box in both the strains grown in galactose shows an array of positioned nucleosomes (Figure 3.4A, array of gray ovals), starting from bp –70 upward, covering the solo δ element and numbered as -1 to -4 (Figure 3.4C). The protection due to upstream nucleosome (-1) spans till -240 bp position giving an ~170 bp size protection. However, owing to the low resolution (± 20bp), it may be less than that and part of the solo δ UAS (at -185 bp position) may fall in the linker region between nucleosomes -1 and -2, embedding the solo δ TATA box in the -2 nucleosome (Figure 3.4C). This mapping agrees with the previously reported chromatin structure of SNR6 where high-resolution mapping showed the spread of the nucleosome array starting from –56 bp upward (Marsolier et al., 1995).

A nucleosomal size protection (+2 nucleosome in Figure 3.4A and C) is seen downstream of B box also in MHY308 (Figure 3.4A; Lanes 3, 4) and UKY403 (Figure 3.4A; lanes 7 and 8) grown in galactose, reported as nucleosome, even elsewhere (Marsolier et al., 1995; Lopez et al., 2001). This protection was lost in UKY403 (Figure 3.4A; Lanes 5 and 6) as well as MHY308 (Figure 3.4A; Lanes 9 and 10) when transferred to glucose. Similar behavior of this nucleosome during growth at 37^0C was reported by Lopez et al (2001). It may be noted, however, that this nucleosome covers the 3' end of a downstream ORF and may be under control of that gene.

The IEL results in the panel A indicate a nucleosome free region of ~100 bp covering the start site and TATA box and flanked by two positioned nucleosomes; one between the boxes A and B on the 3'side and one upstream of TATA box. These results were confirmed by ChIP assay on the cells expressing FLAG-tagged histone H2B. As given in the Figure 3.4B, the region between the boxes A and B shows a high level of occupancy by H2B, proving unambiguously that the region between A and B box is nucleosomal. The H2B occupancy over region covering TATA box and start site was very low while that over the region upstream of TATA box was higher in accordance to the IEL result. Thus, our ChIP assays with MNase treatment could reproduce the IEL data quite well. Thus it is evident that the characteristic feature of SNR6 chromatin structure is a ~ 100 bp nucleosome free region (NFR) flanked by nucleosomes on either side, which resembles

that reported for Pol II transcribed genes. These results also suggest that Pol III probably transcribes the DNA wound over a nucleosome in SNR6.

3.3.2. Repression of SNR6 is associated with chromatin remodeling

Nucleosome positions regulate the accessibility of underlying DNA. Thus, it is possible that in repressed state of the gene, the NFR around TATA box becomes inaccessible. A time course study of H2B (FLAG-tagged) levels over TATA box region under repression, showed a steady increase with time; an approximately 10 fold increase after 40 minutes of repression (Figure 3.5A) indicating an encroachment of the TATA box region by nucleosome. Further characterization of the SNR6 chromatin structure under repression by the IEL method (Figure 3.5B) showed that the upstream nucleosome (gray oval, -70 to –240 bp) is lost and the TATA-A box region, which is exposed (short bar) in the active growth state (lane1), now shows a nucleosomal size protection (+28 to –123, dark gray oval, lanes 2-4) with a 5′ hypersensitive boundary under repressed condition. These observations suggest that the nucleosome which is present upstream of TATA box slides down towards the TATA box when the transcription is repressed, probably destabilizing the PIC. This sliding may lead to further exposure of the solo δ UAS but the nucleosome covering the solo δ TATA box (-2 nucleosome) remains unchanged.

No other change in nucleosomal array in further upstream region is visible. This observation suggests that response of the chromatin structure to repressive signals constitutes altering the position of only one nucleosome in the upstream region of the gene, out of a long array of positioned nucleosomes.

3.3.3. RSC maintains the NFR covering TATA box

The observation that a chromatin remodeling happens on SNR6 upstream region under transcription repression led to the search for the remodeler associated with it. The most probable candidate was RSC because of the reports that RSC is recruited to most of the Pol III transcribed genes (Ng et al., 2002a) and the more recent report on role of RSC in transcription of SNR6 itself (Soutourina et al., 2006). A deletion of 4 amino acids from the C-terminus of RSC4 subunit (RSC4-Δ4) of the RSC complex abolishes RSC interaction with Pol III and this mutant is defective in SNR6 transcription.

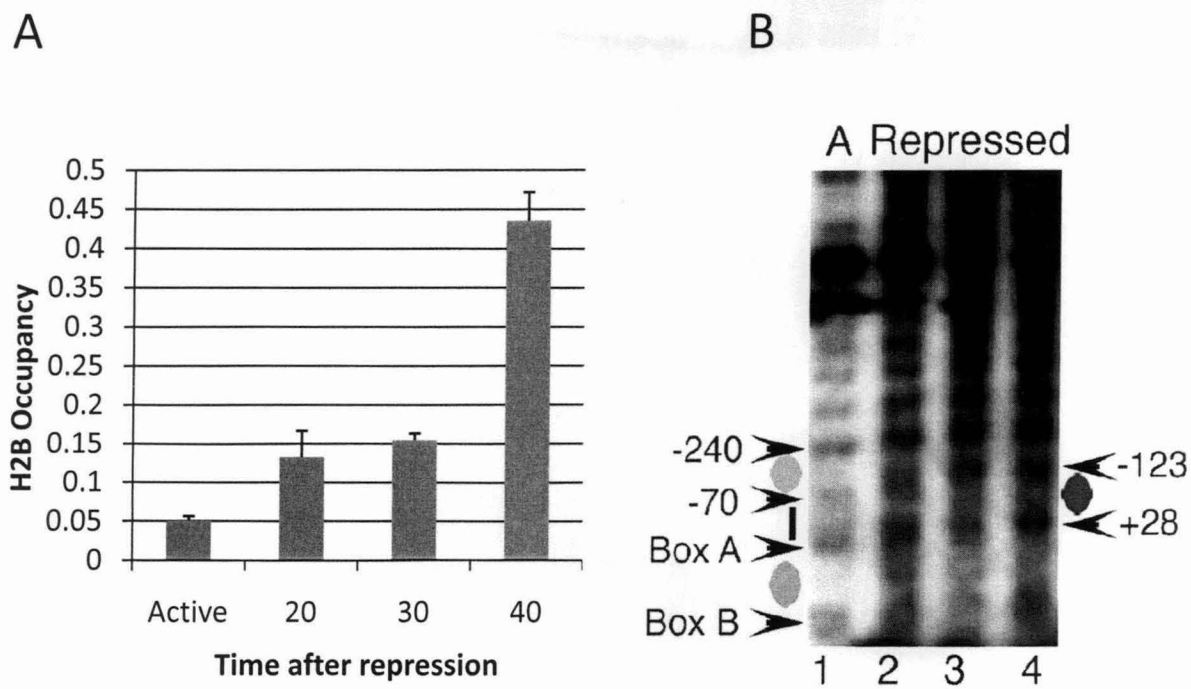


Figure 3.5: Repression of SNR6 is associated with chromatin remodeling

A. Progressive increase in occupancy of the FLAG-H2B over TATA-A box region under repression for 20, 30 and 40 minutes are shown.

B. In vivo IEL analysis of chromatin before (lane 1) and after shifting the cells to 0.15X YEP medium for 1 h (lanes 2 to 4). Gray ovals denote the position of a nucleosomal protection. Arrowheads with numbers indicate the positions of MNase cut sites.

Therefore, the chromatin structure of SNR6 locus in two RSC4-Δ4 mutant strains MW3993 and MW4019 was explored by in vivo IEL method. Strikingly, the entire region encompassing the TATA box and start site in both the mutant strains were found protected from MNase cleavage (Figure 3.6A). This region, exposed in the wild type strain (short bar) becomes nucleosomal in the mutants (Figure 3.6A, dark gray oval, lanes 3-6 vs. lanes 1-2). Similar to repression condition (Figure 3.5B), mapping of the cut sites measured the protection as ~150 bp, from the position +28 to –123 indicating the presence of a positioned nucleosome covering the TATA box and start site. The region upstream of –125 bp position encompasses a hypersensitive region of ~57 bp size (the asterisk, from -123 to –180 bp). Therefore, the ~60 bp region further upstream (from -180 to –240 bp) is probably non-nucleosomal. The nucleosome between the boxes A and B is retained and no other change is seen in the nucleosomal array further upstream of –240 bp.

This observation reveals the possibility that during activation in the wild type cells, RSC slides a positioned nucleosome from TATA box to the upstream region. To examine this, we checked the occupancy of RSC on SNR6 by ChIP assay for Myc-tagged RSC2 subunit of RSC. Figure 3.6B shows that RSC is present on the gene when the gene is active and its occupancy reduces when the gene is repressed. It is known that though the interaction between Pol III and RSC is lost in RSC4-Δ4 mutants, its recruitment to the target genes is unaffected. We checked the occupancy of RSC in RSC4-Δ4 mutants by ChIP assay for Myc tagged Sth1 subunit of RSC. Similar to the wild type RSC, the mutant RSC was also recruited to the gene under active conditions and its occupancy reduced under repression (Figure 3.6B).

3.4. Discussion

3.4.1. Pol III machinery does not leave the gene under repression

Maf1 is the central regulator of Pol III in yeast and is known to interact with Pol III and TFIIIB. Initial studies on Maf1 mediated repression suggested that Maf1 binds to Pol III and Brf1 and prevents their loading on to the genes. The decrease in the occupancy of

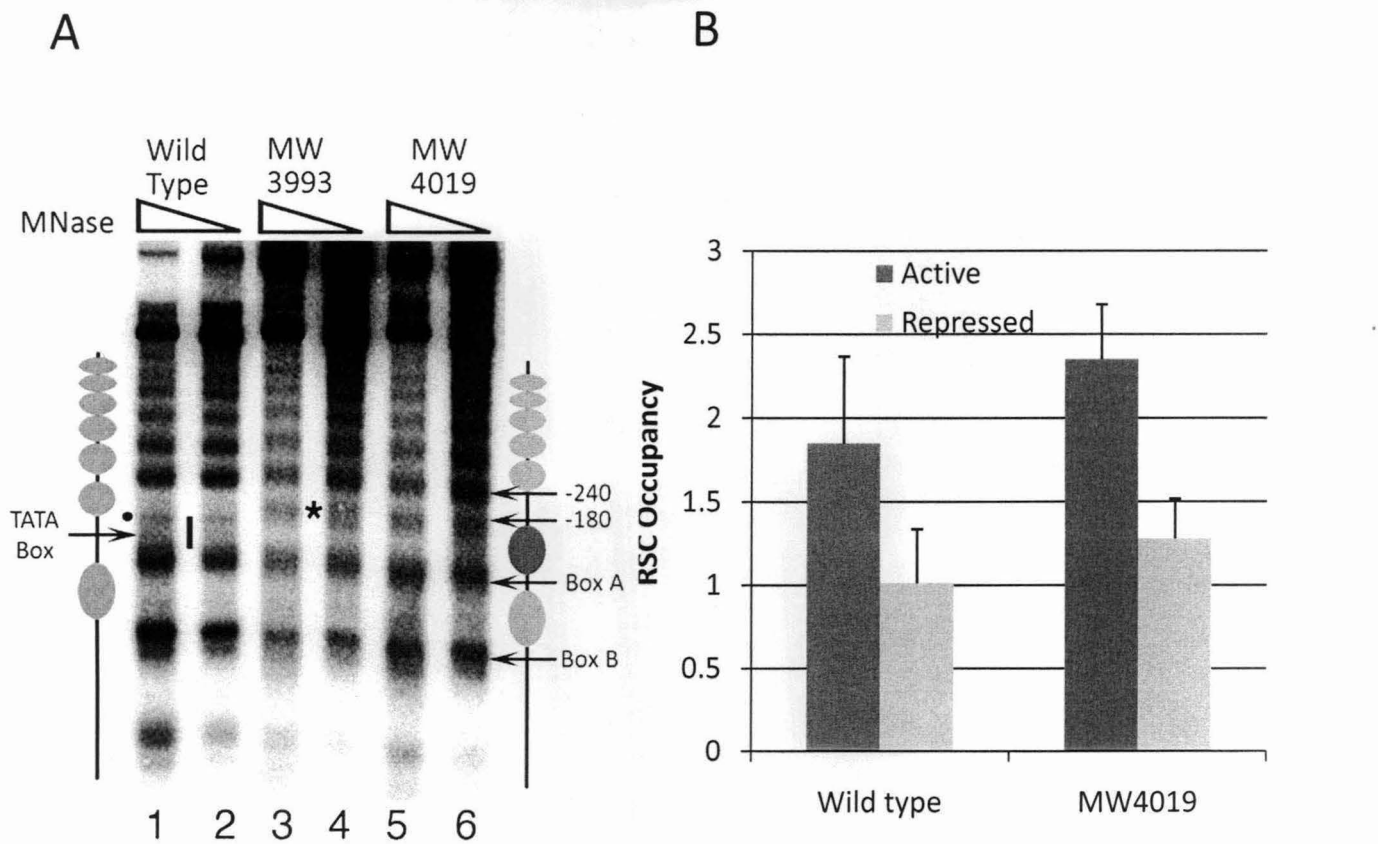


Figure 3.6: RSC maintains the NFR around the TATA box

A. IEL analysis shows the shift of an upstream nucleosome with an RSC4 mutation. The strain MW4019 differs from MW3993 in having the RSC subunit Sth1 as Myc tagged. Yeast cells were grown in YEP medium containing glucose to an A600 of 0.7. Gray ovals denote the nucleosomal size protections, and the bar marks the exposed region in lanes 1 and 2. The dot denotes a cut by MNase at the 70 bp position, and the asterisk denotes the new cut site spanning -123 bp to -180 bp position.

B. Effect of repression on the occupancy of the Myc-tagged RSC subunits, RSC2 (RSC4 wild type), and Sth1 (RSC4 mutant MW4019).

many Pol III subunits on tRNA genes under repression also supported this model. But, unlike Pol III subunits, TFIIIB subunits did not show a major decrease under repression and TFIIIC even showed an increase in the occupancy under repression. Later, the observation of the occupancy of Maf1 on the Pol III genes under repressive conditions suggested that it may even interact with the Polymerase bound to the gene and prevents transcription. Roberts et al (2006) studied the longest Pol III transcribed gene SCR1 (550 bp) in detail and have sown that all polymerase subunits do not behave in the same manner under repression. While occupancy of some subunits decreases, the others remain unchanged. These observations suggested two possible modes of actions for Maf1. It binds to Pol III and Brf1 that are not bound to DNA and prevents their recruitment to the target genes. It can also bind to TFIIIB and Pol III in a PIC and prevent them from doing transcription without making them fall off from the promoter DNA.

Results presented in this chapter provide an additional evidence for the second mode of action of Maf1. The occupancy of 3XHA-RPC160 reduced by 40 minutes of repression on both TATA box region as well as A-B Box region while that of FLAG-RPC128 reduced only over A-B Box, leaving the occupancy over TATA box unchanged. This indicates that Pol III remains bound to the U6 promoter even under repressed condition but stops transcribing the gene. Brf1 occupancy profile also adds weight to this hypothesis (Figure 3.2C). Roberts et al (2006) suggests that Pol III may be in an altered conformation due to Maf1 binding and that may be the reason for reduced occupancy of some of the subunits. Occupancy profile of RPC160 has shown a loss of this subunit of Pol III from all the Pol III transcribed genes under repression (Harismendy et al., 2003; Oficjalska-Pham et al., 2006). Maf1 interacts with the N terminus of RPC160 (Oficjalska-Pham et al., 2006) and the HA epitope tag in these as well as our study is at the N-terminus of RPC160. Therefore, a probable reason for the decreased occupancy of RPC160 under repression may be that the epitope tag at N terminus of RPC160 is masked by Maf1, making it unavailable for ChIP. A probable model for Maf1 action is given in Figure 3.3

3.4.2. DNA between A and B boxes is compacted by a nucleosome in vivo

A non-histone protein like Nhp6 and not a nucleosome was suggested to occupy the DNA between A and B boxes of SNR6 (Gerlach et al., 1995; Kruppa et al., 2001). However, Nhp6 deletion does not show any change in MNase digestion pattern between A and B boxes (Lopez et al., 2001) and recent reports suggest that the major role for Nhp6 in Pol III transcription may be in stabilization of the TFIIIB-DNA complex (Braglia et al., 2007). Our study has provided a strong evidence for the presence of a nucleosome between the boxes A and B of yeast SNR6 in vivo. The study has also revealed the possibility that Pol III transcribes the DNA wound over a nucleosome in SNR6. According to our results, approximately one turn of the nucleosomal DNA (5′ half) is constituted by transcribed region of the gene. Therefore, the read-through of the nucleosome by Pol III during active transcription probably generates an altered structure in its 5′ half generating only a subnucleosomal size protection towards the 3'half of the DNA between A and B boxes, as reported by others (Gerlach et al., 1995). The loss of H2A-H2B dimer or an altered nucleosome structure during active transcription, both can lead to a more exposed nucleosomal DNA. Possibility of a role of FACT complex or other factors involved in exchange of the H2A-H2B dimer during transcription, as in case of Pol II transcribed genes, cannot be excluded at this stage.

3.4.3. Positioned nucleosome in the upstream region of the U6 snRNA genes

In vertebrates, U6 promoter is completely upstream and does not have any B box element. Role of a positioned nucleosome in upstream region of the human U6 gene transcription is well documented (Stunkel et al., 1997; Zhao et al., 2001). The positioned nucleosome on the human U6 brings together two regulatory elements PSE and DSE located upstream at bp positions –70 and –220 respectively (Zhao et al., 2001) while in case of yeast, the positioned nucleosome brings the boxes A and B together in the region downstream of start site (Shivaswamy et al., 2004). Yeast has a PSE like element at –48 to –59 bp position, although it is dispensable for transcription (Eschenlauer et al., 1993). Interestingly, we now find that similar to the human U6, a nucleosome is positioned upstream of the bp –60 to –70 on yeast U6 gene. These observations

indicate that though the promoter structure of yeast and human U6 are entirely different, they have probably evolved through similar chromatin regulatory mechanisms.

3.4.4. RSC maintains the NFR upstream of SNR6

From the time of the discovery that TFIIIC relieves chromatin mediated repression of SNR6 (Burnol et al., 1993a), it was evident that chromatin can block access of TFIIIB to TATA box and TFIIIC is required for placing TFIIIB on the gene (Gerlach et al., 1995). Further studies have shown that TFIIIC mediated chromatin remodeling is required for active transcription of U6 on chromatin templates as well as to produce a chromatin structure on SNR6 which is similar to that in vivo (Shivaswamy et al., 2004; Shivaswamy and Bhargava, 2006). The results presented in this chapter as well as elsewhere (Marsolier et al., 1995) show that the region covering TATA box is nucleosome free, with an array of nucleosome staring from -70 bp upwards. We also show the presence of a positioned nucleosome between the boxes A and B, immediate downstream of the NFR. This chromatin structure resembles that reported for a typical Pol II transcribed gene, which also shows an NFR upstream of the transcription initiation site, flanked on either side by well positioned nucleosomes (Rando and Chang, 2009). Our results show that when RSC is mutated, this NFR containing TATA box is covered by a nucleosome and the transcription is reduced. This suggests that RSC is responsible for maintaining the NFR upstream of SNR6. A very recent genome wide report in yeast shows that the Pol II NFR is also largely maintained by RSC (Hartley and Madhani, 2009). A model for the chromatin structure of SNR6 gene and its maintenance by RSC is given in Figure 3.7.

3.4.5. Chromatin structure upstream and downstream of SNR6

SNR6 is known to organize the chromatin structure of the locus in a TFIIIC dependent way (Marsolier et al., 1995). The gene upstream region contains a solo δ element of Ty1 origin and the results given in this chapter show an array of nucleosomes covering the solo δ. Ty1 and Ty3 retrotransposons are known to target the upstream regions of Pol III transcribed regions (Devine and Boeke., 1996; Chalker and Sandmeyer., 1993) but upstream presence of Ty element has no effect on Pol III transcription though the reverse may not be true. Some tRNA genes have a modest effect on Ty1 transcription (Bolton and Boeke., 2003). The results present in this study show that repression of Pol

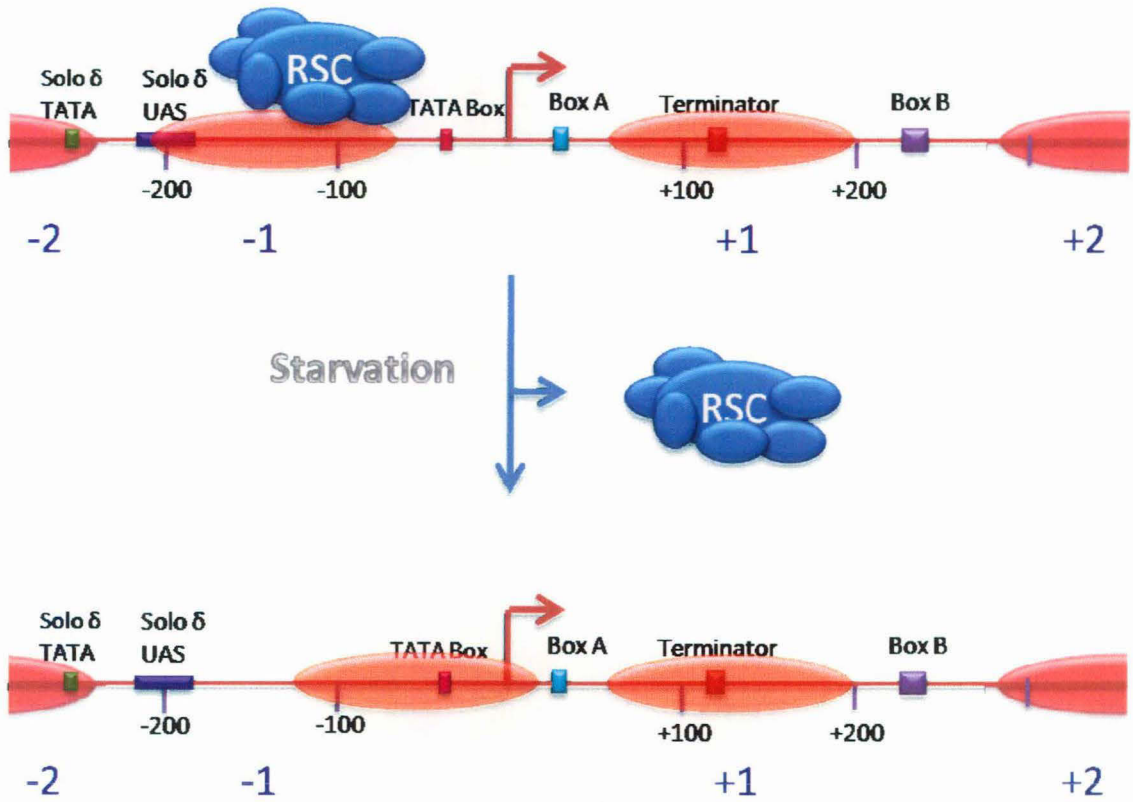


Figure 3.7: A model for chromatin remodeling on SNR6 under repression

When the transcription is repressed, no change in the position of the nucleosome between A and B boxes was observed while the nucleosome upstream of TATA box slides towards TATA Box. Chromatin remodeling complex RSC is required to keep the TATA box region nucleosome free under active conditions as loss of RSC under repression leads to a nucleosome covering the TATA box. Nucleosome sliding due to repression exposes the UAS of solo δ element while it covers TATA box and initiation site of SNR6.

III transcription leads to exposure of the solo δ UAS. This may be significant as Ty1 retrotransposons are activated by starvation (Boeke and Sandmeyer, 1991). Chromatin dynamics may be underlying such an inverse relation between Pol III transcription and Ty1 expression. The nucleosome downstream of box B overlaps the 3' end of a downstream ORF and its dynamics may therefore be related to the regulation of that gene rather than SNR6. Presence of a positioned nucleosome on the 3' end of ORFs is already known by genome wide studies (Lee et al., 2007; Shivaswamy et al., 2008) on Pol II transcribed genes.

Chapter 4

Association of Histone Variant H2A.Z with SNR6

4.1 Overview

Results in the previous chapter have shown that the active state chromatin of SNR6 is characterized by a nucleosome free region (NFR) encompassing TATA box and TSS, flanked by positioned nucleosomes on either side. It was also shown that this NFR is maintained by the chromatin remodeling complex RSC. This type of nucleosomal organization on SNR6 with two well positioned nucleosomes flanking an NFR is similar to that reported on pol II-transcribed genes. NFRs on Pol II genes are also close to TSS, flanked by two well positioned nucleosomes and depend on gene activity (Lee et al., 2007; Schones et al., 2008; Ozsolak et al., 2007). In yeast, the flanking nucleosomes of NFR contain the histone variant H2A.Z and a genome wide study on the occupancy of H2A.Z has shown presence of a H2A.Z containing nucleosome upstream of SNR6 (Raisner et al., 2005; Albert et al., 2007).

Other than the centromeric variant of H3, H2A.Z is the only histone variant present in budding yeast which is highly conserved from yeast to humans (Iouzalen et al., 1996). Though H2A.Z is an essential gene in vertebrates, yeast cells with deletion of H2A.Z gene Htz1 are viable (Dhillon and Kamakaka, 2000; Jackson and Gorovsky, 2000). Functions of H2A.Z are best studied in yeast and have been shown to span a wide range of often contradictory functions. Functions of H2A.Z include prevention of the spread of heterochromatin (Meneghini et al., 2003), gene activation in conjunction with chromatin remodeling (Santisteban et al., 2000), marking an inactive gene for rapid activation by localizing it to the nuclear periphery (Brickner et al., 2007; Gligoris et al., 2007), repression of many euchromatic genes (Meneghini et al., 2003), genomic stability and DNA repair (Krogan et al., 2004; Mizuguchi et al., 2004), chromosome transmission (Keogh et al., 2006) etc. The observations that H2A.Z containing nucleosomes show an increased thermal mobility (Flaus et al., 2004) and have ability to alter the nucleosome positioning (Fan et al., 2002) were of particular interests for this study because of the chromatin remodeling seen on the upstream nucleosome.

H2A.Z-H2B dimers are deposited on the nucleosomes by the ATP dependent chromatin remodeler SWR1 of INO80 family of remodelers (Kobor et al., 2004; Krogan et al., 2003b; Mizuguchi et al., 2004). The histone chaperone Chz1 is specific for H2A.Z-H2B

dimer and helps in the assembly process (Luk et al., 2007). H2A-H2B chaperone Nap1 is also known to play a role in deposition of Htz1 (Park et al., 2005; Mizuguchi et al,. 2004). Presence of H2A.Z is correlated with various histone acetylation marks and it is known that certain histone acetylations are required for deposition of H2A.Z on specific genomic loci (Raisner et al., 2005; Zhang et al., 2005; Shia et al., 2006).

To find whether H2A.Z has any role at SNR6 locus, the occupancy of H2A.Z on SNR6 was confirmed by ChIP assay before following the dynamics of H2A.Z as well as canonical H2A during the transcription repression. The role of H2A.Z in nucleosome positioning and chromatin remodeling was checked by in vivo indirect end labeling (IEL). RNA level of SNR6 in htz1Δ strain was estimated and compared with that of wild type to understand the role of H2A.Z in transcription.

4.2. Dynamics of H2A and H2B differ in the upstream region of SNR6

Histone H2B occupancy was checked on all three regions of SNR6 (Upstream, TATA box and A-B Box) by ChIP assay against FLAG tagged H2B. In contrast to increase in H2B levels over the TATA box region after 40 minutes of repression, no difference is seen in the H2B levels over the upstream region while the level goes up between A and B boxes by 1.8 fold (Figure 4.1A). Similarly, ChIP assays using a strain carrying FLAG tag on both the H2A genes (Figure 4.1B) shows an increase of ~2 fold over A-B boxes and ~10 fold over TATA-A box region, comparable to the increase in H2B levels under repression. This data supports the previous observation that the upstream nucleosome slides to TATA box region under repression (Section 3.3.2; Figure 3.5). Surprisingly, unlike H2B, H2A occupancy for the upstream nucleosome increased 3 fold upon repression. (cf. Figures. 4.1A and B). However, there is a significant overlap between the upstream and TATA box primer positions as well as two alternative positions of the upstream nucleosome, which may be the reason for continued signal of histones from the upstream region under repression.

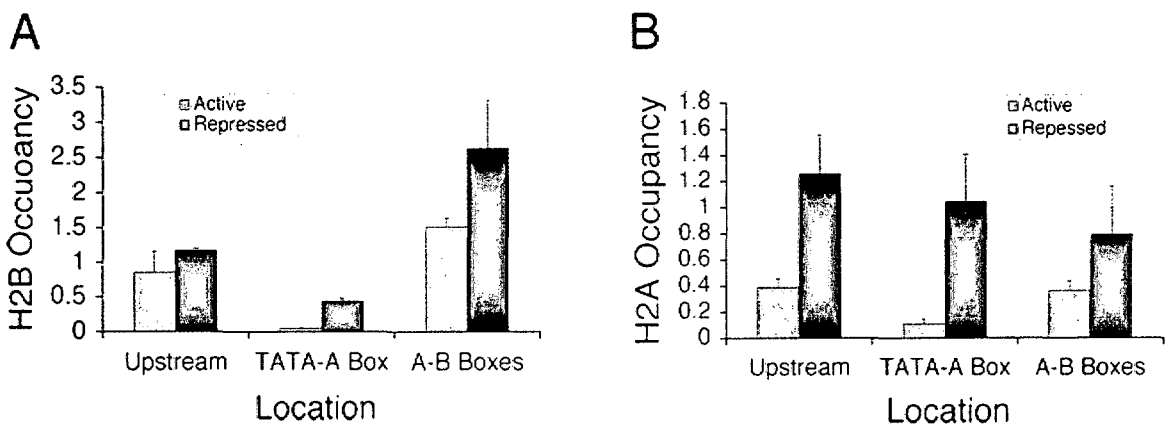


Figure 4.1: H2B and H2A show different dynamics under repression

A. Occupancy of Histone H2B under active condition and after repression for 40 minutes on all three regions of SNR6.

B. Occupancy of H2A under active as well as repressed conditions on three regions of SNR6. Repression was for 40 minutes.

4.3. The upstream nucleosome contains H2A.Z under active conditions

The difference in the dynamics of H2A and H2B only in the upstream nucleosome suggested that the upstream nucleosome may be containing variant histone H2A.Z under active condition which is replaced by canonical H2A when the transcription is repressed. To check this hypothesis, ChIP assay was conducted against flag tagged H2A.Z. As evident from Figure 4.2, the upstream nucleosome contains the histone variant H2A.Z under active condition but its level decreases after repression for one hour. This shows that histone variant H2A.Z is replaced with canonical H2A on U6 under repression.

4.4. Swr1 deposits H2A.Z on the upstream nucleosome

Above results suggest that the histone H2A in the upstream nucleosome is replaced by the variant H2A.Z under the active state. H2A.Z is known to be deposited on to the targets by the chromatin remodeling complex SWR1 of INO80 family. A ChIP assay on SNR6 against TAP-tagged Swr1 subunit of SWR1 complex showed ~2 fold higher Swr1 occupancy over the TATA box region than the upstream nucleosome region (Figure 4.3A), suggesting that the cross-linking of Swr1 to the upstream region of the TATA box is better in the overlapping region of the two amplicons. As expected, the occupancy was low over the A-B boxes region. No significant difference in Swr1 occupancy upon repression is seen anywhere (Figure 4.3A).

To confirm Swr1 mediated H2A.Z deposition, occupancy of TAP tagged H2A.Z on SNR6 in swr1Δ background was checked and compared with H2A.Z-TAP occupancy in wild type cells. As compared to the wild type, Htz1-TAP occupancy in swr1Δ background reduces in the upstream nucleosome (Figure 4.3B), confirming that the SWR1 complex is responsible for the Htz1 deposition near SNR6. These results show that active transcription of SNR6 marks the region upstream of TATA box with a positioned nucleosome containing Htz1 in Swr1-dependent manner.

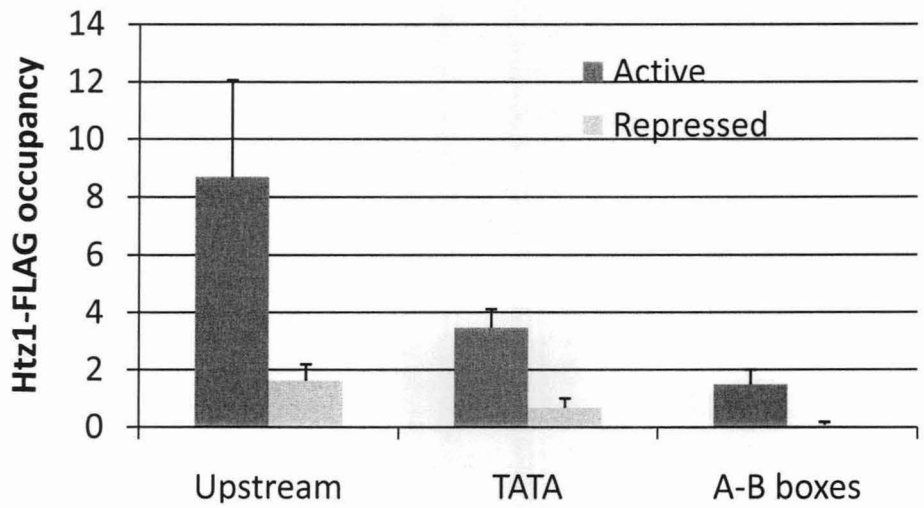


Figure 4.2: Upstream nucleosome of SNR6 contains histone variant H2A.Z

Chromatin immuno precipitation was done against FLAG tagged Htz1 before and after repression for 1 hour.

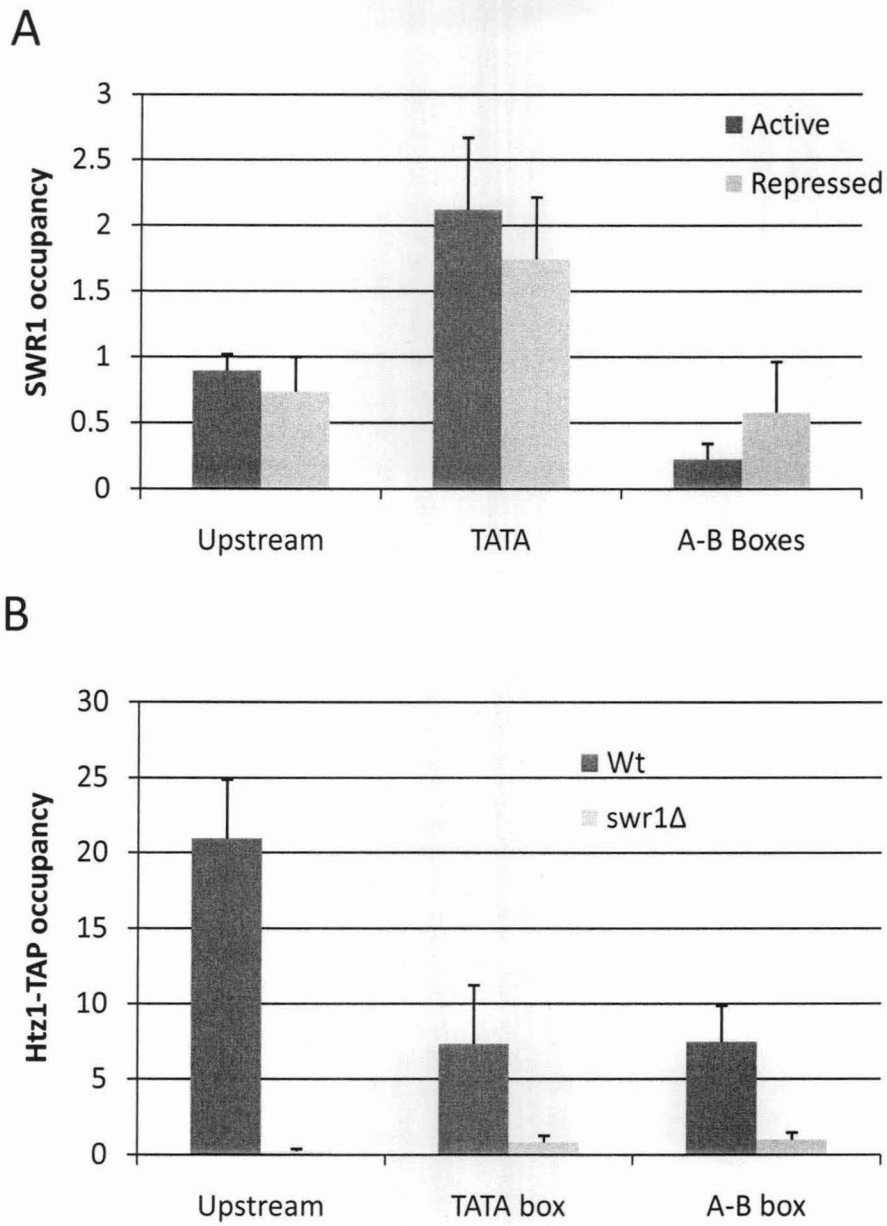


Figure 4.3: Swr1 deposits Htz1 on the upstream nucleosome

A. Occupancy of Swr1 on SNR6. ChIP assay was done against TAP tagged Swr1 in wild type cells. Cells were repressed for one hour, cross linked for 2 hours and lysed by vortexing with glass beads. Cox3 was used as control.

B. Occupancy of Htz1-TAP on SNR6 in wild type and swr1Δ background. Cells were repressed for one hour, crosslinked for 15 minutes and cell lysis was done by vortexing with glass beads. Cox3 was used as control.

4.5. Chromatin structure and transcription of SNR6 are independent of H2A.Z deposition

The activity dependent occupancy of H2A.Z over SNR6 suggested a role for H2A.Z in SNR6 transcription. To know the significance of the presence and dynamics of Htz1 in the SNR6 transcription, the transcript levels in both wild type and htz1Δ cells were checked at different time points of repression up to 4 hours (Figure 4.4A). As expected, the representative gel shows no decrease in U6 RNA level under repression due to its high stability. But comparison of RNA from wild type with htz1Δ cells showed a slight increase in the RNA level of U6 while that of U4 remained the same. The results of the quantification of U6 relative to U4 RNA levels given in Figure 4.4B also show that H2A.Z does not have any positive effect on SNR6 transcription; rather a slight increase in the U6 transcript levels in htz1Δ cells can be seen. Presence of H2A.Z in the active state suggested that it may have positive effect on the transcription of SNR6. But instead of reduced U6 RNA levels in htz1Δ cells, a slight increase was observed. Though the increase was nominal, it persisted all throughout the time course of repression (Figure 4.4A and B) indicating this was a true increase. This may be significant because the high stability of U6 RNA may interfere with detection of reduced transcription but not with that of an increased transcription. Therefore, the Htz1 deposition could be a consequence and not requirement for active transcription by Pol III, suggesting the chromatin structure in the absence of Htz1 may be similar to that in the active state and role of H2A.Z may be limited to keeping U6 levels under check.

Alternatively, it is possible that the H2A.Z dynamics is due to changes in the transcription of solo δ element found in immediate upstream region of SNR6. The expression of solo δ was examined in wild type and htz1Δ cells in both active and repressed conditions. A basal transcription of solo δ was seen irrespective of starvation or Htz1 deletion (Figure 4.4A). This result along with the previous observation that TFIIIC maintains the chromatin structure of the locus (Marsolier et al., 1995) rules out any Pol II transcription mediated changes in the chromatin structure in vicinity of SNR6.

The in vivo chromatin structure of the *htz1Δ* cells in nutrient rich and starvation condition was compared with that of wild type cells by the IEL method (Figure 4.4C).

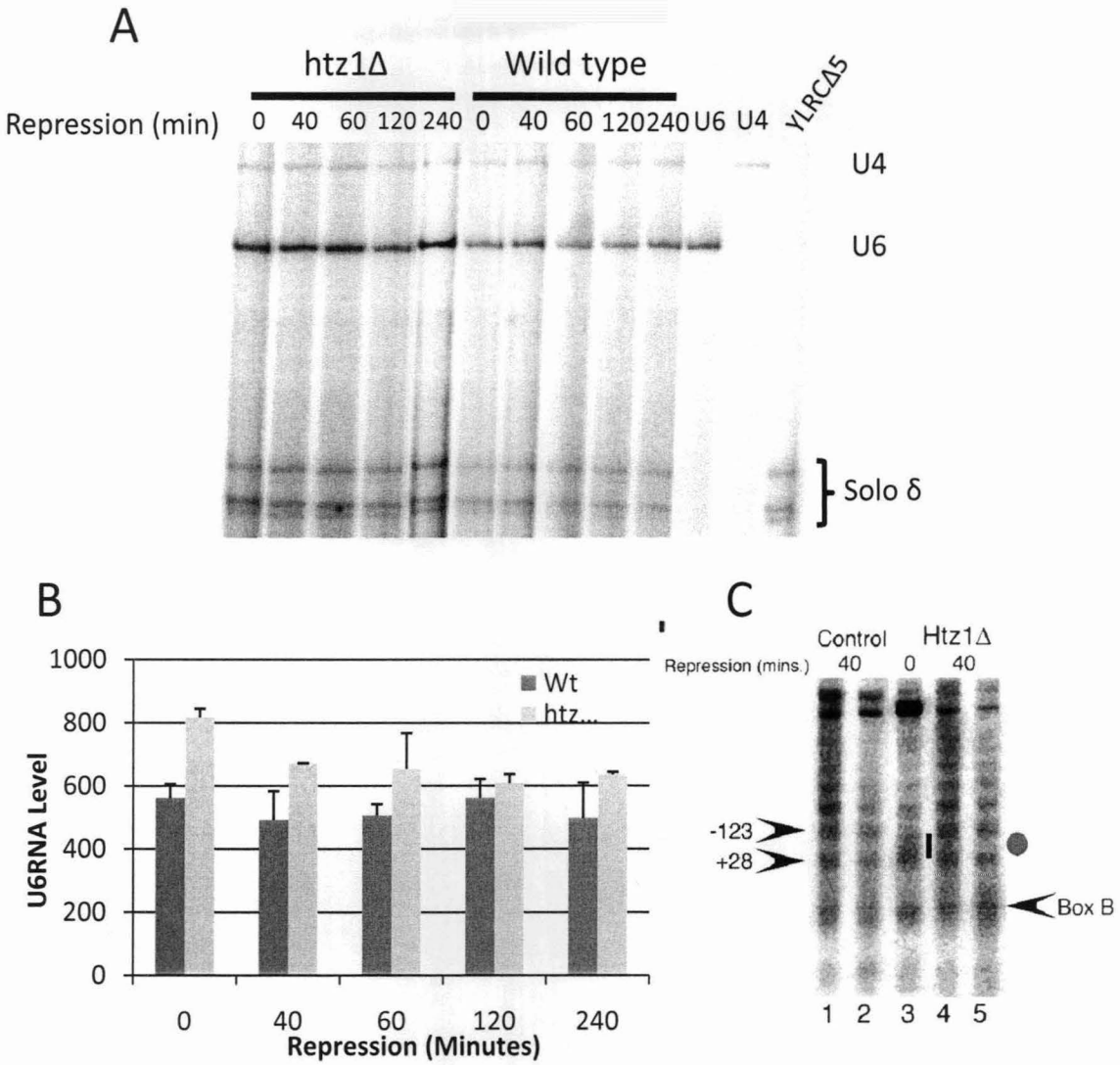


Figure 4.4: Chromatin structure and transcription of SNR6 is independent of H2A.Z

A. Expression of SNR6 and solo δ under repression as well as in htz1Δ background. Total RNA was isolated and subjected to reverse transcription reaction with ^{32}P labeled primers specific for U4 RNA, U6 RNA and solo δ transcript. A representative gel is shown.

B. Quantification of RNA levels in the panel A for U6. Values are expressed against U4. Average from three independent experiments with scatter is given.

C. Chromatin structure of SNR6 is unaffected by H2A.Z. Active and repressed chromatin structure for htz1Δ cells (lanes 3-5) are compared with repressed structure for wild type cells (control; lanes 1&2). Arrow marks on the left indicate the cut sites in repressed chromatin and the arrow mark on right side indicates the B box. Gray oval shows the nucleosome covering TATA box when repressed. Black bar indicates the exposed TATA box region under active condition.

Chromatin structure in both types of cells was indistinguishable under repressed condition, showing the presence of a nucleosome covering the TATA box (gray oval, lanes 4 and 5 as compared to lanes 1 and 2). The TATA box region remains exposed (short bar, lane 3) even in the absence of H2A.Z under active condition, similar to that of wild type indicating H2A.Z does not play a role in the maintenance of chromatin structure of the gene. There was no change visible even in the upstream or downstream regions under different conditions.

4.6. Sas2 acetylates H4K16 under active conditions

H4K16 acetylation and H2A.Z act synergistically to prevent the spreading of telomeric heterochromatin (Shia et al., 2006). Therefore, the H4K16 acetylation level was measured in the upstream region of SNR6. ChIP assay with antibodies specific for acetylated H4K16 showed that in comparison to the A-B boxes region, the upstream nucleosome is enriched with acetylation at H4K16 and similar to H2A.Z, this modification is lost under repressive conditions (Figure 4.5A), suggesting it may be closely related to association of Htz1 with the upstream nucleosome in the active state of the gene. Acetylation of H4K16 in yeast is carried out by Sas2 (part of SAS complex) or by Esa1 (Part of NuA4 complex) (Sutton et al., 2003; Shia et al., 2005). To identify the HAT responsible for acetylating this residue, ChIP assay was done against H4K16Ac specific antibody in sas2Δ cells. In contrast to the wild type cells, no enrichment of this modification was found in the upstream nucleosome, indicating the SAS complex is responsible for this acetylation (Figure 4.5A).

To confirm that SAS is recruited to SNR6, ChIP assay was performed for two subunits of SAS complex using the strains harboring Sas2-TAP or Sas4-myc. As compared to the region between A and B boxes, the results show a clear enrichment of Sas2 (Figure 4.5B) as well as Sas4 (Figure 4.5C) over both the TATA box and the upstream regions. Similar to H4K16Ac, SAS occupancy over both these regions also reduced under repressed conditions (Figure 4.5B and C). Results suggest that in the active state, the upstream nucleosome is marked with H4K16 acetylation by the SAS complex.

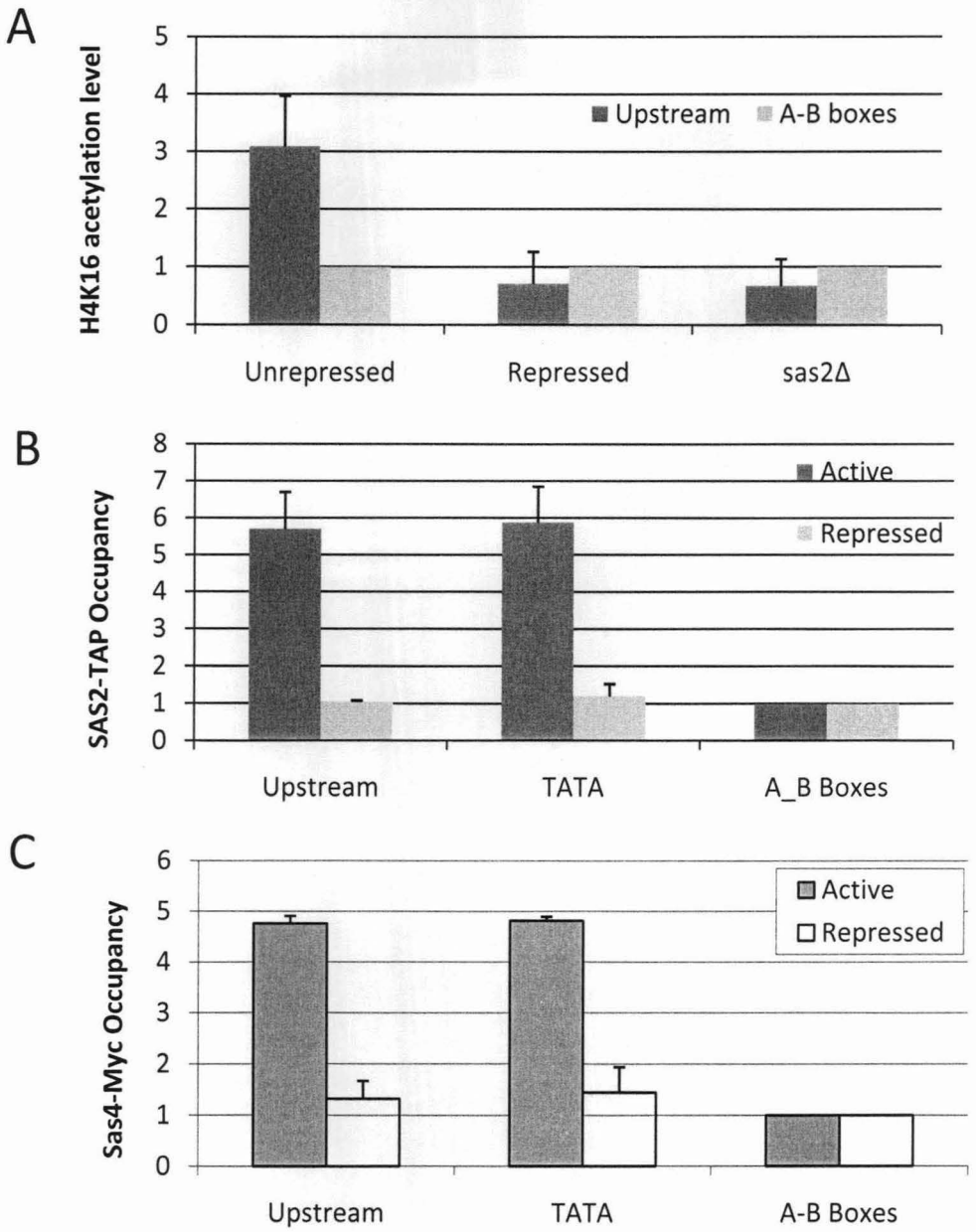


Figure 4.5: Sas2 acetylates H4K16 under active conditions

A. H4K16 acetylation level on SNR6. ChIP assay was done with acetyl H4K16 specific antibody. Since the telomeric region also has H4K16 acetylation, its levels are expressed against the A-B box nucleosome which did not have this acetylation.

B. Occupancy of TAP tagged Sas2 on SNR6 gene. Lysates were prepared by vortexing with glass beads, from cells harboring a TAP tagged Sas2 and a Myc tagged Sas4, after 2 hour crosslinking. DNA was sheared by sonication. Repression was for 1 hour. Values are expressed against the A-B Box nucleosome.

C. Occupancy of Myc-tagged Sas4. ChIP was done with anti-Myc antibodies using the same extract used for the panel B.

4.7. H2A.Z deposition happens prior to H4K16 acetylation

Acetylation of H4K16 is required for subtelomeric incorporation of Htz1 and this correlation is found only in the subtelomeric regions (Shia et al., 2006). The observation that both H2A.Z and H4K16Ac are present on the upstream nucleosome of SNR6 suggested a similar possibility on SNR6. Loss of H4K16 acetylation from the upstream nucleosome in *sas2Δ* strain (Figure 4.5A), provided a good opportunity to study the correlation between H2A.Z and H4K16Ac. Htz1-TAP occupancy in *sas2Δ* background did not show a visible decrease in the occupancy of H2A.Z on the gene. On all three regions examined, the values were similar to that of wild type (Figure 4.6A), showing H4K16 acetylation by SAS complex is not required for H2A.Z deposition on SNR6 gene.

This observation raised the question, what happens to H4K16 acetylation in the absence of H2A.Z. Whether the acetylation happens downstream to H2A.Z deposition? ChIP assays against acetyl H4K16 in htz1Δ cells showed that H4K16 acetylation goes down to the repressed levels even under nutrient rich conditions (Figure 4.6B). These results show that H4K16 acetylation is a downstream event to H2A.Z deposition under active conditions.

4.8. SNR6 transcription is unaffected by H4K16Ac

The findings that H2A.Z did not have a positive effect on SNR6 transcription and H4K16 acetylation happens after H2A.Z deposition, led to the assumption that H4K16 acetylation by SAS also may not have a positive role in SNR6 transcription. To examine this, the U6 RNA level was quantified from wild type as well as sas2Δ strains by primer extension method. The results given in Figure 4.7 show that similar to htz1Δ strains (Figure 4.4A and B), sas2Δ strain also shows a slightly increased U6 RNA level under active condition (Figure 4.7). And as expected, repression did not bring in any change in the RNA levels. These observations suggested that H2A.Z and H4K16 act in the same pathway and they do not have a positive role in SNR6 transcription, if not a negative role.

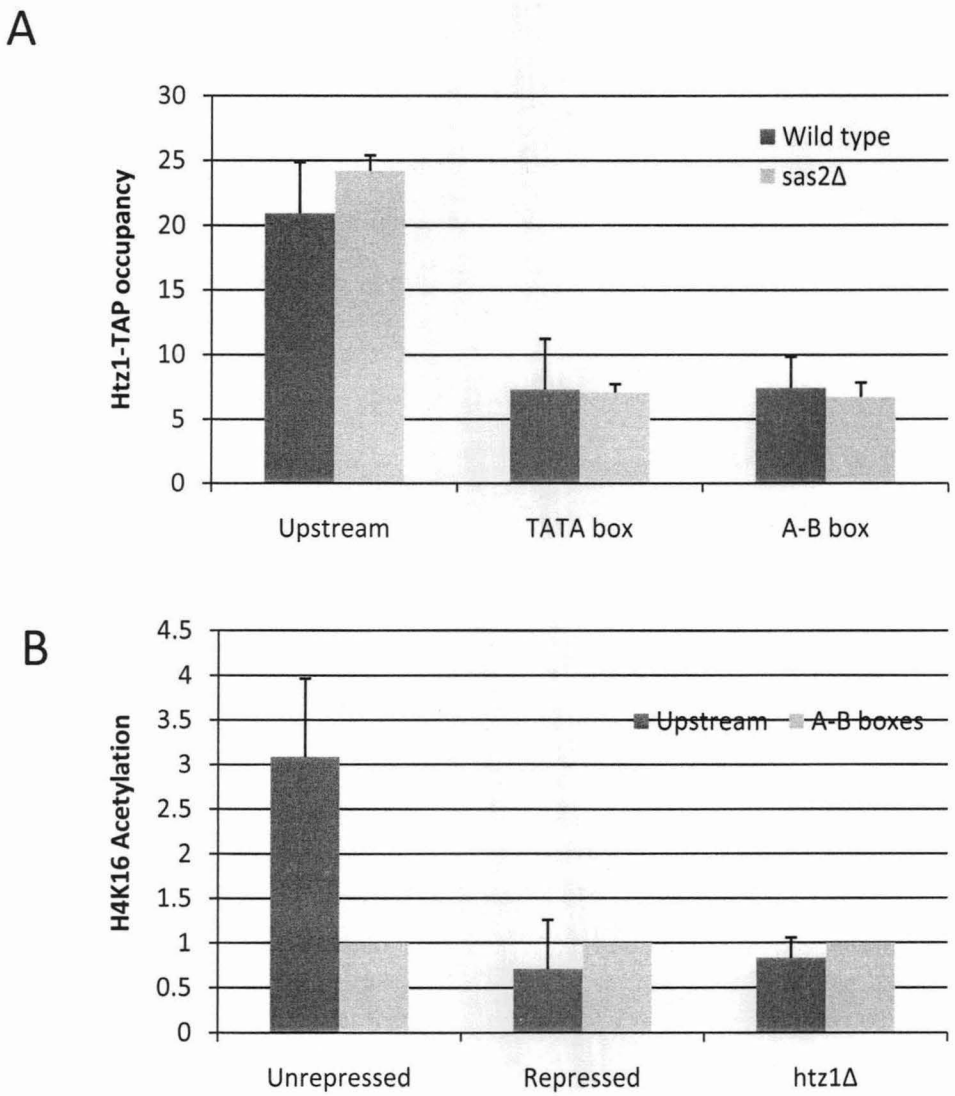


Figure 4.6: H4K16 acetylation on SNR6 depends on H2A.Z deposition

A. Htz1-TAP occupancy in wt and sas2Δ cells. ChIP was done after crosslinking for 15 minutes and lysis by glass beads. Mitochondrial gene Cox3 is used as control

B. H4K16 acetylation in wild type active, repressed and htz1Δ cells.

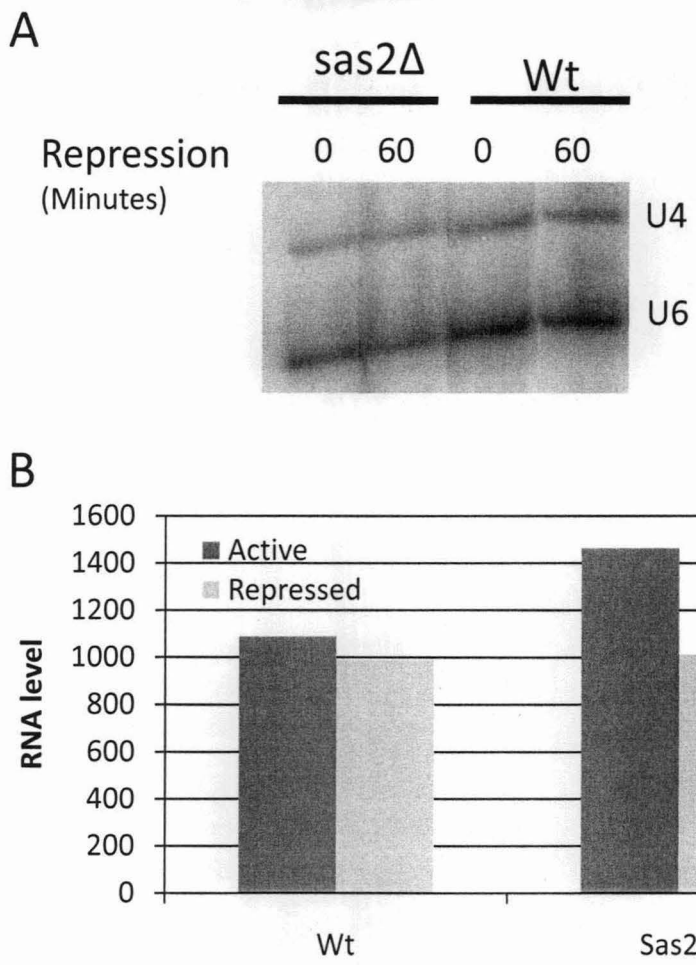


Figure 4.7: SNR6 transcription is unaffected by H4K16 Ac

A. U6 RNA level in wild type and sas2Δ cells. Total RNA was isolated and subjected to reverse transcription with primers specific to U6 and U4. A representative gel is given.

B. Quantification of bands in the gel shown in panel A.

Finally, above results are summarized in a model of the chromatin related events associated with SNR6 as shown in Figure 4.8. When the gene is repressed, RSC, H2A.Z and H4K16 acetylation are absent from the upstream nucleosome (Figure 4.8A). Activation leads to remodeling by RSC and the TATA box covering nucleosome is shifted upward where H2A.Z is deposited (Figure 4.8B). This order is evident from the fact that chromatin remodeling is unaffected in htz1Δ cells (Figure 4.4C). H2A.Z deposition is followed by acetylation of H4K16 by Sas2 thus giving the active state chromatin structure (Figure 4.8C).

4.9. Discussion

4.9.1. H2A.Z and SNR6 transcription

The data given in this chapter show that histone variant H2A.Z is present on the upstream nucleosome but lost under repression. H2A.Z is known to affect transcription in different ways. It poises repressed genes for activation by histone loss (Zhang et al., 2005) and it is required to attach inducible genes to the nuclear pores for their rapid activation when induced (Brickner et al., 2007; Gligoris et al., 2007). It also protects the genes from repression in regions near telomeres by stopping the spread of heterochromatin over them. Though a low resolution microarray study classified Pol III transcribed genes as H2A.Z deficient (Zhang et al., 2007), a recent high resolution study by Chip-sequencing method has shown a weak correlation of Pol III genes and H2A.Z, according to which, many tRNA genes are flanked by H2A.Z containing nucleosomes and the upstream nucleosome of SNR6 also carries this variant (Albert et al., 2007). Present study also shows the presence of an H2A.Z containing nucleosome upstream of SNR6. H2A.Z from the upstream nucleosome is lost during the repression while the nucleosome slides toward the TATA box. Our results suggest that H2A.Z does not have a positive role in SNR6 transcription and it actually may have a negative effect.

A detailed study of relation between transcription and H2A.Z had shown that H2A.Z is present on repressed genes but lost during transcription activation. However, other histones are also lost at the same time indicating it is a nucleosome loss, not mere H2A.Z

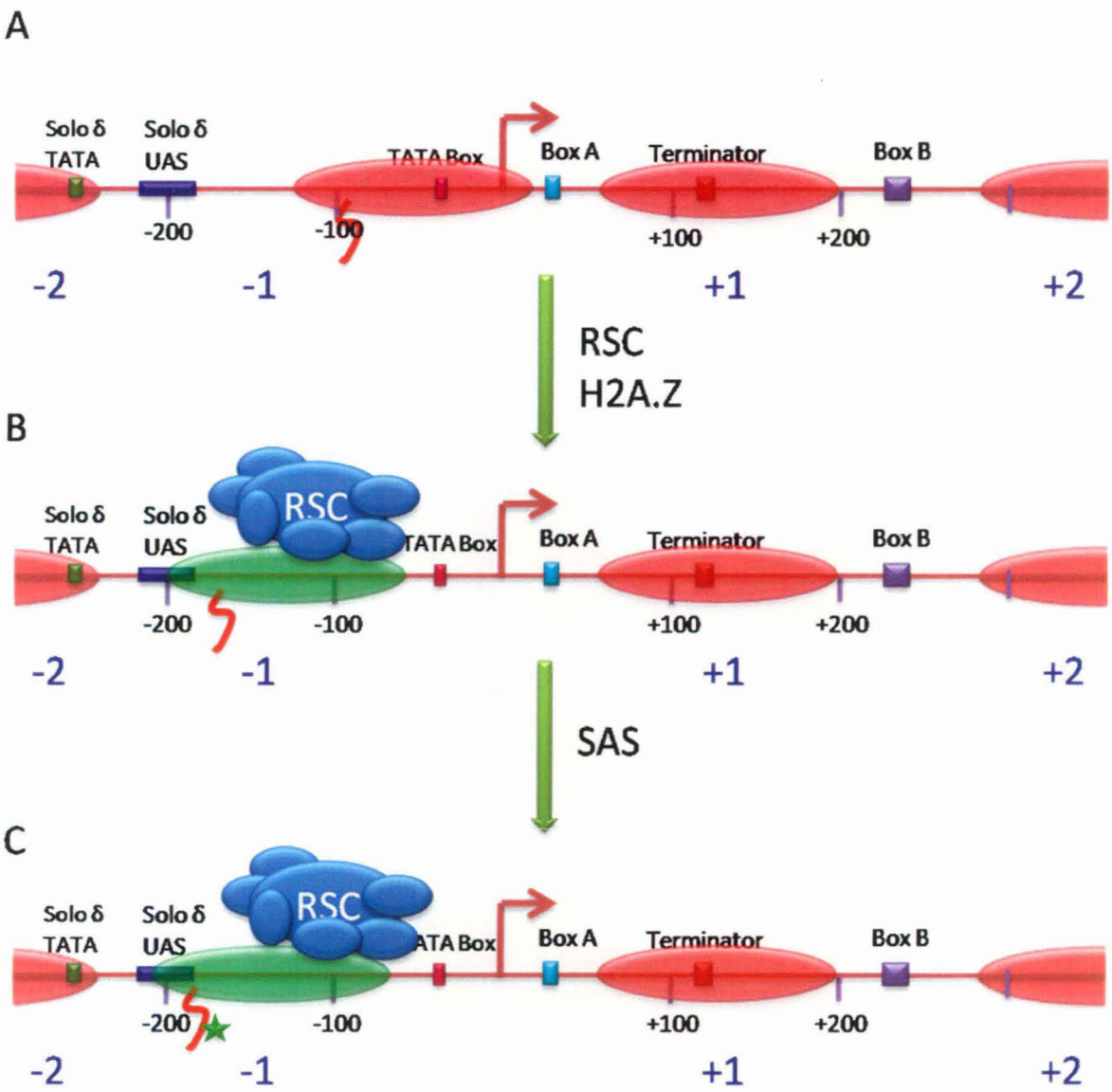


Figure 4.8: A model showing the order of events on SNR6.

Model is similar to that given in Fig 3.7 with additional details

A. Repressed chromatin structure. H2A.Z, RSC and H4K16 acetylation are absent. Red curved line indicates histone H4 tail.

B. Remodeling by RSC and deposition of H2A.Z. Green color indicates a nucleosome containing H2A.Z.

C. H4K16 is acetylated by Sas2 (not shown) after H2A.Z deposition. Green star indicates acetylation.

replacement. Deletion of Htz1 led to attenuation of activation of these genes indicating that H2A.Z poises the genes for activation by marking nucleosome loss/ejection (Zhang et al., 2005). On SNR6, the relation between transcription and H2A.Z is different. It is present when the gene is active and exerts a negative effect on transcription while it is lost when the transcription is repressed. Another difference from Pol II transcribed genes is that H2A.Z is lost but the nucleosome is not ejected. Upstream nucleosome slides toward TATA box and H2A.Z is replaced with canonical H2A (Figures 4.1B and 4.2). This may be the reason for the continued presence of Swr1 on SNR6 even after repression. It is known to deposit H2A.Z-H2B dimer to target loci and may also be involved in replacing H2A.Z with canonical H2A. Thus on SNR6, variant H2A.Z is replaced by canonical H2A, which is a rare example.

4.9.2. H2A.Z and NFR on a Pol III transcribed gene

Pol II genes are known to possess a nucleosome free region upstream of the start site in yeast (Albert et al., 2007; Lee et al., 2007) and in mammals (Ozsolak et al., 2007; Schones et al., 2008). Reports on prediction of nucleosome positioning sequences in yeast have shown that nucleosome positions as well as NFRs are encoded by the genome (Ioshikhes et al., 2006; Segal et al., 2006) but only up to a certain extent. Chromatin modifying machineries like chromatin remodeling complexes and histone variants along with transcription factors may be deciding the final positions of nucleosomes and NFRs. For example, Iswi is known to position nucleosomes only on a subset of available nucleosome positioning signals on a stretch of DNA. Similarly, H2A.Z is known to alter the nucleosome positions (Fan et al., 2002). H2A.Z is also known to act redundantly with chromatin remodeling complexes (Santisteban et al., 2000).

Results in the present study have shown the NFR on SNR6 which is similar to the NFR demonstrated on Pol II genes is maintained by the chromatin remodeling complex RSC. In htz1Δ cells, the NFR was intact and the nucleosome sliding under repression was also identical to that in wild type, suggesting that H2A.Z deposition was not required for the maintenance of NFR and for the remodeling. A very recent study has reported similar conclusions on the maintenance of NFR on Pol II transcribed genes (Hartley and Madhani, 2009). RSC maintains the NFR on Pol II transcribed genes and H2A.Z is not

required for maintenance of NFR. On the contrary, H2A.Z deposition requires the NFR to be established (Hartley and Madhani, 2009; Raisner et al., 2005). It is not clear whether H2A.Z deposition on SNR6 requires the NFR formation.

SNR6 has a dT7 stretch downstream of TATA Box. Such an oligo dT stretch is present in most of the Pol II transcribed genes (Yuan et al., 2005; Iyer and Struhl, 1995). An artificial NFR flanked by H2A.Z containing nucleosomes could be made on the coding region of an inactive gene (PRM1) by placing a stretch of poly dA/dT oligo nucleotides together with a transcription factor (Reb1) binding site (Raisner et al., 2005). SNR6 poly dA/dT stretch is known to be involved in SNR6 transcription and act cooperatively with the increased distance between A and B boxes and Nhp6 (Gerlach et al., 1995; Martin et al., 2001). The role of this poly dA/dT stretch in maintenance of SNR6 NFR needs to be elucidated. Extensive mapping of nucleosome positioning signals on SNR6 have shown a series of positioning signals that are overlapping. A nucleosome can occupy any of these positions when chromatin was assembled in the absence of any other factors but in presence of transcription factors, only a few positions are favored (Vinayachandran et al., 2009).

4.9.3. H4K16 Acetylation and H2A.Z deposition

H4K16 acetylation by Sas2 of SAS complex is required to prevent spreading of telomeric heterochromatin. Spreading of telomeric heterochromatin requires deacetylation of H4K16 by Sir2. This deacetylation facilitates binding of Sir3 protein to unmodified histone H4 tail and the silencing complex spreads through the chromatin. The acetylation/deacetylation gradient formed by the opposing Sas2 and sir2 is required for the boundary formation. So a deletion of Sas2 leads to spreading of heterochromatin towards the nearby euchromatin (kimura et al., 2002; Suka et al., 2002). A similar spreading of telomeric heterochromatin was observed when Htz1 was deleted, suggesting H2A.Z and Sas2 work together to prevent heterochromatin spreading (Meneghini et al., 2003). Further studies revealed that Sas2 mediated acetylation of H4K16 is required for deposition of H2A.Z on subtelomeric regions. But this effect was restricted to within 15-20 Kb from the telomeres and H2A.Z deposition on other regions

was independent of H4K16 acetylation (Shia et al., 2006). But it is not clear how H2A.Z functions to stop the spreading of heterochromatin.

Some of the Pol III transcribed genes are known to act as barrier elements that prevent the spreading of heterochromatin. But SNR6 did not show a significant barrier activity with reporter constructs (Donze and Kamakakka; 2001). Results present in this chapter show that on SNR6, H2A.Z and Sas2 mediated H4K16 acetylations are coexisting. It is interesting that despite all these marks present on SNR6, it does not have a boundary property. The possible reason may be in the details of temporal organization of events. Unlike the subtelomeric regions, on SNR6, the acetylation happens only after H2A.Z deposition. Deletion of either Htz1 or Sas2 led to similar change in RNA level of SNR6 (slight increase in both cases) suggesting that both act in the same pathway with H2A.Z deposition happening first, followed by H4K16 acetylation by Sas2. Thus, relation between H2A.Z and H4K16 acetylation on SNR6 is opposite to that reported for Pol II transcribed genes. H2A.Z shows correlation with many other histone acetylations and some acetylations are required for the deposition of H2A.Z on Pol II transcribed genes, which will be discussed in detail in the next chapter.

Chapter 5

Histone acetylations associated with SNR6 transcription

5.1. Overview

Previous chapters show the involvement of ATP dependent chromatin remodeler RSC and histone variant H2A.Z in SNR6 transcription. Apart from these, covalent modifications of histones are the other epigenetic modes of modifying the chromatin which influence the gene expression. Several proteins are discovered now which carry domains that can read a specific modification mark on a histone (Seet et al., 2006). Most of the chromatin remodeling complexes contain these domains in isolation or in combinations which are required for their activity. In accordance with this, N terminal tails of histones H3 and H4 were shown to positively modulate the activity of RSC as well as SWI/SNF. RSC contains 7 out of 14 bromodomains present in yeast, indicating the importance of lysine acetylation in its function. H3K14Ac is known to enhance nucleosome sliding by increasing the binding of RSC to nucleosome (Logie et al., 1999). An electron microscopic analysis of RSC structure has shown that binding to acetylated histone peptides can alter the conformation of RSC, giving an explanation for the histone modification mediated regulation of chromatin remodeling (Skiniotis et al., 2007). Histone H4 tail is known to be an allosteric activator for Iswi ATPase (Clapier et al., 2001; Hamiche et al., 2001; Clapier et al., 2002; Ferreira et al., 2007). H3K14 acetylation increases the remodeling activity of RSC by enhanced recruitment to nucleosomes and favors nucleosome sliding while the H4 tetra acetylation (at K5, K8, K12 and K16) favors the octamer transfer in trans by RSC, showing how modifications can modulate the function of a remodeler (Ferreira et al., 2007). Like Isw2, yeast Chd1 also requires unmodified H4 tail for its activity although the H4 tail region recognized by Chd1 may be different (Ferreira et al., 2007). On SNR6, RSC slides a nucleosome indicating the possibility of requirement of H3 acetylations for this remodeling.

The importance of acetylation in chromatin remodeling and histone variant deposition prompted us to check whether histone acetylations play any role in regulation of SNR6. In order to understand the role of acetylations, a variety of histone acetylations on the SNR6 locus were checked by chromatin immunoprecipitation assay using specific antibodies. The results are described with their possible implications.

5.2. SNR6 repression is associated with increased acetylation

H2A.Z is known to be associated with certain histone acetylations (Raisner et al., 2005; Zhang et al., 2005). In the previous chapter, it was shown that correlation of H4K16 acetylation with H2A.Z in the upstream nucleosome of SNR6 is different from what is reported for other genes. There are several other H3 and H4 acetylations known to correlate with H2A.Z on Pol II transcribed genes. Therefore, correlation of these acetylations with H2A.Z on SNR6 was checked next.

5.2.1. Histone acetylation pattern on SNR6 is largely unrelated to H2A.Z

The results on levels of all acetylations checked are given in a tabular form and compared with the two previously published studies on correlation of acetylation and H2A.Z, which do not match perfectly with each other (Table 5.1). Among the four histone H3 acetylations checked, H3K9, H3K14 and H3K18 acetylations were present on the upstream nucleosome, while we could not find any enrichment for H3K23. Among H4 acetylations, H4K5 and H4K12 did not show any enrichment while H4K16 and H4K8 were present on upstream nucleosome during active growth. H2B was not acetylated while H2A K7 acetylation could be seen suggesting H2A.Z in this nucleosome carries an acetylation at K7 because the sequence surrounding K7 is same in H2A and H2A.Z.

Zhang et al. (2005) had shown that H2A.Z occupancy correlates well with presence of certain histone acetylation marks while some marks were anti-correlated. Raisner et al (2005) have shown that, H3K9, H3K14, H4K5 and H4K12 are required for deposition of H2A.Z to its target promoters while H4K16 is not. Therefore, these acetylations were checked in htz1Δ background to see a correlation if any between H2A.Z and these acetylations on SNR6 and compared with the conclusions of Raisner et al (2005) and Zhang et al (2005). The results given in Table 5.1 show that all histone acetylations except H4K16 acetylation are unaffected in htz1Δ strain on SNR6. These results show that H3K14 and H3K9 acetylations that are required for H2A.Z deposition on some of the Pol II transcribed genes are present on SNR6 and may be involved in targeting H2A.Z to SNR6.

Acetylation	On SNR6		H2A.Z-acetylation correlation (Zhang et al., 2005)	Requirement for H2A..Z deposition(Raisner et al., 2005)
	Wild type	htz1Δ		
H3K9	Yes	Unaffected	-ve	Yes
H3K14	Yes	Unaffected	+ve	Yes
H3K18	Yes	Unaffected	-ve	-
H3K23	No	-	-	-
H4K5	No	Unaffected	-	Yes
H4K12	No	Unaffected	+ve	Yes
H4K8	Yes	-	+ve	No
H4K16	Yes	Decreases	-ve	No
H2AK7	Yes	-	+ve	-
H2BK16	No	-	+ve	-

Table 5.1: Correlation between H2A.Z and histone acetylation in the -1 nucleosome

All the acetylations checked under active and repressed conditions are listed. Presence and absence of a particular acetylation is denoted by "yes" or "no". Last two columns are the data taken from Zhang et al (2005) and Raisner et al (2005), showing the correlation between Htz1 and acetylation and the requirement of acetylation for H2A.Z deposition. A hyphen indicates that particular acetylation is not checked in the study mentioned.

5.2.2. H3 acetylations increase when transcription is repressed

Acetylations of histones are generally considered active state marks for Pol II transcribed genes (Li et al., 2007). As only some acetylations were seen on SNR6 and they did not show same correlation with H2A.Z as reported for Pol II transcribed genes, their levels on the gene were checked under repressed states. While the A-B Box nucleosome was largely unacetylated, the upstream nucleosome showed interesting pattern of acetylations. As shown in Figure 5.1, acetylations on H3K9, H3K14, H3K18 and H2AK7 (Panels A-D respectively) on the upstream nucleosome surprisingly show an increase under repression. All but H3K14 acetylation showed a slight increase in +1 nucleosome as well. But the levels on the +1 nucleosome are very low compared to that of the upstream nucleosome. This observation was intriguing because all these acetylation are associated with active transcription by Pol II.

5.2.3. Maf1 affects acetylation

Maf1 is the master repressor for Pol III transcription. Repression led to an increase in acetylation; acetylation levels were checked in maf1Δ mutant, which cannot be repressed by nutrient deprivation. Levels of H3K9 and H3K14 acetylations were checked in maf1Δ cells under nutrient rich as well as repressed conditions expecting that the acetylation will remain unaltered. But the acetylation profile of these histones in maf1Δ cells was found opposite to that of wild type strain (Figure 5.2). In maf1Δ cells, H3K9 acetylation level was at very high level in nutrient rich conditions but when transferred to starvation condition, the value reduced to a level similar to that of active state wild type strain (Figure 5.2A). Similarly, H3 K14 acetylation in maf1Δ cells increased by 2 fold under nutrient rich condition but reduced under starvation condition (Figure 5.2B). The results again suggest that increase in acetylation is a direct consequence of repression.

5.2.4. H3 acetylations increase in mutants with decreased SNR6 transcription

The unexpected relationship of acetylations with SNR6 repression suggests a prevalence of high acetylations in mutants with reduced SNR6 transcription. RSC4Δ4 and nhp6ΔΔ are two mutants known to have a reduced SNR6 transcription (Kruppa et al., 2001; Lopez et al., 2001; Soutourina et al., 2006). RSC4-Δ4 mutation leads to reduced SNR6

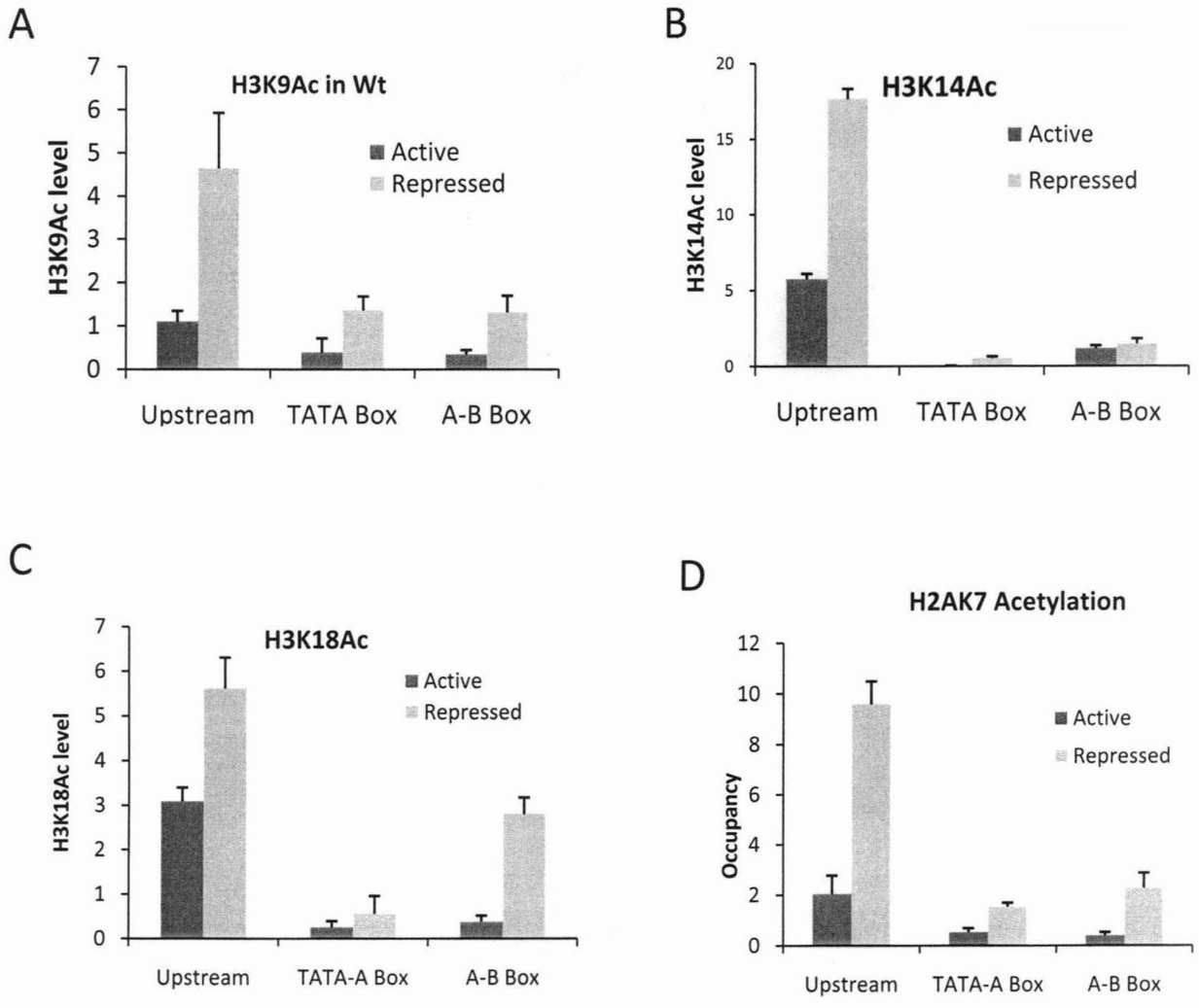


Figure 5.1: Histone acetylations increase on SNR6 under repression

Relative levels of histone acetylations that were found to increase under repression on SNR6 are given. Chromatin immunoprecipitations and Real-Time PCR quantifications are made either in active growth conditions or after 1 hour of repression, using specific antibodies against each modification. Relative level values are normalized against TelVIR.

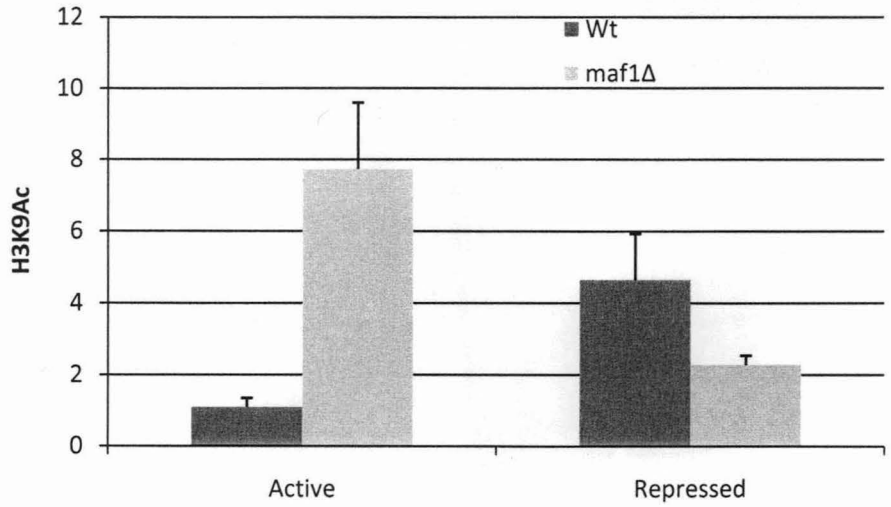
A. H3K9 acetylation level

B. H3K14 acetylation level

C. H3K18 acetylation level

D. H2AK7 acetylation level

A



B

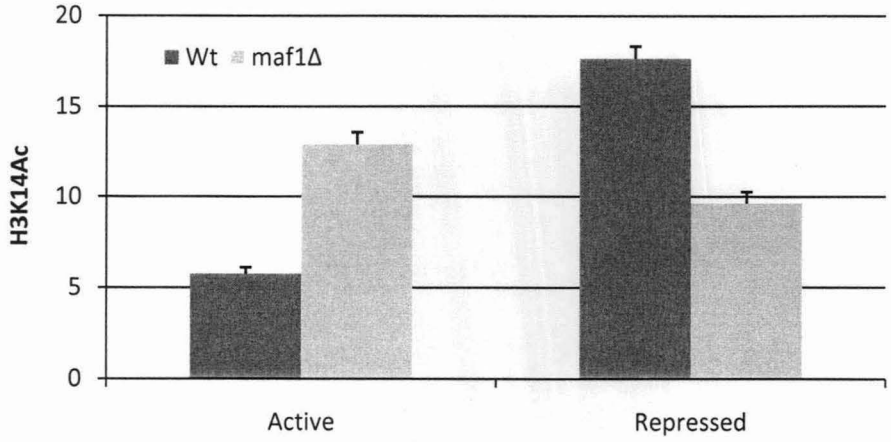


Figure 5.2: Histone acetylations in upstream nucleosome are affected by Maf1 deletion

ChIP assay was done against acetylated H3K14 and H3K9 in maf1Δ cells during active growth and after repression for 1 hour. Only the upstream region (-1 nucleosome) is shown. There were no significant differences on TATA box and A-B Boxes.

A. H3K9 acetylation

B. H3K14 acetylation

transcription (Soutourina et al., 2006) and its chromatin structure resembles that of repressed state (Figure 3.6). Analysis of H3K9, H3K14 and H3K18 acetylation levels in this mutant under active as well as repressed conditions revealed that that the acetylations were very high even under nutrient rich condition and during repression, the levels remained same (Figure 5.3), suggesting a connection between acetylation and RSC action.

Nhp6 is a non histone protein that is required for the expression of SNR6. Deletion of two closely related forms of Nhp6A and Nhp6B (nhp6ΔΔ) leads to an MNase hypersensitivity over TATA box suggesting destabilization of TFIIIB-DNA complex and decreased SNR6 transcription (Kruppa et al., 2001; Lopez et al., 2001). The H3K9, 14 and 18 acetylation levels in this nhp6ΔΔ strain were found to increase in nutrient rich as well as deficient media (Figure 5.3).

Though H3K9 acetylations show a decrease under repression in case of nhp6ΔΔ cells, the decreased level is equivalent to the repressed state of RSC4-Δ4 mutant cells (Figure 5.3B). Other than this, both the mutants show very high acetylation levels under active condition and do not respond to repression.

5.2.5. Increase in acetylation during starvation is specific to SNR6

In order to rule out the possibility that increase in acetylations found on SNR6 is an artefact or a general effect of starvation, H3K14 acetylation and H3K9 acetylation levels were examined on two genomic regions unrelated to Pol III. First, the acetylations on an autonomous replicating sequence (ARS504) on chromosome V, which does not have any Pol III transcribed gene in the vicinity, were analysed. Both H3K14 and H3K9 acetylations were found on the ARS under active condition without any remarkable difference during starvation (Figure 5.4A and B). Similarly, the promoter region of Pol II transcribed gene RPS11b was also examined. There was no H3 acetylation on this promoter under active conditions and it remained same during starvation. ARS504 was found to be nucleosomal while RPS11B is a ribosomal protein gene known to have very little H3 acetylation in the promoter region checked (Reid et al., 2000). These results show that the increase in histone acetylation seen on SNR6 is specific and related to repression of transcription.

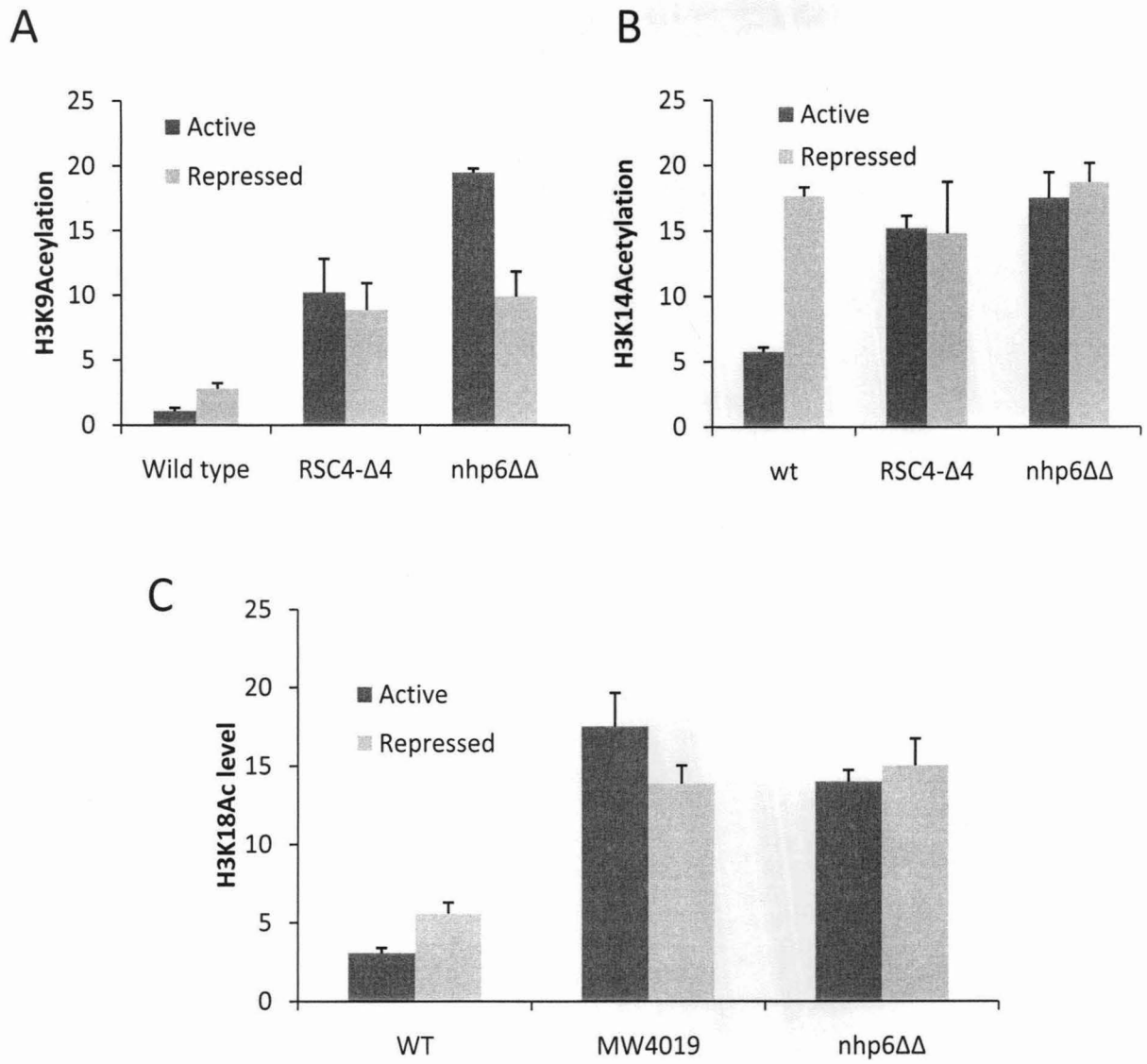


Figure 5.3: Histone acetylations are increased in mutants with decreased SNR6 transcription

Chromatin immuno precipitation was done for the specified modification during active growth and after 1 hour of starvation. Only the upstream region (-1 nucleosome) is shown. There were no significant differences on TATA box and A-B Box regions.

A. H3K9 acetylation

B. H3K14 acetylation

C. H3K18 acetylation

Figure 5.4: Increase in histone acetylation during starvation is specific to SNR6

ChIP assay for representative H3 acetylations on an Autonomous replicating Sequence (ARS504) and on a Pol II transcribed gene.

A. H3K9 acetylation on ARS 504 and RPS11B.

B. H3K14 acetylation on ARS504 and RPS11B.

C. H3K14 acetylation on the solo6 (VLRCdelta5) and down stream to B box.

In order to check whether the increase in acetylation is only on the immediate upstream nucleosome or whether it is present on the upstream array of nucleosomes, H3K14 acetylation was checked on the -2 and +2 nucleosomes (as numbered in Figure 3.4A) as well. The results shown in Figure 5.4C reveal that the -2 nucleosome also shows acetylation dynamics similar to -1 nucleosome while the +2 nucleosome is devoid of acetylation under both active and repressed conditions. This result indicates that the acetylation effect is not specific for the immediate upstream nucleosome (-1) and the whole array may be affected. Interestingly, the region downstream of TATA box, including +1 and +2 nucleosome is devoid of acetylation.

5.2.6. Increased acetylation during starvation is not a general Pol III effect

Histone acetylations were examined on a few tRNA genes to check whether the increased acetylation is a general Pol III effect during repression. Four tRNA genes were examined for H3K14 and H3K9 acetylation dynamics during active growth and starvation (Figure 5.5). Among the four tRNA genes checked, only tI(UAU)L and tD(GUC)K have TATA box upstream of the start site. Similar to SNR6, they can be transcribed in vitro by TFIIIB-TATA box complex in the absence of TFIIIC (Dieci et al., 2000). H3K14 and K9 acetylation patterns on these genes are different from SNR6. Both H3K9 and K14 acetylations were low on tI(UAU)L while tD(GUC)K had a higher level of both acetylations. H3K9 acetylation level shows a decrease on both the genes under repression while H3K14 acetylation levels did not change (Figure 5.5A and B) on tI(UAU)L.

tL(CAA)G1 and tL(CAA)G2 are two genes that code for the same tRNA and both genes are present on chromosome VII. tL(CAA)G2 had significant level of both H3K9 and K14 acetylation while tL(CAA)G1 had no acetylation. While tL(CAA)G2 showed a significant increase in H3K14 acetylation during starvation, that on tL(CAA)G1 did not change (Figure 5.5A). There was no significant difference in H3K9 acetylation on both the genes (Figure 5.5B).

These results suggest that the increase in acetylation observed on SNR6 during repression is a gene specific phenomenon and not a general Pol III effect. Acetylation

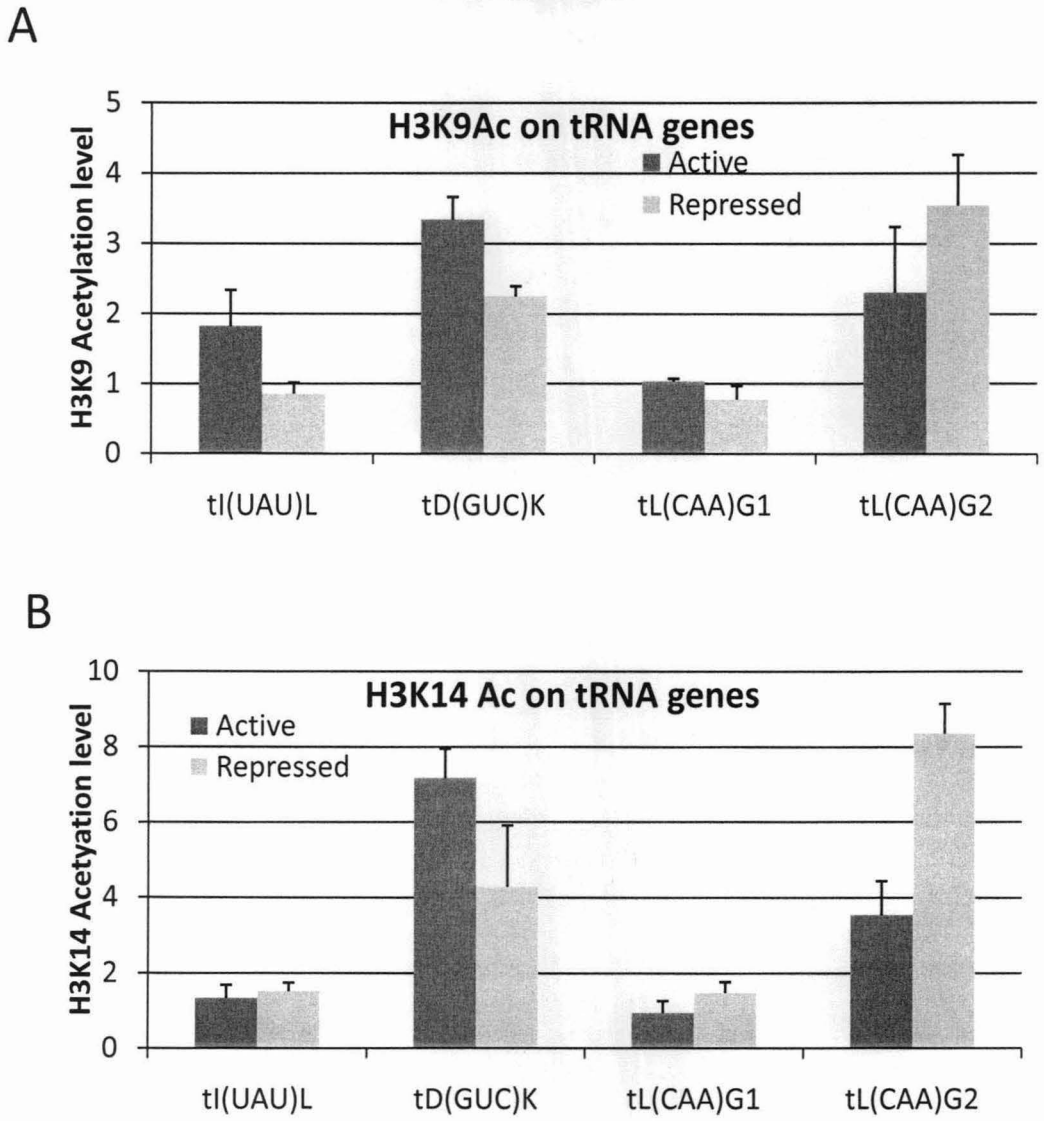


Figure 5.5: Histone H3 acetylations on tRNA genes

ChIP assay was done for acetylated H3K14 and K9 in wild type cells before and after repression for 1 hour. 4 different tRNAs were analyzed for the presence of these acetylations.

A. H3K9 acetylation

B. H3K14 acetylation

dynamics on tRNA genes also indicate that all Pol III genes are not regulated in a similar fashion. The genomic context of the individual gene also takes part in its regulation.

5.3. Chromatin structure and transcription of SNR6 is maintained by Gcn5

Gcn5 is a major HAT, which is part of many HAT complexes like SAGA, ADA and SLIK. It has substrate specificity for H3 and is known to acetylate residues including H3K9 and K14. It can acetylate proteins other than histones also. Gcn5 is known to acetylate proteins not only as part of any of these HAT complexes, but also in isolation.

5.3.1. Gcn5 is required for active transcription of SNR6

In order to check whether Gcn5 is involved in acetylation of H3 on SNR6 under repression, a strain was made in which Gcn5 ORF was deleted. As shown in Figure 5.6A, compared to wild type cells, both H3K14 and H3K9 acetylations were several fold higher in gcn5Δ cells even under nutrient rich condition. H3K14 did not show any difference during starvation while H3K9 acetylation increased further in the mutant. Increase in acetylation when a HAT is deleted was surprising. This observation suggested that Gcn5 may not be the HAT that acetylates histones on SNR6 but it influences the acetylation or else, Gcn5 may help recruit a deacetylase, which will be missing on U6 in gcn5Δ cells. The observation that acetylations are increased in gcn5Δ cells, similar to conditions of decreased SNR6 transcription (Section 5.2) suggested that deletion of Gcn5 probably has adverse effect on SNR6 transcription.

Figure 5.6B shows that native U6 RNA level in gcn5Δ cells is similar to repressed RNA levels in wild type cells. To make sure that whether the decrease is true, Maxi U6 construct was transformed in to gcn5Δ cells and the expression was studied. Figure 5.6C shows that transcription in gcn5Δ cells is reduced to the repressed level of wild type cells even under nutrient rich conditions and does not show much difference during starvation. It is evident from these results that positive effects of Gcn5 on the transcription of SNR6 are via some mechanisms other than histone acetylation.

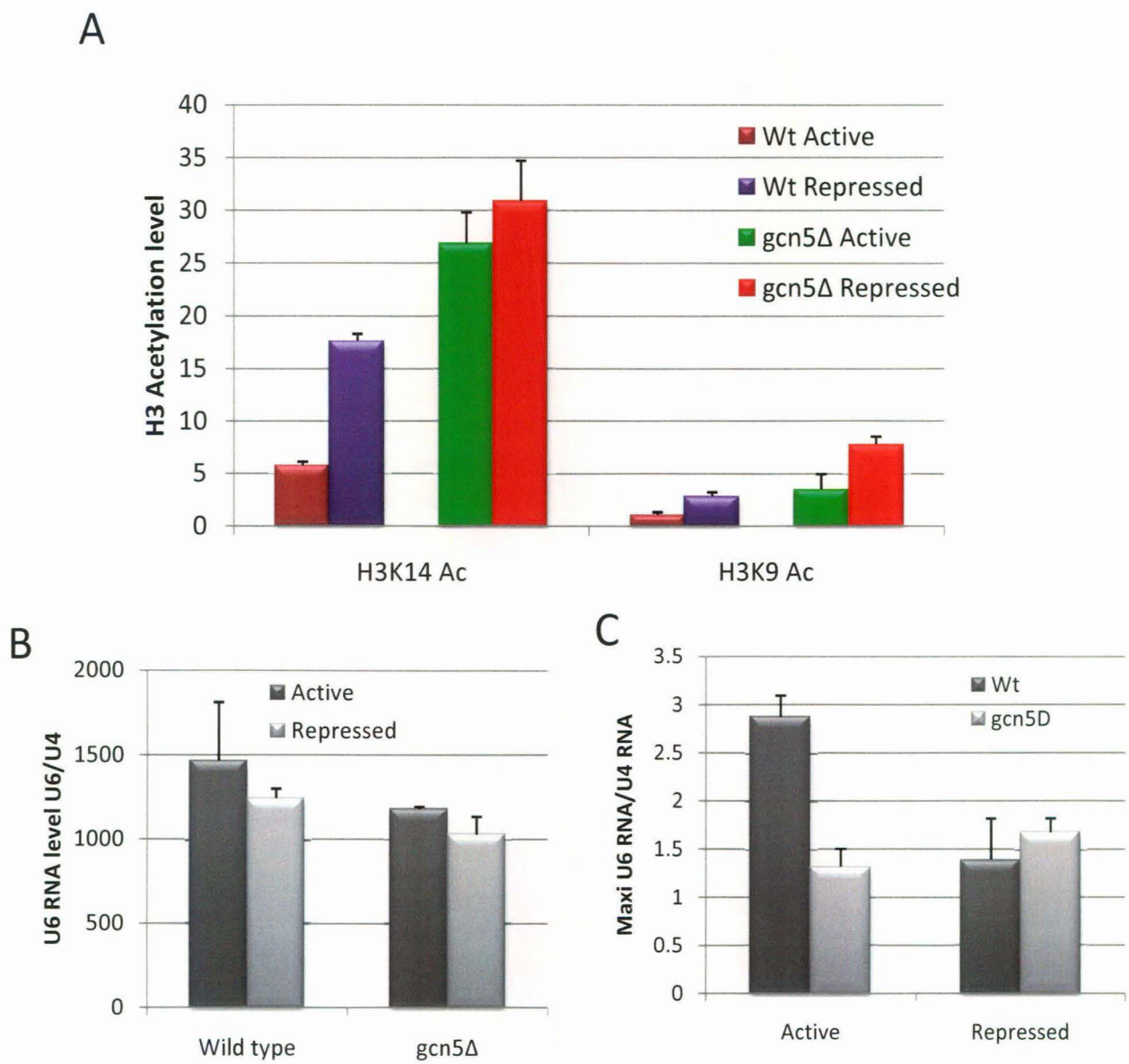


Figure 5.6: Gcn5 is required for active transcription of SNR6

A. ChIP assay showing the dynamics of H3K14 and H3K9 acetylations on SNR6 in gcn5Δ cells. Repression was done for 1 hour. Only the upstream region (-1 nucleosome) is shown. There were no significant difference on TATA box and A-B Box regions. Wt represents wild tye

B. Native U6 RNA level in wild type and gcn5Δ cells before and after 1 hour of repression.

C. Maxi U6 RNA level in wild type and gcn5Δ cells during active growth and after starvation for 1 hour

Therefore, presence of Gcn5 on SNR6 was checked by ChIP assay. However, no Gcn5 could be detected on SNR6 under active or repressed conditions.

5.3.2. Acetyl CoA does not have a positive effect on in vitro transcription of SNR6

One possibility is that Gcn5 acetylates some proteins of the Pol III machinery and absence of this acetylation leads to decreased transcription and increased histone acetylation. If Gcn5 acts in this way, there should be an increase in the in vitro naked DNA transcription with purified proteins when supplemented with a HAT source and acetyl CoA.

Though yeast TFIIIC does not possess HAT activity, previous studies in our lab (Unpublished data) have shown that a HAT activity co purifies with TFIIIC till the penultimate column (MonoQ column) which is lost in the last step of purification on the affinity column. An in vitro transcription assay was done with highly purified Pol III machinery and a plasmid bearing SNR6 (pCS6) to check whether addition of HAT fraction and acetyl CoA has any positive effect on transcription. Figure 5.7A shows that addition of acetyl CoA to a transcription reaction involving a HAT containing TFIIIC fraction from monoQ column did not enhance transcription. A transcription reaction was done with affinity purified TFIIIC (which does not have any HAT activity), supplementing it with a HAT activity containing fraction and acetyl CoA (Figure 5.7B). In this experiment also no enhancement of transcription was seen when HAT and acetyl CoA were added (Figure 5.7B, Compare lane 8 with lane 1). Since the HAT fraction is not characterized, the experiment may not be considered as conclusive, though it demonstrates that acetylation does not have any transcription activation function at least in naked DNA transcription.

5.3.3. Gcn5 is required to maintain the chromatin structure of SNR6

In an attempt to understand the Gcn5 effects further, the chromatin structure of SNR6 in gcn5Δ cells was analysed by IEL in active as well as repressed condition. Lanes 3 and 4 of Figure 5.8 show the chromatin structure in gcn5Δ cells grown in nutrient rich media while lanes 5 and 6 show the structure after starvation for 1 hour. Lanes 1 and 2

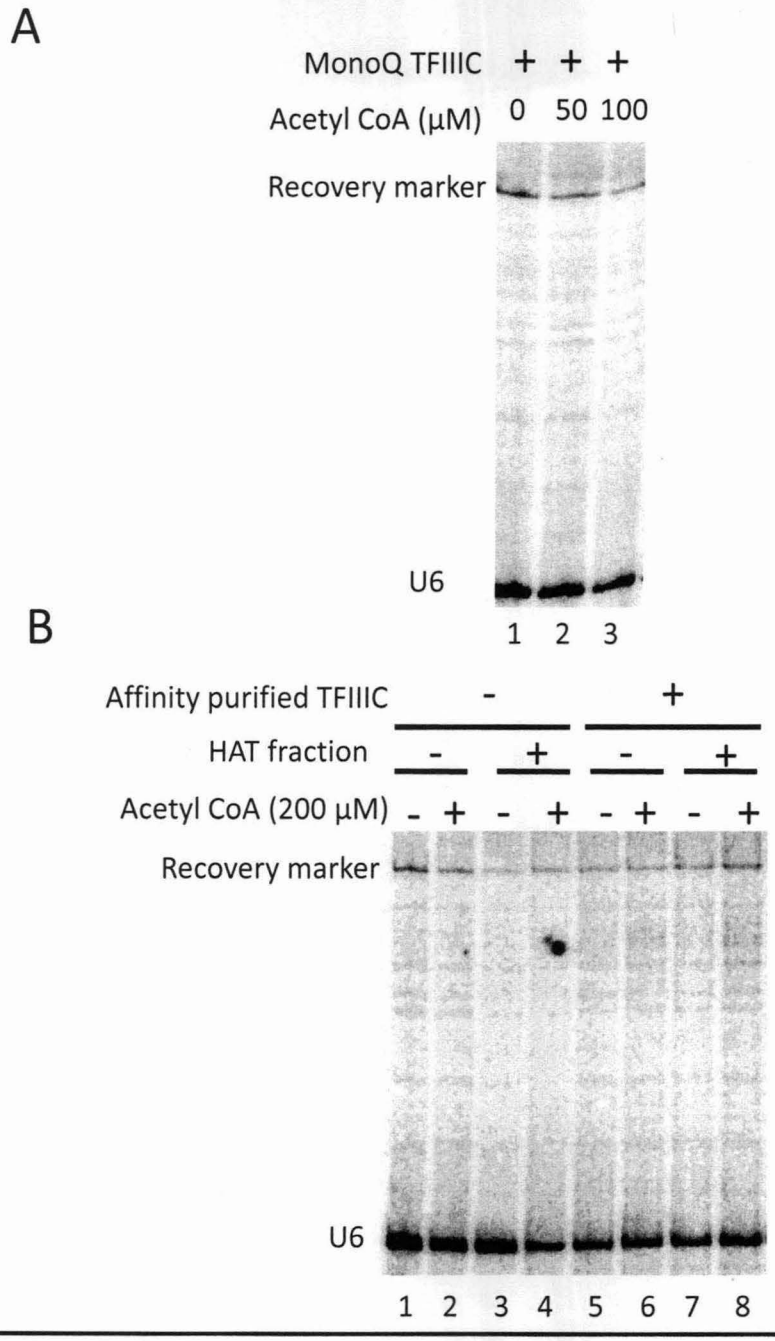


Figure 5.7: Acetylation does not enhance in vitro transcription of SNR6 with purified components

A. In vitro transcription was done with highly purified pol III and TFIIIB. TFIIIC (eluted from MonoQ column) has a HAT activity co purified with it. 50 μM (lane 2)or100 μM (lane 3) was added to analyze the effect of acetyl CoA on SNR6 transcription. Recovery marker is a known amount of ^{32}P labeled DNA added to the reaction for the purpose of quantification.

B. In vitro transcription was carried out as in Figure 5.8A, with the difference that the monoQ TFIIIC was further purified by passing through the Box B+ affinity column. HAT activity associated with MonoQ TFIIIC is lost in flow through fraction of this column. The reaction is supplemented with the flow through fraction showing HAT activity and acetyl CoA in the presence (Lane 8) or absence (lane 4) of TFIIIC.

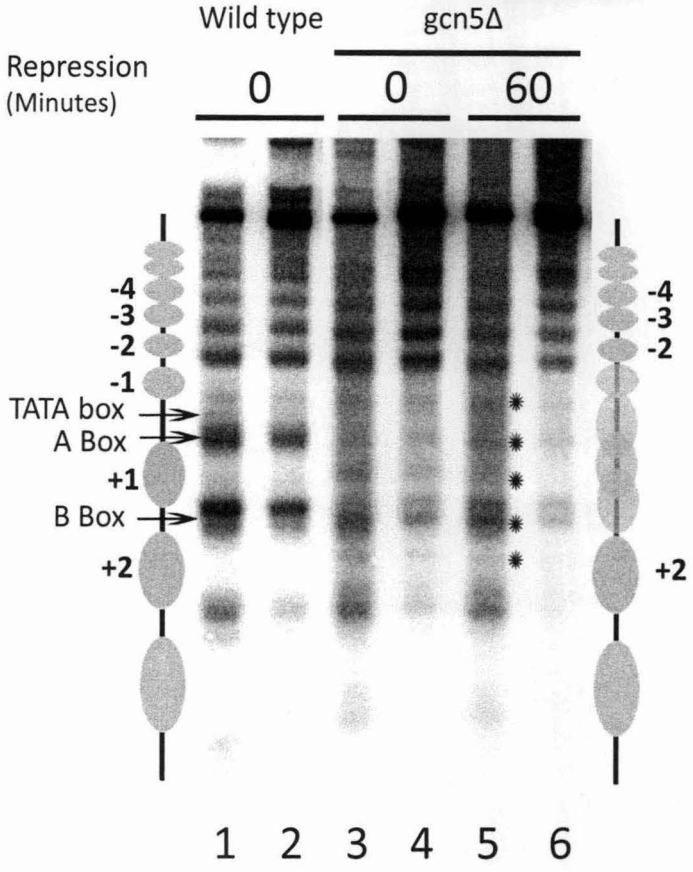


Figure 5.8: Gcn5 is required to maintain the chromatin structure of SNR6

In vivo IEL showing the chromatin structure of Wild type (Lanes 1, 2) and gcn5Δ (Lanes 3-6) strains grown in nutrient rich media (Lanes 1-4) and after starvation for 1 hour (Lanes 5-6). TATA box, A box and B box are marked with black arrows. Gray ovals on the left hand side of the gel show the positioned nucleosomes in wild type and the same on the right hand side show positioned nucleosomes in gcn5Δ cells. The overlapping ovals shown on right hand side indicate the possible multiple positions of nucleosomes in gcn5Δ. Red stars indicate the bands that are formed or altered by deletion of Gcn5.

represent chromatin from wild type cells under active condition. The array of upstream nucleosomes starting from -2 are unaffected by the deletion of Gcn5. But the nucleosome organization on the gene looks completely disturbed in gcn5Δ cells without any further change under repression (Compare lanes 5 and 6 with 3 and 4). The downstream (+2) nucleosome is also lost in gcn5Δ cells. The upstream thin boundary and downstream hypersensitive boundary of the TATA box NFR in wild type cells show similar nuclease sensitivity in gcn5Δ cells indicating loss of the demarcation of NFR. Among the two bands flanking the B box, MNase hypersensitivity of the upper band in wild type indicates the boundary of the +1 nucleosome. In gcn5Δ cells, increased sensitivity of the lower band suggests a change in nucleosome positions even as a new band appeared between A and B boxes. Appearance of 4 bands on the gene region and the distance between them suggest that instead of a single nucleosome positioned between A and B boxes in wild type, a number of rotationally phased nucleosomes may be occupying the whole gene region in gcn5Δ cells (Overlapping ovals on the right side of the gel), which look similar to the positions in the absence of any factor bound to the gene (Vinayachandran et al., 2009). These observations imply that Gcn5 is required for chromatin remodeling on SNR6 that leads to the nucleosome positioning, which generates the typical chromatin structure of SNR6 found in wild type cells.

5.4 No specific HAT involved in acetylation of SNR6

One possibility of increased acetylation under repression is that Pol III machinery is bringing a HAT complex to the gene under repressed condition, which specifically acetylates H3 and H2AK7. As Gcn5 could not be found on SNR6, a variety of HAT deletion mutants and two strains in which putative HATs are deleted were generated and screened for H3K14 acetylation on the upstream nucleosome of SNR6. The first candidate was Sas3, which is part of the NuA3 Hat complex and known to acetylate H3K14. But to our surprise, sas3Δ cells had not only this acetylation but also its increase under repression (Figure 5.9). All the other mutants that were checked also showed the same result, though the absolute values varied (Figure 5.9). This result may imply that more than one HAT complex can acetylate the upstream nucleosome in a redundant

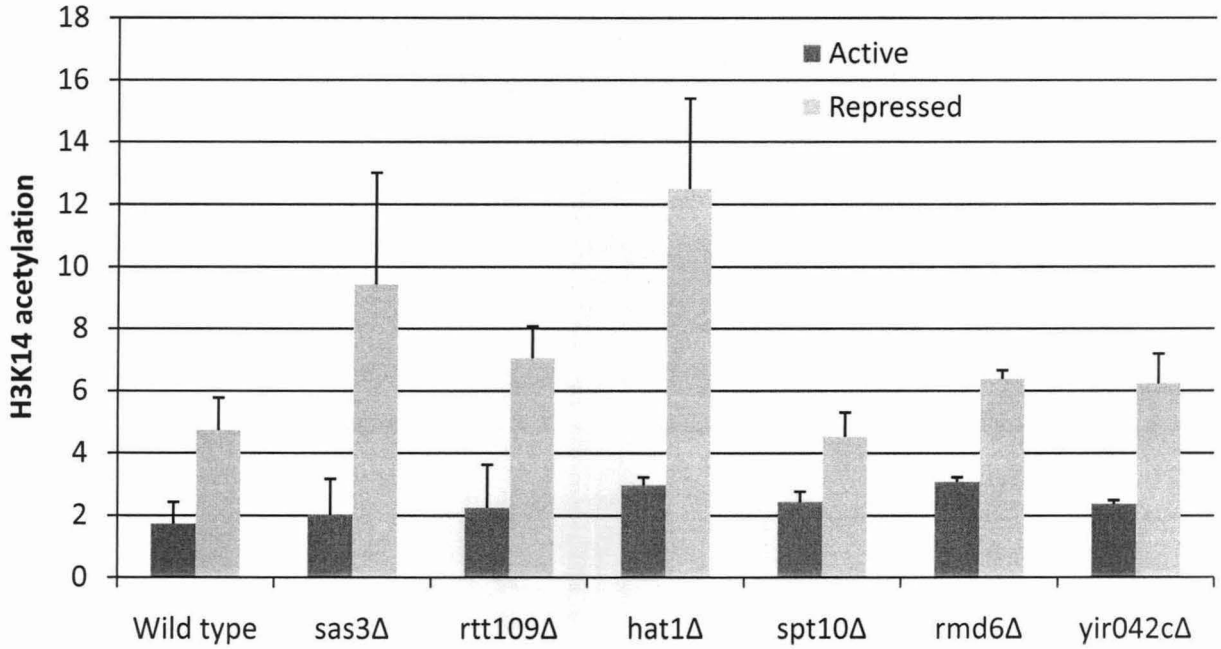


Figure 5.9: H3K14 acetylation in HAT mutants

ChIP assay was carried out in different yeast strains bearing deletions of known HATs. Rmd6 and Yir042C are two proteins with HAT domains. Repression was for 1 hour.

manner and deletion of one HAT does not show any effect because another HAT complex can take over its function.

5.5. Rpd3 deacetylates H3K9 and H3K18 but not H3K14

Histone acetylation levels observed in vivo are the end results of the actions of the acetylases and deacetylases on a target. Presence of histone acetylations on the upstream nucleosome and their increase under repression could also be due to imbalance in a site specific acetylation or deacetylation by these enzymes. It is also reported that deacetylase activity dominates in generating and maintaining an acetylation pattern.

The search for a HAT that hyperacetylates the upstream nucleosome under repression was not fruitful indicating possible involvement of the histone deacetylation. Rpd3 is a major histone deacetylase that can deacetylate acetyl lysines of all four core histones (Taunton et al., 1996). Monitoring of H3K9, H3K14 and H3K18 acetylation levels in rpd3Δ cells revealed a striking behavior of Rpd3. Both H3 K9 and K18 acetylations were higher than the wild type cells even under active conditions in rpd3Δ while H3K14 acetylation was unaffected (Figure 5.10A). This kind of site specificity for Rpd3 is not common. This effect may be due to difference in accessibility of the acetylated residue to Rpd3. Acetyl H3K14 is known to bind to the RSC4 bromodomain (Kasten et al., 2004) which may prevent its deacetylation by Rpd3.

Increased acetylation levels of H3K9 and K18 in rpd3Δ cells may affect transcription of SNR6. As native U6 RNA level did not show much difference from wild type cells (Figure 5.10B), rpd3Δ cells were transformed with Maxi U6 plasmid and its expression was studied. As shown in Figure 5.10C, U6 Maxi RNA shows only a slight decrease in rpd3Δ cells. The level further decreased under repression indicating that the gene is repressible further in the absence of Rpd3. This result indicate that H3K14 acetylation, which is unaffected by Rpd3 deletion may be more important for SNR6 regulation and strengthens the possibility of H3K14 binding by RSC4 on SNR6.

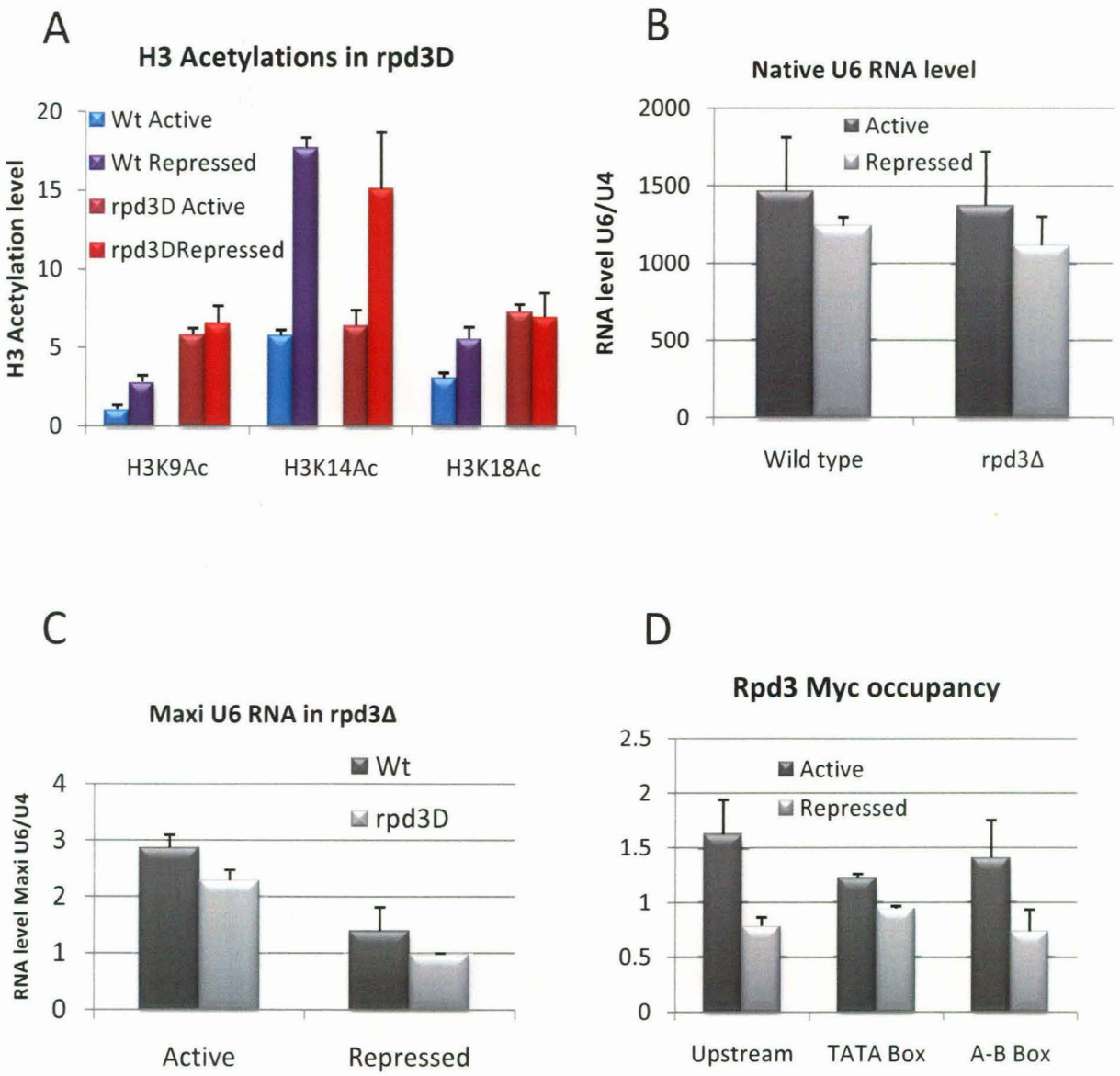


Figure 5.10: Rpd3 deacetylates H3K9 and K18 but not K14

A. ChIP assay for acetylated H3K9, K14 and K18 in rpd3Δ background. Repression was for 1 hour. Only the upstream region (-1 nucleosome) is shown. There was no significant difference on TATA box and A-B Boxes.

B. Native U6 RNA level in rpd3Δ and wild type during active growth and after starvation for 1 hour.

C. U6 Maxi RNA level in rpd3Δ and wild type during active growth and after starvation for 1 hour.

D. Rpd3-Myc occupancy on SNR6 during active growth and after starvation for 1 hour.

Presence of Rpd3 was checked on all three regions of SNR6 under active as well as repressed conditions. Though Rpd3 was found on SNR6 under active conditions, it was lost during repression for 1 hour (Figure 5.10D), probably contributing to increase in acetylation levels.

5.6 Discussion

5.6.1. Increase in acetylation is associated with decrease in transcription of SNR6

Results presented in this chapter reveal a striking and unexpected correlation between transcription repression and increase in acetylation. Though histone acetylation is correlated well with active transcription and deacetylation with repression on Pol II transcribed genes, there are some examples known for histone deacetylases involved in transcription activation. One such example is Hos2, which is associated with and is required for proper transcription of a number of genes (Wang et al., 2002). It is known that PU.1, a regulator of hematopoiesis is repressed by HDAC inhibitors and an H4 HDAC activity is required for its proper activation (Laribee and Klemsz, 2001; Laribee and Klemsz, 2005). Rpd3 is recruited to osmostress and heatshock genes leading to deacetylation of H4 when activated. Activation of these genes is impaired in rpd3Δ (de Nadal et al., 2004). An earlier study on SNR6 had shown that mutation of H3K9, 14, 18 and 23 to glycine increases transcription from some of the promoter mutants while transcription of wild type gene is unaffected (Marsolier et al., 1995). In agreement with this, the present study shows that acetylation of these same residues increases under repression, implying a negative role for these acetylations on SNR6 transcription.

The increase in acetylation seen on SNR6 is not a general effect of pol III transcription because on most of the tRNA genes studied, the pattern was different. Pol III transcribes ~285 genes with a very similar promoter structure, especially in the case of tRNA genes. 42 species of tRNA genes are encoded by 274 genes scattered throughout the genome. Each species has number of isogenes with almost identical coding sequence and promoter elements A and B boxes (Dieci et al., 2007). It is

technically difficult to determine the expression levels of individual genes of the same species. A previous study relying on the levels of a particular species of tRNA than individual genes (Ciesla et al., 2007) concluded that different species of tRNAs may be regulated differently. This may have physiological importance. It is not known whether the isogenes will be regulated identically or they are regulated depending on their genomic location. Our results on the acetylation levels on different tRNA genes show that even the isogenes behave differently. The differences among isogenes with identical coding region is known for the heterochromatin barrier activity of tRNA genes (Donze and Kamakaka, 2001). The results presented here provide further evidence that the flanking region of tRNA genes may be important in their regulation.

5.6.2. Correlation between H2A.Z and histone acetylation

H2A.Z is known to be associated with certain histone acetylations. Two published studies on this aspect have shown that H3K14 acetylation, H2AK7 acetylation, H4K8 acetylation and H4K12 acetylation are positively correlated with H2A.Z (Raisner et al., 2005; Zhang et al., 2005). While Zhang et al (2005) found a strong negative correlation between H2A.Z and H3K9Ac, Raisner et al (2005) found that it is required for deposition of H2A.Z to NFR flanking nucleosomes on Pol II transcribed genes. In short, histone acetylation has a role in targeting H2A.Z to various genomic loci. Histone acetylation is believed to promote H2A.Z deposition due to the binding of Bdf1 (*B*romo*d*omain *f*actor *1*), a subunit of Swr1 to acetylated histone H4 tails. Mutations in Bdf1 and a closely related protein Bdf2 cause a reduction in H2A.Z deposition (Raisner et al., 2005). Bdf1 has substrate specificity for acetylated H4K12 and its genome wide occupancy anticorrelates with acetyl H4K16 indicating this particular modification may prevent binding of Bdf1 to H4 tail. (Kurdistani et al., 2004). Our study shows that H4K12 acetylation is absent in the SNR6 upstream nucleosome but H4K16 is present during active conditions. On subtelomeric regions, acetyl H4K16 is known to be required for H2A.Z deposition. The exact mechanism of acetyl H4K16 requirement in H2A.Z deposition is not known. On SNR6, H4K16 is acetylated but this acetylation happens after H2A.Z deposition. This implies that H2AZ deposition on SNR6 utilizes some other mechanism.

5.6.3. Gcn5 mediated maintenance of chromatin structure of SNR6

Gcn5 is a HAT that acetylates primarily histones H3 and H2B (Fukuda et al., 2006).But Gcn5 deletion leads to an increase in H3 acetylations on SNR6. The increase was similar to that seen in nhp6ΔΔ and rsc4-Δ4. Similar to nhp6ΔΔ and rsc4-Δ4, transcription was also reduced in gcn5Δ. It will not be wise to conclude that acetylation increased because transcription is reduced. The in vitro transcription also did not show any positive effect of acetyl CoA on transcription. And the chromatin structure in gcn5Δ shows lack of nucleosome positioning, indicating a lack of chromatin remodeling. Results in the third chapter (section 3.3.3) suggest that RSC is the chromatin remodeler for SNR6. There are evidences that link H3K14 acetylation, RSC and Gcn5. H3K14 acetylation is known to enhance nucleosome sliding by RSC (Ferreira et al., 2007). Histone acetylation also helps chromatin remodeling by RSC to allow pol II to transcribe nucleosomal DNA (Carey et al., 2006). The tandem bromodomains in the N terminus of RSC4 subunit of RSC complex are essential for cell survival and the second bromodomain is shown to bind to the acetylated H3K14 (Kasten et al., 2004; VanDemark et al., 2007). It was shown by an in vitro study that a lysine residue in RSC4 at position 25 (RSC4 K25) can be acetylated by Gcn5, which leads to its binding by the first bromodomain of RSC4. Interestingly, binding of acetyl K25 to first bromodomain inhibits the binding of acetyl H3K14 to the second bromodomain indicating a possible auto regulation of RSC recruitment and action (VanDemark et al., 2007). However, genetic studies indicate that none of the known complexes of Gcn5 are involved in the acetylation of RSC4 K25 indicating Gcn5 may be responsible for this acetylation either alone or as part of a yet to be identified complex in vivo. Histone H3 tail acetylation becomes essential in the absence of RSC4K25 acetylation and it is the loss of this acetylation in the absence of Gcn5 which makes gcn5Δ synthetically lethal to another histone H3 acetyl transferase deletion condition, sas3Δ.

The positive charge neutralization of H3 tail may be the important aspect of acetylation, necessary for nucleosome mobilization by RSC (Choi et al., 2008). One possible explanation for the increased acetylation on SNR6 under repression may be that RSC4 binds to acetylated H3K14 and masks the residue from deacetylation by Rpd3 as well as from detection by antibody. Under repressed conditions, RSC is removed

from the gene (Figure 3.6) exposing the residue and that was detected as an increase in acetylation. In case of rsc4-Δ4 mutant, the mutation in the RSC4 C-terminus may be affecting the binding of RSC4 second bromodomain to acetyl H3K14 and as a result, acetylation level remains high even under repressed conditions. It should be noted that C terminus of RSC4 is required for its incorporation in to the RSC complex (Kasten et al., 2004). Similarly, the chromatin remodeling on SNR6 is lost in gcn5Δ indicating a loss of function of RSC. This might have led to an increase in acetylation level.

Observations in rpd3Δ cells support this model. H3K9 and H3K18 acetylations are increased in rpd3Δ cells while H3K14 acetylation level is similar to that of wild type suggesting Rpd3 deacetylates H3K9 and K18 during active growth. This was quite surprising because Rpd3 is a nonspecific deacetylase that can target almost all residues. One possibility is that RSC binding to H3K14 prevents deacetylation of H3K14 by Rpd3, which is associated with the gene only in active condition. Rpd3 may be lost due to the lack of active transcription in RSC4-Δ4, leading to an increased acetylation of other residues like H3K9 and K18. Though the acetylation levels of H3K9 and K18 were high in rpd3Δ cells, it had only a slight effect on transcription indicating that H3K14 acetylation is more important for transcription.

Chapter 6

Conclusions

6.1. Summary

Several studies on *Xenopus* oocyte and somatic 5S rRNA genes have shown the role of chromatin structure in developmental stage regulation of these genes many years back. But the progress made on the role of chromatin structure in regulation of Pol III transcription has been negligible. Most of the studies relating transcription and chromatin in recent years were for Pol II. Owing to the small size of Pol III genes (around a hundred base pairs), even many of the genome wide studies do not have sufficient resolution to make conclusions possible for Pol III-transcribed genes. However, during the course of this study, evidences showing the association of chromatin modifying machinery with Pol III transcription have started accumulating. However, these studies do not reveal the mechanisms by which chromatin modifying machineries may modulate the transcription by Pol III.

Yeast U6 snRNA (SNR6) gene was used in this study as the model gene to study the chromatin mediated regulation of Pol III transcription because of the evidences showing requirement of chromatin remodeling for activation of this gene in vitro. SNR6 transcription is repressed by chromatin due to the inaccessibility of TATA box for TFIIIB binding (Burnol et al., 1993a). TFIIIC binding to box B brings a chromatin remodeling activity which positions a nucleosome between A and B boxes and allows TFIIIB binding (Shivaswamy et al., 2004). TFIIIB binding to the TATA box leads to positioning of another nucleosome upstream of TATA box (Shivaswamy et al., 2006). The present study was carried out to elucidate mechanism of chromatin associated regulation of SNR6 in vivo.

SNR6 is a constitutive gene which is actively transcribed during favorable growth conditions. Starvation can repress Pol III transcription in general. To begin with, starvation led repression of SNR6 was confirmed by comparing the transcription and RNA polymerase occupancy during active growth as well as starvation. Interestingly, even under repressed condition, Pol III and Brf1 were not displaced from the gene suggesting that Maf1, the negative regulator of Pol III probably binds to the promoter bound PIC disengaging it from transcription (Chapter1).

Chromatin footprinting coupled to nucleosome depletion revealed that the DNA between A and B box has a positioned nucleosome (+1 nucleosome). The active state chromatin structure of SNR6 is characterized by an NFR flanked by two positioned nucleosomes: A-B box nucleosome being the downstream boundary and an upstream nucleosome (-1 nucleosome) starting from ~-70 bp position as the upstream boundary. In cells carrying a mutant of RSC4 subunit of RSC complex, where the transcription of SNR6 was reduced (Soutourina et al., 2006), a nucleosome is found occupying the TATA box region (Chapter 3) suggesting that RSC is the remodeler that maintains the NFR during active condition and keeps the TATA box free for TFIIIB binding and subsequent formation of PIC. Repression of transcription led to a decrease in RSC occupancy and the upstream nucleosome was found to slide towards TATA box.

The upstream nucleosome (-1 nucleosome) was shown to have histone variant H2A.Z during active growth while it was replaced with H2A during repression. ATP-dependent chromatin remodeling complex SWR1 was found necessary to deposit H2A.Z in the upstream nucleosome. SAS mediated histone H4K16 acetylation is also present on the -1 nucleosome and is lost during repression. Unlike the subtelomeric regions, where H4K16 acetylation is required for H2A.Z deposition; on SNR6, H4K16 acetylation depends on H2A.Z deposition. Both H2A.Z and H4K16 acetylation had a negative effect on transcription, evident from the increased RNA levels in htz1Δ and sas2Δ strains. Moreover, H2A.Z deletion did not cause any difference in the chromatin structure of SNR6 suggesting that H2A.Z is not required for the maintenance of the NFR (chapter 4).

The results in chapter 3 and chapter 4 suggested that the NFR on SNR6 is maintained by RSC and H2A.Z deposition is not required for this. A recent genome wide study on budding yeast has also observed that RSC is required for maintenance of NFR on Pol II transcribed genes and H2A.Z present on the flanking nucleosomes is dispensable for the maintenance of NFR (Hartley and Madhani, 2009).

Histone acetylations are known to be associated with active transcription by Pol II. But reverse was observed on SNR6 in this study. Higher levels of histone H3 acetylations that are normally associated with active transcription were found on SNR6 under repression (Chapter 5). This increase was specific to SNR6 when compared with

genomic regions unrelated to Pol III transcription. It was not a general effect of Pol III transcription as well because acetylations on different tRNA genes behaved differently during repression. The mutants with a reduced SNR6 transcription showed an increase in acetylation even under nutrient rich conditions. Histone deacetylase Rpd3 was found to associate with SNR6 under active conditions and deacetylate H3K9 and H3K18. But specific HAT responsible for acetylation could not be found indicating redundant action of more than one HATs in acetylation of the -1 nucleosome. Deletion of the HAT Gcn5 was found to increase the acetylation levels drastically and further studies revealed that Gcn5 is required for active transcription of SNR6 as well as maintenance of the chromatin structure. The exact mechanism by which Gcn5 activates SNR6 transcription is yet to be found.

6.2. A model for the chromatin associated events on SNR6

Based on the results presented in Chapters 3 to 5, a model for the formation and maintenance of chromatin structure of SNR6 is proposed (Figure 6.1). For the ease of understanding, Pol III machinery is excluded from the model. However, it may be noted that the whole process starts after binding of TFIIIC. The events happen in the following steps.

Step1. In the absence of chromatin remodeling, the gene region is covered by a number of rotationally phased nucleosomes (Figure 6.1A).

Step2. Biding of TFIIIC and TFIIIB generates the repressed chromatin structure. Gcn5 or Sas3 or some other HAT may acetylate these nucleosomes at this stage.

Step3. With formation and stabilization of PIC during activation, RSC binds to the H3K14 slides the nucleosome upward. RSC is speculated to bind acetyl H3K14 protecting it from deacetylation while other H3 acetylations (K9 and K18) are deacetylated by Rpd3 (Figure 6.1B). SWR1 deposits H2A.Z on the -1 nucleosome followed by SAS mediated acetylation of H4K16 (Figure 6.1C)

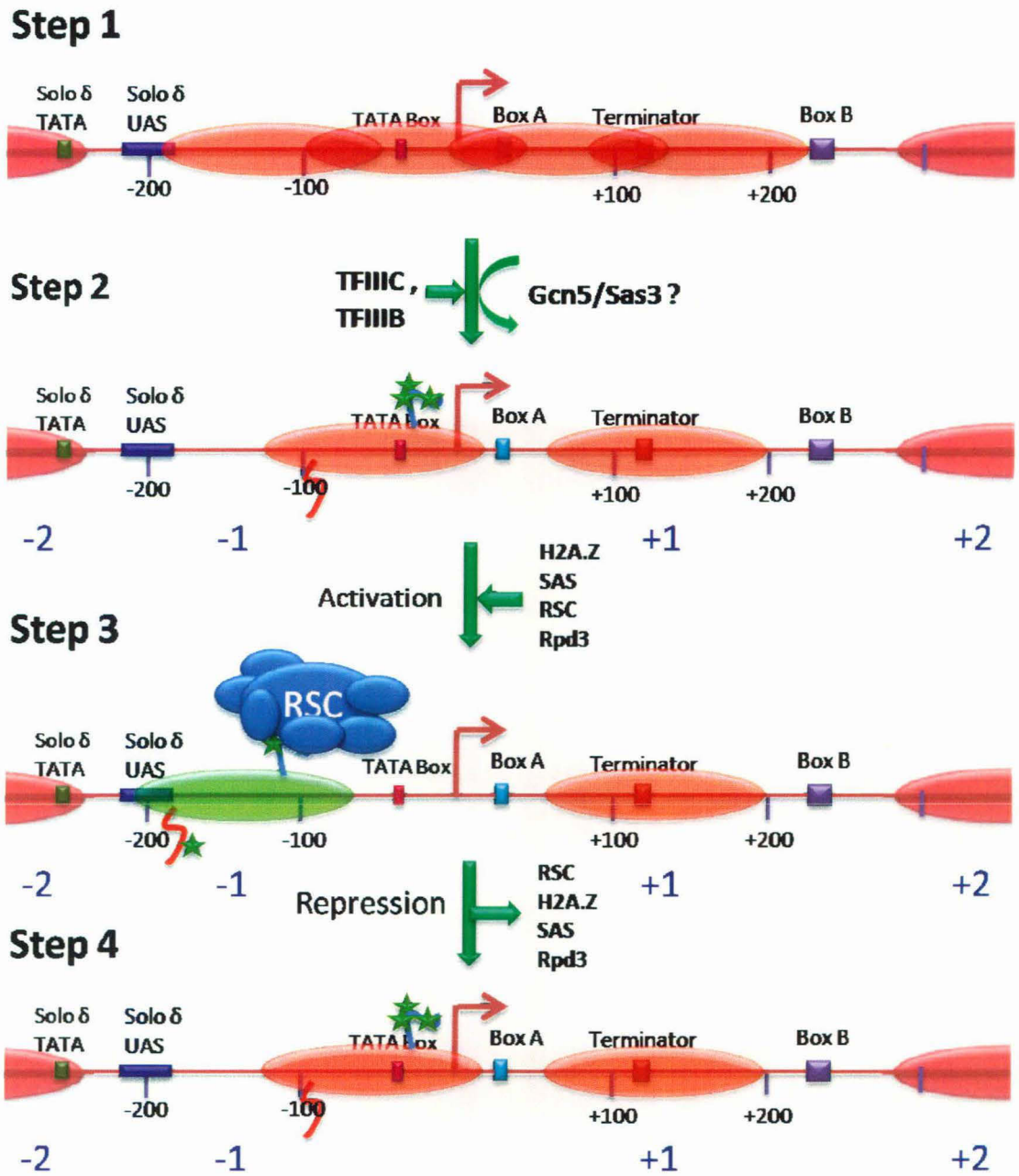


Figure 6.1: Formation and maintenance of chromatin structure on SNR6

A model showing the proposed order of events leading to the establishment and maintenance of chromatin structure of SNR6. Markings are similar to Figure 4.8. The blue line indicates H3 tail and green stars indicate acetyl group

Step4. During starvation, RSC, H2A.Z and SAS leave the locus and the -1 nucleosome slides back toward TATA box (Figure 6.1D), generating a structure as in step 2 wherein nucleosomes can be acetylated again.

6.3. Future directions

This study could elucidate mechanisms of various chromatin mediated regulatory processes and the sequence of events on SNR6 gene to some extent. However, many unanswered questions have still to be answered to fully understand regulation of SNR6. The presence of a positioned nucleosome between A and B boxes gives an opportunity to study how Pol III tackles a nucleosome in vivo. The observation that H4K16 acetylation is not required for H2A.Z deposition and the acetylations targeted by Bdf1 are absent, suggest that SWR1 may be targeted to SNR6 in a Pol III-specific manner. The exact role played by H2A.Z on SNR6 transcription is unknown. Studies associating H2A.Z and H4K16 acetylation may reveal that. Similarly, it will be interesting to see whether the NFR formation is required for H2A.Z deposition.

Another interesting aspect is the relation between histone acetylation, RSC, Nhp6 and Gcn5 on SNR6. Mutation of any of these proteins leads to an increase in the acetylation level as well as decrease in transcription. Therefore, it would be interesting and important to ask whether these three proteins act in the same pathway. A study on the occupancy of one of the protein in the absence of other on SNR6 will tell whether they are inter-related.

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