Chapter I

INTRODUCTION
1.1 OVERVIEW

This chapter gives an overview of literature and the recent advances made in the field of transcription in eukaryotes in general and in S. cerevisiae in particular. RNA synthesis involves the copying of a template DNA strand by RNA polymerase. Though several different types of RNA polymerases are known, all catalyze the following basic reaction,

\[ \text{RNA polymerase} + \text{template DNA} \rightarrow \text{RNA} + \text{PPi} \]

using the rules of complementarity (A-T, G-C, C-G, and U-A), where the first base of each pair is a ribonucleotide and the second is a deoxyribonucleotide.

A single RNA polymerase catalyzes the synthesis of all three classes of RNA in E.coli: mRNA, rRNA, and tRNA. This was shown in experiments with rifampicin, an antibiotic that inhibits RNA polymerase in vitro and blocks the synthesis of mRNA, rRNA, and tRNA in vivo (Wehrli et al., 1968). In contrast, the transcription of nucleus-encoded genes in eukaryotes is performed by three distinct RNA polymerases termed I, II, and III. Each of the nuclear RNA polymerase contains a dozen or more subunits (Sentenac et al., 1992). The enzymes differ in their sensitivity to inhibition by α-amanitin, a toxin from the poisonous Amanita phalloides mushroom. RNA polymerase II (pol II) is inhibited at low concentrations, RNA polymerase III (pol III) is inhibited at high concentrations, and RNA polymerase I (pol I) is quite resistant to α-amanitin (Seifart et al., 1969; Kedinger et al., 1970).

In contrast to prokaryotic systems, all of the eukaryotic RNA polymerases require additional protein factors (transcription factors) in order to bind to a promoter and initiate transcription (Kadonaga, 1998; Tan and Richmond, 1998; Green, 2000; Pugh, 2000; Eick et al., 1994). Transcription factors play a major role in determining selectivity in the transcription of genes.

The isolation of genes encoding subunits of eukaryotic RNA polymerases from a wide spectrum of organisms have confirmed previous biochemical and immunological data indicating that all three enzymes are closely related in structures and have been conserved in evolution (Guilfoyle et al., 1983; Archambault and Friesen, 1993). Each RNA polymerase is an enzyme complex composed of two large
subunits that are homologous to the two largest subunits of prokaryotic RNA polymerases and are associated with smaller polypeptides, some of which are common to two or to all three eukaryotic enzymes (Sweetser et al., 1987; Allison et al., 1985) (Fig.1.1). Transcription in eukaryotes is a more complex process than in prokaryotes. Not only is there much more discrimination in what is to be transcribed and what is not, but transcription is precisely programmed during development and tissue differentiation. Furthermore, the transcription machinery must deal with the complicated levels of structure in eukaryotic chromatin.

Enzyme - RNA polymerase I (also known as RNPase A) is a complex enzyme, containing 14 subunits totaling over 600,000 Da (Valenzuela et al., 1976). It is responsible for synthesizing the large 45S pre-rRNA transcript that is later processed into mature 28S, 18S, and 5.8S ribosomal RNAs (rRNAs). At least two transcription factors are known to be required, but there may not be a need for an elaborate transcriptional apparatus characteristic of pol II transcription, because only a single kind of gene is transcribed.

All of the protein-coding genes in eukaryotes are transcribed by RNA polymerase II (also called RNPase B). This enzyme also transcribes some of the small nuclear RNAs involved in splicing. Like its counterparts, pol II is a complex, multi-subunit enzyme (12 subunits), but not even its numerous subunits are sufficient to allow accurate transcription initiation on a eukaryotic promoter. The minimal unit involves the TATA binding protein, (TBP), but in vivo formation of the complex probably always uses TFIIID, a multi-subunit structure incorporating both TBP and TBP associated factors (TAFs)

RNA polymerase III (also called RNPase C) is the largest and most complex of the eukaryotic RNA polymerases. It involves about 17 subunits, totaling ~700,000 Da. All of the genes it transcribes are small, they are not all translated into proteins, and their transcription is regulated by certain sequences that lie within the transcribed region. The major targets for pol III are the genes for all the tRNAs, and for the 5S ribosomal RNA. Like the major ribosomal RNA genes, these genes are present in multiple copies, but they are usually not grouped together in tandem arrays, nor are they localized in one region of the nucleus. Rather, they are scattered over the genome and throughout the nucleus. The yeast RNA polymerase III system is probably the best-characterized eukaryotic transcription system. Nearly all of the components have been identified and the genes for them cloned. Many of the
Fig. 1.1: Subunit compositions of *S. cerevisiae* RNA polymerases (Sentenac, 1999)
interactions within initiation complexes are coming to light. Considering the many parallels between Pol III transcription and the other polymerase systems, findings in the Pol III system can act as predictions for Pol II and Pol I transcription.

1.2.1 RNA POLYMERASE III TRANSCRIPTION AND THE CLASS III GENES

The known pol III targets are genes encoding structural RNAs required for translation (all tRNAs, 5S ribosomal RNA, 7SL RNA), tRNA processing (RPR1; the RNA of the ribonuclease P complex), and splicing (i.e., U6). Though pol II has received most attention, it contributes to only 20% of the cellular transcription. The pol III transcription machinery is highly conserved in eukaryotes; it consists of the multi-subunit polymerase, and its two complex factors (Transcription Factor IIIB; TFIIIB and Transcription Factor IIIC; TFIIIC) required for promoter recognition and/or initiation. An additional factor (Transcription Factor IIA; TFIIIA) is required only for 5S rDNA transcription.

1.2.2 The enzyme RNA polymerase III:

The RNA polymerase III appears to be the most complex of the nuclear RNA polymerases as it contains the largest number of sub-units (Fig.1.1). Out of the 17 unique genes that encode these sub-units, 16 genes are essential and the role of the remaining one gene has not yet been specified (Sentenac et al. 1992; Chedin et al., 1998a; Geiduschek and Kassavetis, 2001; Ferri et al., 2000). Of these, the following five subunits form the core of the pol III and they are related to sub-units of the other two polymerases.

- Subunit C160 is ~160kDa and is evolutionarily related to A(I)190 and B(II)220 and also to the largest sub-unit of archaeal (A) and bacterial (B') RNA polymerases.
- The C128 subunit is related to A110, B150, archaeal B and bacterial β subunits.
- C40 (AC40) is common to RNA pol I and pol III and is evolutionarily related to pol II B45 (Rpb3), bacterial α and archaeal D sub-units.
- AC19 is related to B12.5 (Rpb11), bacterial α and archaeal L subunits.
ABC23 (Rpb6) is related to bacterial \( \omega \) and archaeal K subunits (Minakhin et al., 2001).

Of the 17 *S. cerevisiae* (sc) pol III sub-units, 5 are shared among pol I, II and III. They are ABC27, ABC23, ABC14.5, ABC10\( \alpha \) and ABC 10\( \beta \). Two sub-units are shared with pol I (AC19 and AC40). Eight sub-units seem to be pol III specific, out of which three have paralogues in pol II and/or pol I. From protein-protein interaction studies in yeast (Flores et al., 1999) it is known that five sub-units: C82, C34, C31, C17 and C10\( \alpha \) are capable of interacting with transcription initiation factors. BLAST search has shown that *S. pombe* has homologues of all 17 sc pol III sub-units (Huang and Maraia, 2001). The summary of known subunit homologies of pol III is given in Table 1.1.

Homologues of C82, C34 and C31 in humans (hRPC32, hRPC29, hRPC62) are involved in promoter-dependent transcription initiation in conjunction with accessory factors and this sub-complex directs RNA pol III binding to TFIIIC-DNA complex via the interactions between TFIIIB and hRPC39 (Wang and Roeder, 1997). Photoaffinity probing of pol III initiation and elongation complexes in *S. cerevisiae* (Persinger and Bartholomew, 1996) has shown that nine subunits crosslink with DNA. Of these, three RNA polymerase III specific subunits - C82, C34 and C31 form a sub-complex (Brun et al., 1997); C34 could be crosslinked farthest upstream on the DNA with both C82 and C31 located close to C34. The two largest subunits show crosslinks over almost the entire length of the complexes (Bartholomew et al., 1993). In the pre-initiation complexes, C128 was cross-linked to nucleotide positions \(+16/+17\) whereas C160 cross-links were both upstream and downstream from this C128 contact site. Also, the C53 (pol III specific subunit) was shown to contact TFIIIC. Though this contact may not be required for initiation, it might contribute to recruitment and correct positioning of pol III (Flores et al., 1999). The protein-protein interactions of pol III with TFIIIB and TFIIIC are summarized in Fig1.2.

One interesting finding by Hockman and Schultz (1996) was that Casein Kinase II is required for efficient transcription by RNA pol III on 5S rDNA templates in yeast. But, unlike RNA pol II, any phosphorylated component(s) in RNA pol III remain(s) unidentified.
<table>
<thead>
<tr>
<th>S. pombe pol III</th>
<th>S. cerevisiae homologue</th>
<th>Human homologue</th>
<th>Relationship to pols</th>
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<tbody>
<tr>
<td>Subunit</td>
<td>Mass (kDa)</td>
<td>Length (amino acids)</td>
<td>Subunit</td>
</tr>
<tr>
<td>Rpc158</td>
<td>158</td>
<td>1405</td>
<td>C160</td>
</tr>
<tr>
<td>Rpc130</td>
<td>130</td>
<td>1165</td>
<td>C128</td>
</tr>
<tr>
<td>Rpc66</td>
<td>66</td>
<td>571</td>
<td>C222</td>
</tr>
<tr>
<td>Rpc39</td>
<td>39</td>
<td>348</td>
<td>AC40</td>
</tr>
<tr>
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<td>330</td>
<td>C53</td>
</tr>
<tr>
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<td>242</td>
<td>C37</td>
</tr>
<tr>
<td>Rpc34</td>
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<td>Rpc24a</td>
<td>24</td>
<td>210</td>
<td>C31</td>
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<td>16</td>
<td>142</td>
<td>ABC23</td>
</tr>
<tr>
<td>Rpc24b</td>
<td>24</td>
<td>210</td>
<td>ABC27</td>
</tr>
<tr>
<td>Rpc25</td>
<td>23</td>
<td>203</td>
<td>C25</td>
</tr>
<tr>
<td>Rpc19</td>
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<td>125</td>
<td>AC19</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>ABC10α</td>
</tr>
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<td>8.3</td>
<td>71</td>
<td>ABC10β</td>
</tr>
<tr>
<td>Rpc17</td>
<td>14.9</td>
<td>129</td>
<td>C17</td>
</tr>
</tbody>
</table>

nd: not determined

Table 1.1: Comparison of pol III subunits of S. pombe, S. cerevisiae and humans.
(Huang and Maraia, 2001)
Fig. 1.2: Subunit interactions between *S. cerevisiae* RNA polymerase III, TFIIIB and TFIIIC:

Interactions deduced from two-hybrid analysis are marked with red dots, and those deduced indirectly from the interactions with the homologous large subunits of pol I are marked with green dots. Black arrows mark interactions inferred from multicopy suppression of temperature sensitive mutations (the arrowhead pointing to the suppressed mutation).

(Geiduschek and Kassavetis, 2001)
1.2.2 Promoters of pol III transcribed genes:

The pol III promoters as a class can be distinguished from pol I and pol II promoters because of the preponderance of gene-internal promoter elements i.e. they are downstream of the +1 (transcription initiation) site and within the transcribed region. These promoter elements are usually discontinuous and consist of blocks separated by non-essential regions. These intragenic control regions are categorized into several different consensus sequences, denoted as A, B and C blocks.

The Class III genes can be divided into different types based on the structure of their promoter elements (Fig. 1.3).

1.2.2.1 Type I promoter elements:

The 5S rRNA promoter is the only example for a type I RNA pol III promoter. The *X. laevis* 5S gene promoter consists of an A box, an intermediate element (IE) and a C box that are conserved in the 5S promoters of different species (Bogenhagen et al. 1980; Sakonju et al. 1980). The transcription factor that binds the promoter elements is the nine-zinc finger DNA-binding transcription factor (TF) IIIA. The promoter is usually intolerant to changes in spacing between individual elements (Pieler et al., 1987). This type of Internal Control Region (ICR) is found in 5S rRNA genes of many lower organisms including *Drosophila melanogaster* and *Saccharomyces cerevisiae*. In the *S. cerevisiae* 5S genes, only the “C” box is required for transcription (Challice and Segall, 1989).

1.2.2.2 Type II promoter elements:

This is the most common promoter arrangement and present in many genes transcribed by pol III, like tRNA genes, adenovirus Ad2 VA1 and VA2 genes, and 7 SL RNA gene (Galli et al. 1981; Hofstetter et al., 1981; Sharp et al., 1981; Allison et al., 1983). The type II promoter consists of two boxes: A and B present within the transcribed region. These form the binding sites for the transcription factor (TF) IIIC. These sequence elements are highly conserved in different tRNA species, probably because they encode the tRNA D and T loops which are important for tRNA function. The separation between A and B boxes in the tRNA genes are variable over a wide range. A separation of about 30-60 bp is optimal for simultaneous occupancy of both boxes by TFIIC, but wider separations can be tolerated (Baker et al. 1987; Fabrizio et
Fig. 1.3: Different types of RNA polymerase III promoters:

The type 1 promoter of the *X. laevis* 5S RNA gene consists of an internal control region (ICR), which can be subdivided into A box (+50 to +60), intermediate element (IE, +67 to +72), and C box (+80 to +90).

The type 2 promoter of the *X. laevis* tRNA<sub>Leu</sub> gene consists of an A box (+8 to +19) and a B box (+52 to +62).

The type 3 promoter of the *H. sapiens* U6 snRNA gene consists of a distal sequence element (DSE, -215 to -240) that enhances transcription and a core promoter composed of a proximal sequence element (PSE, -65 to -48) and a TATA box (-32 to -25).

The *S. cerevisiae* promoter is a hybrid promoter consisting of a TATA box (-30 to -23), an A box (+21 to +31), and a B box located downstream of the U6 coding region (from +234 to +244 relative to +1 site).

(Adapted from Schramm and Hernandez, 2002)
al. 1987). Type I and type II A boxes are structurally related and functionally interchangeable in *X. laevis* (Ciliberto et al., 1983) because of sequence similarity.

### 1.2.2.3 Type III promoter elements:

In contrast to the typical Class III\textsubscript{ICR} genes, several pol III genes lack ICRs. This can be associated with specialization of metazoan pol III transcription machinery. The U6 small nuclear RNA genes from several vertebrate species (Krol et al., 1987, Das et al., 1988; Kunkel and Pederson, 1988), and the human 7SK gene (Murphy et al. 1986) do not require intragenic promoter elements for transcription \textit{in vitro} and \textit{in vivo}. Their promoters are gene-external. They are located in the 5' flanking region of the gene and contain a proximal sequence element (PSE), a TATA box located at a fixed distance from PSE and a distal sequence element (DSE). These genes share certain promoter elements and transcription factors with pol II-transcribed snRNA genes (Hernandez and Lucito 1988, Kunkel and Pederson 1988, Lobo and Hernandez 1989). The presence of a TATA box helps the U-class genes to be discriminated by pol II or pol III. The U6 gene that contains a TATA box is transcribed by pol III and U2 gene that lacks TATA box is transcribed by pol II. The other genes with PSE include human genes encoding the RNA component of RNase P, MRP RNase (helps in maturation of RNA primer of mitochondrial DNA replication) as well as some RNAs of unknown function called Y1 and Y3 RNAs (Yuan and Reddy, 1991; Baer et al., 1990; Farris et al., 1996).

Although the vertebrate U6 gene lacks the gene internal promoter elements, in case of *S. cerevisiae* U6 snRNA gene, in addition to upstream TATA box, there is a gene internal A box and a B box downstream to the terminator. The structure of this gene is discussed in greater detail later in this chapter (Section 1.3).

Recently, some tRNA genes have been found that contain a TATA box in addition to boxes A and B (Dieci et al., 2000.). In *S. pombe*, the U6 snRNA gene contains gene-internal promoters and nearly all tRNA and 5S genes also contain a TATA box upstream to +1 that is required for transcription (Hamada et al., 2001).

### 1.2.3 Basal transcription apparatus of RNA pol III: Transcription Initiation Factors

The first transcription factor to be purified and cloned was Transcription factor (TF) IIIA from Xenopus (reviewed in Wolffe and Brown 1988). But, most information
regarding the pol III transcription initiation factors is available for *S. cerevisiae* and human systems. Ten essential genes seem to encode the sc pol III transcription initiation factors. They are genes encoding for TFIIIA, six subunits of TFIIIC and three subunits of TFIIIB.

1.2.3.1 Transcription Factor IIIA:

TFIIIA is the prototypical member of the zinc finger family of nucleic acid binding proteins. It was the first eukaryotic transcription factor to be purified that was necessary for the accurate transcription of a eukaryotic gene in vitro, and has become one of the most intensely studied eukaryotic DNA binding proteins (Engelke et al., 1980). The TFIIIA of *S cerevisiae* has been cloned and sequenced (Archambault et al., 1992). The TFIIIA contains nine zinc fingers and binds extensively to >50bp of 5S rDNA. The yeast protein is 429 amino acids long. DNase I footprinting showed that TFIIIA bound and protected a 50 bp region which spanned +45 to +95 of the 5S rRNA gene (Engelke et al. 1980). Because of the small size of the protein (37kDa) and the large size of the protection, it was initially thought that more than one protein bound to each of the 5S rDNA. But titration experiments showed that the 30kDa DNA binding domain associates with the DNA in a linear fashion along the entire 50bp protected region (Smith et al., 1984). TFIIIA can also bind 5S transcripts and is involved in feedback regulation of 5S rRNA synthesis in vivo (Andrews and Brown, 1987; Rollins et al. 1993; Brow and Geiduschek, 1987).

Comparison of the sequences of *S. pombe* (sp)TFIIIA, *S. cerevisiae* (sc)TFIIIA and human (h)TFIIIA (Wolfe et al., 2000) show that spTFIIIA is 30% identical and 45% similar to scTFIIIA and is 35% identical and 49% similar to hTFIIIA and that this similarity is limited to their zinc-finger domains. The C terminal zinc finger of scTFIIIA is separated from its nearest neighbor by an 89-amino acid loop in contrast to *Xenopus* TFIIIA, in which the spacers between the zinc finger domains are only 4-8 amino acids long (Miller et al., 1985). The unique feature of spTFIIIA is that it contains 10 C\(_2\)H\(_2\)C zinc fingers in contrast to nine fingers in other organisms. It is interesting to note that the *Xenopus* TFIIIA can be substituted for the human factor.
1.2.3.2 Transcription Factor IIIC:

The general transcription factor TFIIIC binds to the box A and box B promoter elements of the tRNA genes (and other genes with similar promoter elements) and to TFIIIA-DNA complex in case of 5S rRNA genes. From proteolytic studies, it is known that scTFIIIC is organized into two domains separated by a flexible linker. The domains are called $\tau_B$ (binds strongly to boxB) and $\tau_A$ (binds weakly to boxA) (Marzouki et al., 1986) and the flexible linker seems to be able to accommodate variously spaced box A and boxB.

The six subunit of *S. cerevisiae* TFIIIC (adding up to a mass of 520kDa) have been cloned and shown to be essential for cell viability (Willis et al., 1989; Lefebvre et al., 1992; Marck et al., 1993; Arrebola et al., 1998; Manaud et al., 1998; Deprez et al., 1999). The genes encoding these sub-units are TFC1, TFC3, TFC4, TFC6, TFC7 and TFC8 (Table 1.2). $\tau_B$ is composed of Tfc3 (132kDa), Tfc6 (75kDa) and Tfc8 (68kDa). Tfc8 serves as the linker between $\tau_B$ and $\tau_A$. Tfc6 and Tfc3 co-operate in binding DNA. Tfc6 is known to contact the transcriptional terminator, at the downstream end of the protein-DNA complex. $\tau_B$ complex forms the protease resistant core of TFIIIC and retains the ability to bind to its box B DNA site.

The upstream DNA-binding $\tau_A$ domain is made up of Tfc1 (74kDa), Tfc4/Pce1 (120kDa) and Tfc7 (49kDa). The Tfc4 is present upstream in the TFIIIC-DNA complex (Bartholomew et al. 1991) and possesses eleven tetratricopeptide repeat (TPR) motifs that have been implicated in the protein-protein interactions (Marck et al. 1993).

The location of the TPR repeats in the Tfc4 is given in Fig.1.4. Nine of the TPR repeats are present in the N-proximal half of Tfc4 as arrays of five and four repeats, and other two TPR repeats are located at the C-proximal half of Tfc4 as two separate repeats. Tfc4 subunit of TFIIIC helps in recruiting TFIIIB to the upstream region through its interactions with both Brf1 and Bdp1 (Moir et al., 1997; Moir et al., 2000; Chaussivert et al., 1995).

In the absence of TFIIIB, the TPR repeats of Tfc4 get internally folded forming antiparallel $\alpha$ helices and generate the globular structure of $\tau_A$, but when Tfc4 interacts with TFIIIB, these repeats unfold and Tfc4 bridges the gap between box A and TFIIIB binding site (Moir et al., 1997).
### Table 1.2: *S. cerevisiae* TFIIIC components and orthologues in *S. pombe* and *H. sapiens*  
(Schramm and Hernandez, 2002)

<table>
<thead>
<tr>
<th><em>S. cerevisiae</em></th>
<th><em>S. pombe</em></th>
<th><em>H. sapiens</em></th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFIIIC</td>
<td>TFIIIC</td>
<td>TFIIIC2a</td>
<td></td>
</tr>
<tr>
<td>Tfc3/Tfc3/t138</td>
<td>Sfc3</td>
<td>TFIIC220/TFIIIC</td>
<td>Tfc3 and Sfc3 are related, but neither shows sequence similarity to TFIIC220. Tfc3 cooperates with Tfc6 for binding to DNA. Fragments of TFIIC220 and TFIIC110 form a subcomplex capable of binding to the B-box.</td>
</tr>
<tr>
<td>Tfc4/ Sfc4</td>
<td>TFIIC102/TFIIIC</td>
<td>Tfc4 protrudes upstream of the start site. TPR repeats present. Tfc4 contacts ScBrfl, ScBdp1, and ABC10. Most conserved of the TFIIIC subunits. TFIIC102 associates with HsBrfl, HsTBP, TFIIC63.</td>
<td></td>
</tr>
<tr>
<td>Tfc1/ Sfc1</td>
<td>TFIIC63/TFIIIC</td>
<td>tRNA A-box binding. Tfc1 and Tfc7 associate and can form a distinct complex. TFIIC63 associates with TFIIC102, HsBrfl, HsTBP, and HsRPC62.</td>
<td></td>
</tr>
<tr>
<td>Tfc6 (t91) Sfc6</td>
<td>TFIIC110/TFIIIC</td>
<td>Binds terminator. HMG-I and HMG-Y motifs, WD-40 repeats. Similarity between Tfc6 and TFIIC110 apparent only through Sfc6. Tfc6 cooperates with Tfc3 for binding to DNA. TFIIC110 and TFIIC220 form a subcomplex capable of binding to the B-box. Full-length TFIIC110 absent in TFIIC2b. TFIIC110 possesses HAT activity.</td>
<td></td>
</tr>
<tr>
<td>Tfc8 (t60) Sfc9</td>
<td>TFIIC90/TFIIIC</td>
<td>Tfc8 bridges τB and τA domains as well as TFIIB. Associates with ScTBP. Similarity of Tfc8 and Sf9 limited to short C-terminal segment. No similarity between TFIIC90 and the yeast proteins, but TFIIC90 binds to TFIIC220, TFIIC110, HsBrfl, HsRPC62, and HsRPC39, and thus may be a functional homolog of the yeast proteins. TFIIC90 displays HAT activity for histone H3 Lys 14.</td>
<td></td>
</tr>
<tr>
<td>Tfc7 (t33)</td>
<td>none</td>
<td>none</td>
<td>tRNA A box binding. Tfc7 and Tfc1 associate and can form distinct complex.</td>
</tr>
</tbody>
</table>
Fig. 1.4: Structure domains of budding yeast Tfc4 / τ131 and its homolog hTFIIIC-110:

Tetratricopeptide repeats (TPR) are marked in green, basic domains in blue, acidic domains in red, and helix-loop-helix domains in brown. (Geiduschek and Kassavetis, 2001)
**S. pombe TFIIIC:**

Five subunits of *S. pombe* TFIIIC have been identified by BLAST searches, out of which four of them have been confirmed to be TFIIIC subunits using immunoaffinity purification of tagged TFIIIC from cells (Huang et al. 2000). They are Sfc1 (homologous to TFC1), Sfc3 (homologous to TFC3), Sfc4 (homologous to TFC4), Sfc6 (homologous to TFC6) and Sfc9 (no homologue in *S. cerevisiae*). Sfc4 consists of multiple TPR repeats and therefore may have functions similar to Tfc4. Sfc3, Sfc6, Sfc1 may also have similar functions as their homologues in *S. cerevisiae*. A putative fifth *S. pombe* TFIIIC subunit is Sfc9 that has homology with human TFIIIC90, shares sequence homology with the *S. cerevisiae* Tfc8 subunit only within a short C-terminal segment. The identification of *S. pombe* subunits is very interesting, as in some cases it clarifies the relationship between *S. cerevisiae* and human TFIIIC subunits.

**Human TFIIIC**

In contrast to scTFIIIC, human TFIIIC seems to be more complex and contains several functions; some of which have not been characterized completely. In a chromatographic analysis of hTFIIIC, Yoshinaga et al. (1987) found that it separated into two fractions named as TFIIIC1 and TFIIIC2. The TFIIIC2 sub-complex has been characterized to have 5 subunits and functional similarity with yeast TFIIIC. It exhibits box B binding. hTFIIIC1 stimulates binding by hTFIIIC2 and is required for transcriptional activity (Wang and Roeder, 1996; Kovelman and Roeder, 1992). The TFIIIC2 complex is present in two forms; TFIIIC2a and TFIIIC2b. The active form: TFIIIC2a (80-90% of total TFIIIC2) consists of subunits TFIIIC220, TFIIIC102, TFIIIC63, TFIIIC110 and TFIIIC90. The TFIIIC220 subunit helps TFIIIC2a to bind to box B albeit weakly. Interestingly, amino acid sequence of hTFIIIC220 is highly divergent and does not show homology with either scTfc3 or Sfc3 though they have similar function. The TFIIIC102 subunit is homologous to scTfc1 (Hsieh et al., 1999a). hTFIIIC220, hTFIIIC110 and hTFIIIC90 subunits of TFIIIC2a have intrinsic HAT activity and acetylate both free and nucleosomal histones (hTFIIIC 90 acetylates H3 Lys 14, hTFIIIC110 acetylates H3, H4 and nucleosomal H2B; Hsieh et al., 1999b and Kundu et al., 1999). The hTFIIIC2b (10-20% of total population of hTFIIIC2) in
actively dividing HeLa cells does not contain the TFIIIC110 subunit and contains an uncharacterized 77 kDa subunit (Hsieh et al., 1999b).

The holo TFIIIC also contains the hTFIIIC1 sub-complex that has 4 subunits with masses 70, 50, 45 and 40 kDa. The TFIIIC1 sub-complex strengthens the interaction of TFIIIC2 to box B but one paradox is that TFIIIC1 can have a direct role in recruiting RNA pol III even in the absence of TFIIIC2. In case of the human 7SK gene, in vitro transcription can take place in the absence of TFIIIC2 but needs the presence of TFIIIC1 (Yoon et al., 1995).

Thus, human TFIIIC is divergent from S. cerevisiae TFIIIC in all but two of its subunits (in TFIIIC 2a) that are located close to transcription start site and are involved in interaction with TFIIIB.

1.2.3.3 Transcription Factor IIIB

The scTFIIIB is made up of three proteins that have been well characterized: They are TBP (TATA box binding protein), Brf1 (TFIIB related factor 1) and Bdp1 (B’). TBP and Brf1 are associated with each other (even in the absence of DNA) and form a very stable complex called B’. Bdp1 or B” associates with B’ complex less stably and can be separated by chromatography (Kassavetis et al., 1991).

TBP

TBP is required for transcription by pol I, pol II and pol III. TBPs from different species are very highly conserved in their C-terminal regions but the N-terminal regions are more species specific. The TBP residues that are important in its interaction with Brf are located in its conserved region (Shen et al., 1998; Hernandez, 1993; Colbert et al., 1998). The human TBP cannot substitute for S. cerevisiae TBP but S. pombe TBP can; as the residues important for its interaction with Brf are conserved in the two yeast TBPs but not in the human TBP (Cormack et al., 1994; Teichmann et al., 1997). TBP binds to DNA and bends it sharply. This bend is retained even when it is complexed with Brf (Braun et al., 1992). In yeast, TBP is involved in transcription of both TATA containing and TATA-less gene. In case of TATA containing genes, TBP can recognize the strong TATA box and bind to DNA directly. Alternatively, TBP in TFIIIB can be recruited to the upstream site with the help of transcription initiation factor TFIIIC.
**scBrf**

The scBrf (referred to as Brf1, Willis 2002) is a 70 kDa protein that displays 23% identity and 44% similarity to TFIIB in its N-terminal 320 residues (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez de Leon et al., 1992). Though Brf1 has extensive homology with TFIIB the principal TBP binding domain of Brf1 is outside the region that is conserved with TFIIB (Kassavetis et al., 1998a, Kassavetis et al., 1997; Colbert et al., 1998; Shen et al., 1998). There are two separate TBP binding domains in Brf1 that contact opposite faces of TBP DNA complex. Brf1 also plays a critical role in holding TBP and Bdp1 together through a 110 amino acid homologous stretch conserved in Brf related protein from fungi to humans (reviewed in Geiduschek and Kassavetis, 2001). Other than TBP, scBrf (Brf1) interacts with B", TFC4, subunits C34 and C17 of pol III (Kassavetis et al., 1997). The Brf1-TFC4 interaction is required for TFIIIB recruitment on class III promoters, and the Brf1-C34 interaction is required for pol III recruitment.

**scBdp1**

The gene encoding Bdp1 of yeast was cloned simultaneously by Kassavetis et al. (1995, B"), Roberts et al. (1996, TFIIIB90) and Ruth et al. (1996, TFC7P). It encodes a 594 amino acid protein (67,688 Da); though it migrates as a 90 kDa “band” on a denaturing SDS gel. The scBdp1 has been biochemically well characterized. It functions in the assembly of pre-initiation complex (PIC) and is required for the formation of heparin resistant TFIIIB-DNA complex indicating its requirement for the stability of the pol III transcription initiation complex (Kumar et al., 1997). Bdp1 serves as a scaffold that forms a clamp on one side of TBP-DNA complex with the other side being clamped by Brf1. This makes the TFIIIB-DNA complex stable (Persinger et al., 1999). Bdp1 contains a domain related to a Myb repeat (Fig.1.5A) known as the SANT domain (was identified in SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and yeast TFIIIB Bdp1). The SANT domain is required for TFIIIC dependent RNA pol III transcription (Kumar et al., 1997). A region upstream of SANT domain (see Fig.1.5A) is required for transcription from linear but not supercoiled templates (Kassavetis et al., 1998b). In addition to pol III recruitment and TFIIIB complex stabilization, scBdp1 helps in upstream promoter melting in a pol III dependent manner.
Fig. 1.5: Schematic representation of the domains in Brf and Bdp1:

(A) Comparison of the ScBdp1 and HsBdp1 polypeptides. The proteins contain a SANT domain (maroon box). The region upstream of SANT domain (indicated in orange) is quite conserved and is required for transcription from linear, but not supercoiled, templates. The percentages indicate amino acid identities between ScBdp1 and HsBdp1 in the region bracketed by dotted lines.

(B) TFIIIB, Brf1 and Brf2 form a family of related transcription factors. The location of the structure zinc ribbon as modeled in ScTFIIIB and ScBrf1 and that of HsBrf1 and HsBrf2 is indicated in green. The location of the structured core domain of TFIIIB and that of the corresponding regions in the other proteins is indicated in blue. The percentages below the sequences indicate percent identities between the different proteins within the region of highest conservation.

(Schrann and Hernandez, 2002)
TFIIIB from humans and other organisms:
Humans, *Xenopus*, and silk moth have TFIIIB complexes similar in constitution and properties. Amongst these, the human TFIIIB has been very well characterized.

**hTFIIIB**

The human TFIIIB can be separated into two components α and β (Teichmann and Seifart, 1995). The type 1 and 2 promoters use different components in the TFIIIB fraction than type 3 promoters. Type 1 and 2 promoters require TBP containing complex (hTFIIIBβ) (Lobo et al., 1992; Teichmann and Seifart, 1995) that contains TBP and a homolog of scBrf1 called HsBrf1 (Wang and Roeder, 1995; Mital et al., 1996). The type 3 promoters use a protein related to Brf1 initially known as BRFU (or TFIIIB50) (Schramm et al., 2000; Teichmann et al., 2000), and now referred to as HsBrf2 (Willis 2002).

Fig. 1.5 B shows the structure of TFIIIB and Brf from humans and *S. cerevisiae*. All of them contain an N terminal zinc-binding domain similar to TFIIIB and a core domain consisting of two imperfect repeats. The Brf1 and Brf2 contain C terminal domains containing three regions I, II and III that are absent in TFIIIB and conserved in the different yeasts. The human Brf1 has only conserved region II and III (Mital et al., 1996). The C terminal of HsBrf2 does not show any homology to HsBrf1.

The HsBdp1 ORF was identified by database searches for sequences similar to scBdp1 SANT domain that is relatively conserved in *D melanogaster*, *S. pombe*, and *C. elegans*. The HsBdp1 was found to be a large protein (1388 amino acid residues), with 43% identity to the SANT domain of scBdp1. The region just upstream and downstream of SANT domain shows 21% and 17% identity with scBdp1 and the rest of the protein is not conserved. The C-terminal of HsBdp1 has 9.5 repeats of 55 amino acid residues with potential phosphorylation sites. The *Xenopus* TFIIIB seems to contain TBP and two proteins with molecular weights 75 and 92 kDa but their relationship with Brf and Bdp1 are not yet known.

**TRFs**

TRFs are TBP related factors and share sequence homology with TBP in their DNA binding core repeat domain. Two types of TRFs are found: TRF1 is found in
Drosophila and has homology to hBrf. TRF2 (also called TLF for TBP like factor) is found in most multicellular animals but not found in plants and fungi (Ohbayashi et al., 1999; Rabenstein et al., 1999; Teichmann et al., 1999; Berk, 2000). The Drosophila TRF1 is associated with pol III transcription of 5S RNA, U6 gene and several tRNA genes; and immuno depletion of this complex makes transcription of these genes defective. Recent in vivo studies imply that TLF works near promoters and is associated with pol II in a development dependent manner (Kaltenbach et al., 2000) but its role in pol III transcription is not known.

1.2.4 Transcription Complex Assembly on pol III Promoters

Type I and II promoters: The assembly of the transcription factors on type I and II promoters happens in a very well defined way. TFIIIC performs the following functions
(a) It recognizes promoter elements, directly in the case of type II promoters and with the help of TFIIIA in the case of type I promoter
(b) It recruits TFIIIB
(c) It helps in recruiting RNA pol III

On the SUP4 tRNA^Tyr gene, photo cross-linking experiments have shown that the scTfc3 subunit crosslinks just upstream of box B and scTfc6 cross-links at the terminator (Bartholomew et al., 1990, 1991). The Tfc1 and Tfc7 crosslink on opposite sides of the DNA helix in the vicinity of box A and are involved in a weaker and less sequence selective interaction of TFIIIC at that site. The Tfc4 is conformationally flexible and projects upstream of transcriptional start site to contact scBrf1 and scBdp1 subunits of TFIIIB. The scTfc8 does not cross-link with DNA (see Fig. 1.6).

In humans, the scTfc3 counterpart, TFIIIC220 can be cross-linked to box B (Yoshinaga et al., 1989; Kovelman and Roeder, 1992). N-terminal region of TFIIIC220 interacts with TFIIIC110 (homolog of scTfc6) and generates the DNA binding surface of TFIIIC2a complex. The TFIIIC90 subunit is the functional homolog of scTfc8 and interacts with TFIIIC220, TFIIIC110, TFIIIC63 and HsBrf1 (of TFIIIB) (Hsieh et al., 1999b). Therefore, Tfc8/TFIIIC90 seems to act as a bridge between \( \tau_B \) and \( \tau_A \) domains of TFIIIC, which therefore extends over the entire gene.
Fig. 1.6: A schematized cartoon of transcription factors assembled onto a tRNA gene:

(A) The Transcription Initiation complex on a prototype Pol III promoter in S. cerevisiae

(B) The cartoon reflects the relative evolutionary conservation of individual TFIIB and TFIIC subunits. The positions of the A box and the B box promoter elements are indicated. The arrow indicates the direction and start site of transcription. ‘T’ indicates the site of transcription termination. The minimal number of T residues required for efficient termination by S. cerevisiae (6, S.c), S. pombe (5, S.p) and human (4, H.s) polys III are indicated schematically.

(Huang and Maraia, 2001)
The ortholog of scTfc4 is TFIIC102 and it interacts with TFIIC63, TBP and HsBrf1 (Hsieh et al., 1999).

On the 5S RNA genes, the scTFIIIA cross-links to boxA strongly and extends over a large portion of the gene (Braun et al., 1992). The TFIIC in turn binds to TFIIIA to generate a rigid structure. The TFIIC contacts on 5S rRNA gene seem shifted downstream with cross-links at 3' end of C box. Tfc6 contacts the terminator even in the 5S rRNA gene.

The main function of TFIIC after binding to the promoter elements is to recruit TFIIIB. The TFIIC-DNA complex initially interacts with Brf1 component of TFIIIB (Kassavetis et al., 1992) through the Tfc4 subunit. The weak interaction of Brf1 with TFIIC-DNA complex is stabilized by TBP binding due to TBP generated structural transition in Brf1. The binding of Bdp1 to the B' complex further stabilizes the TFIICB-DNA complex and makes it extremely resistant to dissociation by poly ions such as heparin. Once recruited, yeast TFIIIB occupies a region ~ 40bp upstream of +1. It subsequently recruits pol III to the promoter and positions it over the +1 region. In humans, TFIIC102 and TFIIC63 associate with both HsBrf1 and HsTBP; TFIIC90 associates with HsBrf1 and this network of protein-protein interaction helps in recruiting TFIIIB during transcription initiation.

Several TFIIC subunits interact with pol III subunits and these interactions are required for pol III recruitment. In S. cerevisiae, Tfc4 subunit interacts with C53 and ABC 10 α subunits of pol III. In humans, TFIIC90 interacts with HsRPC62 and HsRPC39 that are required for transcription initiation. Thus, the above interactions suggest that not only TFIIIB, but also TFIIC is involved in recruiting RNA pol III to the initiation site.

Type III promoters: The type III promoters of vertebrates (7SK and U6 snRNA genes) contain binding sites for TFIIIA and TFIIC2 and require transcription factors distinct from most vertebrate pol III transcribed genes. The promoter structure of vertebrate U class snRNA genes includes a gene-upstream distal sequence element (DSE) and a proximal sequence element (PSE). All members of the U class genes have similar promoters but some of them are transcribed by pol II while others are transcribed by pol III.
The PSE is recognized by a factor called SNAPc (the snRNA activator protein complex) or PTF (the proximal element transcription factor). SNAPc is made up of five different subunits SNAP190, SNAP50, SNAP45, SNAP43 and SNAP19 (reviewed in Schramm and Hernandez, 2002; Hernandez, 2001; Geiduschek and Kassavetis, 2001).

In addition to PSE, which forms the core sequence, the DSE enhances transcription from the core promoter. The DSE contains an SPH element that recruits transcription factor STAF and an octamer sequence which recruits transcription factor Oct 1 (reviewed in Hernandez, 2001). STAF is a seven-zinc finger protein and Oct 1 is a POU domain protein with a bipartite DNA binding domain made up of two helix-turn-helix containing DNA binding modules. TBP is also required for the human U6 gene transcription and the SNAPc complex helps TBP to bind TATA box (Mittal and Hernandez, 1997).

The S. cerevisiae type 3 promoter is very different from that of human and other metazoans in having a gene internal box A element and a downstream box B element. Transcription factor assembly on this gene is discussed greater detail later in this Chapter (in Section 1.3)

1.2.5 A minimal RNA pol III transcription system from human cells:

Hu et al. (2003) have shown that a highly purified pol III complex combined with the recombinant transcription factors SNAPc, TBP, Brf2 and Bdp1 directs multiple rounds of transcription initiation and termination from the human U6 promoter. The pol III complex contained traces of CK2 that associated with the U6 promoter region in vivo. The CK2 phosphorylation of pol III was required for transcription and phosphorylation of TBP, Brf2 and Bdp1 combined was inhibitory for transcription.

1.2.6 Transcription elongation, termination and recycling:

The genes having internal promoter elements usually have their transcription complex bound to the transcribed regions and therefore obstruct the RNA pol III during transcription. The ability of pol III to get past these obstacles can be a rate-limiting step and can lead to the formation of abortive products. But, on a SUP4 tRNA\textsuperscript{Tyr} gene, it was observed that the amount of abortive products produced was relatively
low in yield and were principally di- and tri-nucleotides (Bhargava and Kassavetis, 1999). The first round of transcription by pol III is much slower than the subsequent rounds, so, it has been proposed that pol III never leaves its template but recycles directly from termination to re-initiation on the same gene (Dieci and Sentenac, 1996). Each molecule of pol III undergoes only about three rounds of abortive initiation before going into elongation phase. It has been observed that both TFIIIA and TFIIIC present only a negligible kinetic barrier to the elongating pol III (Matsuzaki et al., 1994).

The RNA pol III is different from other eukaryotic polymerases in having the ability to recognize short stretch of T residues as the termination signal. \( T_4 \) is enough for *Xenopus* and human pol III (Bogenhagen and Brown, 1981); \( T_4\text{-}T_5 \) for *S. pombe* pol III (Hamada et al., 2000) and \( T_6 \) for *S. cerevisiae* pol III (Allison and Hall, 1985); the efficiency of termination is dependent on the flanking sequence. The C11 subunit of pol II seems to be involved in efficient termination of transcription (Chedin et al., 1998b).

In higher eukaryotes (eg. humans) a number of factors have been implicated in helping efficient termination and RNA pol III recycling. These include TFIIIC1, topoisomerase 1, PC 4, NF1 and La protein. The La protein binds to poly U tail at the end of the pol III transcripts and helps transcript release and re-initiation of transcription (Maraia, 1996). La also participates in the 5' and 3' tRNA end processing and helps in assembly of snRNPs in yeast and in humans (Fan et al., 1998; Xue. et al., 2000). The TFIIIC1 fraction contains a polypeptide called NF1 which can either homodimerize and recognize a consensus sequence close to the T residues in the VA1 gene or with TFIIIC220 and TFIIIC110 of human TFIIIC2 (Wang et al., 2000) and prevent the formation of read through transcripts. But the exact mechanism of action of this factor is not known. Both topo 1 and PC4 are present in trace amounts in the TFIIIC holo enzyme and have been implicated in suppressing read-through transcript *in vitro* (Wang and Roeder, 1998). Their role *in vivo* is not defined.

Experiments with *S. cerevisiae* tRNA gene suggest that RNA polymerase recycling on pre-assembled tDNA-TFIIIC-TFIIIB complexes is much faster than the initial transcription cycle (Dieci and Sentenac, 1996). After the first transcription cycle on a template having PIC, pol III seems to be committed in a termination dependent manner to reinitiate and more rapidly transcribe the same template in the subsequent rounds.
1.3 THE *S. cerevisiae* U6 sn RNA GENE

The *S. cerevisiae* U6 sn RNA gene (SNR6) was first isolated and characterized by Brow and Guthrie in 1988 (Fig.1.7). They found that the U6 gene is in a single copy, is essential and has two upstream sequence elements that are also found upstream of mouse and human U6 genes. RNA sequencing revealed that the 3' end of SNR6 is heterologous and contains 4, 5, 6 or 7 contiguous U residues. In contrast, primer extension of total yeast RNA showed that the 5' end of SNR6 is homogenous and the yeast U6 comprises a set of RNAs 112-115 nucleotides in length. When the SNR6 coding region was compared with mammalian U6 snRNA, about 60% identity was found over the entire sequence. Upon analysis of sequences upstream of SNR6 gene, they found two regions similar to those implicated in transcription of the vertebrate U6 gene. One was centered at -30 with respect to the initiation site and contained a consensus TATA box (with a 10 bp perfect match between the mouse and yeast genes). Another sequence was found further upstream, in the region between -40 and -60, in the yeast and human genes with a 11 bp perfect match.

The U6 snRNA is the only spliceosomal snRNA synthesized by RNA pol III; the rest of snRNAs are transcribed by pol II. Brow and Guthrie (1990) conducted gross mutational analysis of the SNR6 promoter to look for distinctive properties. They found that in addition to the TATA like element at -30 and a PSE at -50, the SNR6 gene has an conveniently placed intragenic box A beginning at position +21 that is identical to the A box consensus at six out of the nine non-degenerate positions and an essential promoter element (box B) positioned 120-130 bp downstream of its 3' end. The DNA sequence of SNR6 box B matches closely to that of consensus box B internal control element that forms the primary binding site for TFIIIC in tRNA genes. Deletion of 2 bp that are essential for TFIIIC binding destroyed the U6 promoter function both *in vitro* and *in vivo*. Therefore, the yeast U6 gene is different from its vertebrate counterpart and from other yeast pol III transcription genes in the following two features.

(i) Unlike vertebrate U6 gene, it uses the conventional pol III promoter elements

(ii) Its essential promoter element (box B) is downstream of the gene

In addition to the box A at +21 position, the SNR6 gene also has a "cryptic A box" (at ~ +145) that has been responsible for the production of 235 to 295 nucleotide transcripts from SNR6 clones in whole cell extracts with initiation at ~ +125 position.
Fig. 1.7: Structure of *S. cerevisiae* U6 snRNA gene

The *S. cerevisiae* U6 snRNA gene is a single copy essential gene containing a TATA box present between \(-30\) and \(-23\), an A box present between \(+21\) and \(+31\) and a gene-downstream B box present between \(+234\) to \(+244\) relative to the start site of transcription. The terminator consists of a stretch of T residues starting from position \(+113\).
downstream of the U6 gene. The PSE like element located ~ -50 bp was found to be dispensable for the cell and could be replaced without diminishing viability or affecting transcription in vivo or in vitro (Eschenlauer et al., 1993). Margottin et al. (1991) showed that purified TFIIIB supplemented by either purified TBP or bacterial extracts expressing recombinant TBP could initiate transcription by pol III in a box B independent manner that is not seen in case of most tRNA genes. These results suggest that TFIIIB is capable of recruiting pol III to the SNR6 promoter in vitro on naked DNA templates without the help of either TFIIIA or TFIIIC.

In contrast, TFIIIC has been shown to be essential for SNR6 transcription in vivo and on chromatin templates reconstituted either using Xenopus egg extract or by salt dialysis method (Burnol et al., 1993a). When nucleosomes are assembled on SNR6 gene, the TFIIIB directed transcription is blocked and TFIIIC can relieve this repression in a box B dependent manner. In tRNA genes, increasing the box A-box B distance reduces transcription in vitro but in the case of SNR6 it has been observed that reducing the distance between boxes A and B weakens transcription in vivo (Eschenlauer et al., 1993). These results suggest that in vivo, and on assembled chromatin, box B might be placed much closer to box A due to chromatin mediated foreshortening of the intervening DNA and this might have a role in allowing TFIIIC to bind to both promoter elements (boxes A and B) and assemble TFIIIB onto the SNR6 TATA box.

Electron microscopic studies of DNA bound TFIIIC showed that the two domains of TFIIIC (τA and τB) were mainly found in 2 different conformations (a) as separate entities (dumb bell shaped complexes) or (b) as a single, large complex associated with standard size tRNA genes (Burnol et al., 1993b). Though TFIIIC could undergo major conformational changes, the distance between the two domains was within 25 nm (about 70 bp) and seemed to be physically linked by a thin, undetectable connection arm.

Another interesting observation was that when the box B was inverted, the polarity of the τA binding in the dumb bell shaped complex changed suggesting that despite its palindromic sequence, B box does not act as a symmetric element. Burnol et al. have also shown that mutations in the A box interfered with the transcription of SNR6 gene both in vitro and in vivo implying that box A is an important promoter element.
The role of TFIIC in placing TFIIB on SNR6 in vivo was studied by Gerlach et al. (1995). They found that though TATA box helps in selection of box A (A1 vs. A2), the precise selection of start site is done by box A. Their experiments suggest that TFIIC binds to box A, interacts with TFIIB and stabilizes it on the upstream region, covering the TATA box. However, Gerlach et al. did not observe a protection over the box A region, indicating that the affinity for TFIIC to box A is relatively low, either because of its sequence, or due to the large distance between box A and box B. Interestingly, Burnol et al. (1993b) have found that increase in the SNR6 box A consensus (A up mutation) increases TFIIC dependent transcription of SNR6 gene in both crude yeast extract and with purified proteins. Also, A up mutation allows transcription even in the absence of box B in a TFIIC dependent system.

Therefore, yeast SNR6 gene is an ideal example in which three weak promoter elements (block B being functionally weakened by its position with respect to box A) appear to cooperate to activate SNR6 transcription.

1.4 TRANSCRIPTION ON CHROMATIN TEMPLATES

The compaction of ~2m of DNA into the nucleus has severe consequences for processes that require access to DNA. This compaction is achieved by the binding of histones that can mediate successive orders of DNA folding. The nucleosome, the basic subunit of chromatin consists of 146 bp of DNA wrapped around the outside of an octamer of histones (two copies each of H2A, H2B, H3 and H4) by ~1.75 left handed super helical turns. The length of the linker DNA (the DNA between two adjacent nucleosome particle) varies on an average of ~15-60 bp depending on the species (van Holde, 1989; Wolffe, 1999).

The amino terminal tails of the four histones protrude beyond the confines of the nucleosome, with H2B and H3 tails passing between the DNA strands on the surface of the nucleosome and H2A and H4 tails projecting from the sides of the nucleosome (Luger et al., 1997). The histone N terminal tails are flexible and can adopt various conformations, and influence the behavior of the nucleosomal DNA. The nucleosomal DNA can also fold into higher order structure and the histone tails seem to play an important role in forming these structures (Carruthers et al., 2000). In addition to the histone octamer, the linker histone called H1 is required to stabilize the nucleosomal structure. These histones are small, lysine rich proteins that bind to the...
external surface of the nucleosome at a ratio of about 1 molecule per nucleosome but, interestingly unlike the core histones, they are not essential for cell viability.

Though packaging of DNA into chromatin is beneficial to the cells in terms of gene regulation by constraining interaction with specific DNA sequences, it also acts as a major obstacle to protein factors that need to access DNA. Activation of transcription is usually accompanied by a reorganization of chromatin structure facilitating the access of the required transcription factors to the DNA. Although chromatin structure is decondensed in actively transcribed DNA, nucleosomes are still present throughout the transcribed region. Therefore, it is easy to comprehend that the cell must make use of multiple mechanisms to render important regulatory elements accessible. They may be achieved by:

(a) Exclusion of histones from key DNA sequences (like promoters)
(b) Specific arrangement of the relevant surface of the DNA helix on the histone core such that it is accessible to the trans-acting factors.

Both conformations or conditions can be generated by the precise positioning of nucleosomes around the eukaryotic promoter and the gene region. There are many in vivo examples of positioned nucleosomes (Flaus and Richmond, 1998; Puig et al., 1999; Schild et al., 1993; Komura and Ono, 2003; Baumann et al., 2003; Svaren and Horz, 1993; Ioshikhes and Trifonov, 1993 for nucleosomal DNA sequence database). Nucleosome positioning can occur through specific positioning sequences on the DNA or through the interaction with trans-acting factors both acting as passive boundaries, or by specific contact between histones and other proteins. Positioning of nucleosomes can be defined using two parameters.

(a) **Translational positioning**: this defines the region of DNA (a defined bp length) within a given sequence that is wrapped around the histone octamer.
(b) **Rotational positioning**: this refers to the angular orientation of the individual base pairs on the nucleosomal surface (or) which face of the double helix is in contact with or exposed away from the histone core.

Analysis of DNA sequence preferences for histone octamer suggests that deformable DNA such as T:A sequences usually lie about +/- 1.5 and 3.5 turns from the dyad axis. AA/TT sequences at the minor groove usually face histone octamer and GC/GC sequences in the minor groove face away from the histones. The DNA at the dyad axis is usually relatively straight. The accessibility of factors to their cognate binding site in the chromatin is dependent on both translational and rotational positioning.
Translational positioning would determine whether the binding site is within the nucleosomal particle or in the linker region. If present within the nucleosome, the rotational orientation of the site would influence the transcription factor binding.

A classical example of positioned nucleosomes is the 5S rRNA gene sequence of the sea urchin *Lytechinus variegatus*. Multiple nucleosome positioning is an inherent property of this DNA sequence. The central 50-60 bp of the gene are responsible for the translational positioning of the nucleosome (Fitzgerald and Simpson, 1985). This region contains the sharp bends and structural discontinuities in DNA observed within the nucleosome. These positioning signals are recognized primarily by the (H3-H4)$_2$ tetramer (Hayes et al., 1991; Dong and van Holde, 1991) the (H2A-H2B) dimer does not recognize the positioning signal. The Zn finger protein TFIII A continuously contacts two 10 bp segment within the 5S rRNA gene (from bp +51 to +61 and +81 to +91). However, this interaction with DNA cannot happen with the simultaneous wrapping of DNA around the histone core. Functional studies have shown that prior association of a positioned histone octamer with a 5S rRNA gene inhibits transcription but a positioned histone tetramer (H3-H4)$_2$ allows transcription (Tremethick et al., 1990; Almouzni et al., 1991; Clark and Wolffe, 1991). Modification of histone octamers by acetylation could also facilitate assembly of TFIII A. Therefore, the cell has many different events that contribute synergistically to establish transcriptionally active states depending on specific promoters.

Once transcription initiation occurs, the RNA polymerase has to encounter nucleosomes during its elongation phase. Various models have been proposed to describe what might happen to a nucleosome as polymerases pass. They are based on dissociation, octamer transfer, and partial release of histones or unfolding (reviewed in van Holde et al., 1992). Various investigators have done experiments to clarify which of the above four scenarios occur during elongation but none can be either unambiguously excluded or rigorously affirmed by the existing evidence. One conclusion that can be made is that various genes adopt different methods depending on the trans acting factors and polymerase they are associated with.
1.5 CHROMATIN REMODELING IN EUKARYOTIC GENE EXPRESSION

The control and regulation of eukaryotic transcription requires interplay between various large enzymatic complexes known as chromatin remodelers that are recruited by sequence specific promoter or enhancer binding proteins. Chromatin remodelers alter the structure of chromatin such that the DNA is either made more, or less accessible for transcription factors. The regulation could be exerted at all the steps of the process, including chromatin recognition, covalent modifications of the histones, basal transcription factors and co-activator recruitment and the formation of elongation competent transcription complex that can read through nucleosomomal array.

1.5.1 Chromatin remodeling complexes:

Chromatin modifying and remodeling complexes act by targeting nucleosomes either through perturbation of histone DNA interactions, or through covalent modification of histone tails. The remodeling complexes contain a DNA dependent ATPase subunit and are ATP dependent. The modifying complexes contain one or more histone acetyl transferase (HAT) or histone deacetylase (HDAC) proteins.

1.5.1.1 ATP dependent nucleosome remodeling complexes

All ATP dependent chromatin remodeling factors identified till date are multi subunit complexes that contain an ATPase subunit, which belongs to the Swi2/Snf2 ATPase super family. There are four different classes of ATPase subunits: the SWI2/SNF2, ISWI, CHD and INO80.

The SWI2/SNF2 class

The SWI2/SNF2 class includes yeast SWI/SNF (originally identified in mating type switching and sucrose non-fermenting mutations), yeast RSC, Drosophila Brahma complex, the human BRM (hBRM) and human BRG1 (hBRG1). The conserved ATPase subunits in the above complexes are Swi2/Snf2, Sth1, Brm, hBRM and BRG1 respectively. All of them have a homologous ATPase domain, a bromo domain in the C-terminal region and two other conserved regions of unknown function called domain 1 and 2. The yeast SWI/SNF contains 11 subunits and is able
to increase accessibility of nucleosomal DNA in an ATP dependent manner (reviewed in Vignali et al., 2000).

The catalytic subunit of RSC (remodels structure of chromatin) complex is Sth1 and is essential for cell viability. RSC complex is more abundant than SWI/SNF in yeast cell (thousands of molecules vs. 100-200 of SWI/SNF. Chromatin immuno precipitation (ChIP) followed by microarray analysis has shown that RSC is localized at many promoters and is involved in both positive and negative regulation of transcription (Damelin et al. 2002, Ng et al., 2002).

The ISWI class
ISWI stands for “imitation switch” and the distinguishing feature of this class is the presence of two SANT-like domains in the C-terminal third of the enzyme. In Drosophila, there are 3 ISWI containing complexes namely NURF (nucleosome remodeling factor), ACF (ATP utilizing chromatin assembly and remodeling factor) and CHRAC (chromatin accessibility complex). NURF is able to facilitate initiation of transcription from repressed chromatin templates in vitro, because of its ability to increase access of promoter sequences to transcription factors (Mizuguchi et al., 2001).

ACF complex consists of ISWI and Acf1 (a bromo domain and PHD finger protein). ACF catalyzes nucleosome sliding, increases chaperone dependent nucleosome deposition on DNA, and facilitates the regular spacing of nucleosomes in an array. CHRAC is very similar to ACF. It induces nucleosome sliding and facilitates conversion of irregularly spaced nucleosomes to a regular array.

The CHD class
The CHD class of ATPases (eg. Chd1 in yeast, Tran et al., 2000) are characterized by the presence of a pair of chromodomains. This class of factors contains Mi2 that is present in complexes containing HDAC subunits and methylated DNA binding proteins. Therefore, they are believed to be repressive for transcription (Wade et al., 1999; Zhang et al., 1998).

The INO80 class
INO80 is a new class of ATPases first identified in S. cerevisiae and characterized by the presence of a unique ATPase domain that is split into two sub domains (Shen et
al., 2000). Ino80 complex (Ino80.com) from budding yeast is a 1.0-1.5 MDa complex containing DNA helicases called Rvb1 and Rvb2 that are homologous to bacterial RuvB, which is involved in DNA recombination and repair. The presence of Rvb proteins in INO80.com suggests a coupling between chromatin remodeling and DNA repair (Shen et al., 2003).

1.5.1.2 COVALENT MODIFICATION OF HISTONES

Histone acetyl transferases
HATs function by transferring an acetyl group from acetyl CoA to the ε-amino group of certain lysine residues within the N terminal tail regions of the histones. The HATs can be divided into the following five families: (reviewed in Carrozza et al., 2003; Sterner and Berger, 2000; Brown et al., 2000).

Gcn 5 related acetyl transferases (GNATs):
The HATs belonging to this super family show sequence and structural similarity to Gcn5 (Neuwald and Landsman, 1997). This group includes the HAT Gcn 5, its close relatives and also chromatin assembly related Hat 1 complex, the elongator complex subunit Elp 3, the mediator complex subunit Nut 1 and Hpa 2 (reviewed in Roth et al., 2001). They share functional domains that include an N-terminal region of variable length, an acetyl transferase domain, a region that interacts with Ada2 and a C-terminal bromodomain. Members of the GNAT family are important for the regulation of cell growth and development probably due to their role in transcription and DNA repair.

MYST (for MOZ, ybf2/Sas3, Sas2 and Tip 60)-super family:
The MYST family is composed of a group of HATs that are involved not only in transcription activation and DNA repair but also have roles in cell cycle and growth control, transcription activation, gene silencing and DNA repair. The MYST domain includes the acetyl-CoA binding motif as well as a C2HC zinc finger that is important for HAT function. This family includes human Tip 60, MOZ, yeast Sas2 and Sas3, Esa1, human HBO1 and MORF, Drosophila MOF and mouse Querkopf (Utley and Cote, 2003).
p300/CBP HATs:
p300/CBP is a large protein (first discovered form HeLa nuclear extracts) of about 300 kDa. p300/CBP can acetylate amino tails of all the 4 core histones. p300/CBP can also acetylate various transcription related proteins other than histones (Ogryzko et al., 1996). Therefore, it is a versatile acetyl transferase and acts as a global co-activator in higher eukaryotes.

TBP-associated factor TAFII250:
One of the subunits of the general transcription factor TFIID (TAFII250 in humans, TAFII230 in Drosophila and TAFII145/130 in S. cerevisiae) shows HAT activity in vitro (Mizzen et al., 1996). The HAT activity of TAFII250 and its homologs may be required for facilitating binding of TBP to TATA box by acetylation of histone tails.

Nuclear receptor coactivators:
HAT proteins may mediate transcription activation by hormone signals. Human co-activators such as ACTR and SRC1 that interact with nuclear hormone receptors have HAT activities, and can stimulate ligand dependent activation by numerous nuclear receptors by acetylating H3 and H4. Both of them are known to interact with CBP and PCAF and are acetylated by CBP (Chen et al., 1997; Leo and Chen, 2000).

Histone Deacetylase Complexes (HDACs)
HDACs fall into three main classes (reviewed in Narlikar et al., 2002). The class I HDAC family contains HDAC1 and HDAC2 present in the Sin3 and NuRD complex. The members of the class II HDACs are yet to be purified. The class III HDAC contains Sir2 and is involved in heterochromatin silencing at silent mating loci, telomeres and ribosomal DNA. NuRD contains Mi2 implying that, remodeling by Mi2 increases the accessibility of the histone tails for HDAC1 and 2 (Tong et al., 1998). Therefore, the presence of a ATP dependent remodeling activity and histone deacetylase activity in NuRD suggests a cooperation between these two classes of activity.

1.5.2 The Histone Code Hypothesis
Decondensation of the chromatin fiber can occur due to histone acetylation through neutralization of positive charge on the lysine residues or through histone
phosphorylation, by the addition of a negative charge. But there have been clues to suggest that a single histone modification does not function alone. The “histone code hypothesis” says that multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions (Fig.1.8). So how is the histone code translated? The modification marks on the histone tails provide binding sites for effector proteins. There are evidences to show that bromodomain containing proteins interact with acetylated lysines in the histone N-terminal tails and chromodomains are the targeting modules for methylation marks for H3 at Lys 9 (Winston and Allis, 1999; Bannister et al., 2001; Nakayama et al., 2001). There are various interplays between different histone tails. One example is that histone H3 N-terminus exists in two distinct modification states regulated by a “switch” between Lys 9 methylation and Ser 10 phosphorylation. Ser 10 phosphorylation inhibits Lys 9 methylation but is coupled with Lys 9 and/or Lys 14 acetylation during mitogenic and hormonal stimulation in mammalian cells. H3 phosphorylation is required for mitotic chromosome condensation. But Lys 9 methylation antagonizes Ser 10 phosphorylation and this is known to cause mitotic chromosome dysfunction (Rea et al., 2000). Also, deacetylation of Lys 14 at H3 is required for subsequent Lys 9 methylation by Clr4 histone methyl transferase. A single H3 Lys 9 methyl epitope recruits HP1 in heterochromatic region; acetylation of Lys 12 in H4 is repressive in nature and brings about a silent chromatin state.

Therefore, these observations indicate that one histone modification can influence other modifications either in a synergistic or an antagonistic way (reviewed in Jenuwein and Allis 2001; Strahl and Allis 2000; Zhang and Reinberg 2001; Ng and Bird 1999).

1.6 INTERACTIONS BETWEEN TRANSCRIPTION FACTORS AND CHROMATIN REMODELERS

Most genes are regulated by mixing and matching different types of co-activators and repressors in a coordinated fashion (Fig.1.9). Consistent with this notion, many transcriptional cofactors are multi subunit complexes, containing different TAFs that can interface with diverse enhancer and promoter factors, thus accommodating a limited number of multi subunit co-regulator complexes. Transcriptional co-regulators can be classified into (Lemon and Tjian, 2000):
Fig. 1.8: The "histone code" hypothesis:

Distinct H3 (red) and H4 (black) tail modifications act sequentially or in combination to regulate unique biological outcomes. Relevant proteins or protein domains that are known to interact or associate with distinct modifications are indicated. (Strahl and Allis, 2000)
Fig. 1.9: Schematic representation of potential linkages between Chromatin and Transcription
(Kadonaga, 1998)
(i) Activator and repressor targets present in the core machinery such as TAFs, TFIIA, NC2, PC4.

(ii) Activator and repressor adaptors that modulate DNA binding and target other co-regulators eg. VP16, E1A.

(iii) Multi functional co-regulators that interact with pol II and other activators (eg. yeast: mediator complex, SRBs; humans: TRAP, DRIP, PC2 etc.).

(iv) Chromatin modifying activators and repressors, acetyl transferases and deacetylases that act on histones, histone related proteins, activators, regulators and the core transcription machinery (eg. CBP/p300, GCN5, HDAC1 and 2, Sir2).

(v) ATP dependent chromatin remodelers such as SNF2, ISWI ATPases.

How are these complexes assembled and targeted to specific promoters? This can occur in a stepwise way where co-operative remodeling events by ATP-dependent remodelers and HATs can lead to nucleosome shifting which can make the DNA accessible to other transcription factors and core machinery. Alternatively, a pre-assembly model has also been proposed because of the evidence that RNA pol II co-purifies with subsets of basal machinery along with co-regulators including chromatin remodeling factors such as SWI/SNF and CBP (Parvin and Young, 1998). The model suggests targeted recruitment of a pre-assembled holo enzyme containing chromatin remodeling factors, multiple coregulators, RNA polymerase, core initiation machinery and RNA processing factors. The advantage of this model versus the previous one is to facilitate rapid responsiveness to arrayed regulators that can cooperatively recruit the transcriptional machinery via targeting of multiple interfaces.

1.6.1 A role for RNA polymerases in chromatin remodeling:

From recent studies, it has become apparent that RNA polymerases can cause mobilization of nucleosomes in a way similar to ATP dependent remodelers. The eukaryotic RNA polymerases seem to deal with chromatin in different ways in vitro and in vivo. Nucleosomes are disrupted on active 5S rRNA gene which is transcribed by pol I but the chromatin remodeling mechanism is not known (Dammann et al., 1993).

Many RNA polymerases (E. coli, pol II, pol III and bacteriophage SP6 and T7) can transcribe through nucleosome in vitro (reviewed in Thoma, F, 1991). SP6
and pol III can transcribe through intact nucleosomes (Studitsky et al., 1997) without removing them. Studitsky et al. found that when pol III transcribes through single nucleosomes assembled in vitro, a direct internal nucleosome transfer occurs in which the histones never leave the DNA template. This transfer can occur either by displacement of histones and recapture by the DNA or directly transferred out of the path of the advancing polymerase (O'Donohue et al., 1994). Experiments by Clark and Felsenfeld (1992) suggest that this transcription dependent nucleosome mobilization involves DNA bulging during transcription. An advancing polymerase enters the nucleosome, DNA is partially dissociated from the octamer forming a loop and because of uncoiling of DNA due to transcription, a bulge is formed that can rotate over the surface of the octamer, resulting in nucleosome translocation in cis. Since SP6 RNA pol functions in a similar way (Studitsky et al., 1994), there does not seem to be a requirement of specialized mobilizing activity to carry out the above process.

By contrast, pol II transcribed genes are known to retain chromatin structure. Bhargava (1993a and b) used time-resolved fluorescence emission spectroscopy to show that during read-through of the template by the pol II, histone octamers do not fall off the DNA. Only minor conformational changes within the histone octamer take place to accommodate the transcribing polymerase.

Kireeva et al. (2002) have shown that when pol II transcribes through mononucleosomal templates, one H2A-H2B dimer is displaced from the histone octamer and results in conversion of a nucleosomes to hexasomes. It was also observed that nucleosomes form a greater barrier to pol II than to pol III and SP6 in vitro. Since pol II transcribes long nucleosomal arrays in vivo, one might expect it to have functions that would help it transcribe through nucleosomes. The higher nucleosomal barrier for pol II makes it necessary to have factors that facilitate chromatin transcription. These factors include pol II elongation factors, ones that bind to histone proteins and chromatin remodelers.

Orphanides et al. (1998) have characterized one such factor called FACT (facilitates chromatin transcription). FACT can stimulate transcription through chromatin by RNA pol II in a highly purified system in vitro. Saunders et al. (2003) have shown that in vivo, FACT is associated with actively transcribed pol II genes and displays kinetics of recruitment and chromosome tracking similar to pol II driven transcription by destabilizing nucleosomal structure such that one histone H2A-H2B
dimer is removed during the enzyme passage. FACT can interact with all the 4 core histones in vitro and possesses intrinsic chaperone activity that allows it to deposit core histones to DNA. Thus FACT may help pol II by inducing disruption of nucleosome and re-assembling intact octamers behind the transcribing enzyme. Spt16/Pob3 (yFACT); the yeast equivalent of FACT promotes transcription by pol III through its interactions with Nhp6 (Brewster et al., 2001; Formosa et al., 2001).

1.7 AIM OF THE THESIS WORK

Packing of DNA as chromatin generates gene-specific architecture that can regulate transcription. Chromatin is generally repressive for transcription; but this repression can be overcome with the help of protein complexes that can modulate chromatin structure. ATP dependent chromatin remodeling factors and histone acetyl transferases have been studied mainly in the context of pol II genes, and hardly any have been found to be specific to pol III genes. Chromatin has been shown to play a very important role in pol III gene transcription (Paule and White, 2000; White, 2004; Wolffe, 1999). Nearly the entire stretch of the pol III genes are covered with their "own" transcription factors suggesting a competition between transcription factors and occupancy by nucleosomes in this region.

The human U6 promoter contains a core sequence element called PSE and an upstream enhancer element (present ~150bp upstream) called DSE that serves to enhance transcription from the core promoter. SNAPc and Oct1 bind co-operatively to their two spatially separated PSE and DSE sites. A positioned nucleosome in the upstream region between PSE and DSE was shown to bring the two promoter elements into greater proximity to allow co-operative binding of Oct-1 and SNAPc to result in activated transcription (Stunkel et al., 1997; Zhao et al., 2001). This is one example of how a positioned nucleosome can have a role in activated transcription.

The role of chromatin in transcription of the yeast U6 snRNA (SNR6) gene could be very interesting because of several characteristic features associated with it. The single copy gene has a strong TATA box to which yeast transcription factor III B can bind directly in the absence of a chromatin background. Therefore, transcription initiation on naked DNA templates in vitro can occur in the absence of TFIIC. In addition to the TATA box, SNR6 has a box A sequence in its canonical location ~20bp downstream to the start site of transcription and an extragenic box B located
~200bp downstream of box A (Fig 1.7). It is easy to conceive that the large separation between the two boxes may make it difficult for TFIIIC to bind both boxes simultaneously. Nevertheless, transcription of SNR6 in vivo and in crude extracts in vitro was shown to be absolutely dependent on TFIIIC. TFIIIC was shown to alleviate repression due to chromatin structure in the SNR6 gene region in vitro (Burnol et al., 1993), suggesting that TFIIIC has an important role to play in chromatin transcription of this gene.

The study of relationship between chromatin structure and transcriptional activity of the SNR6 gene in vivo started about a decade ago, when Marsolier et al. (1995) reported that the transcriptionally active U6 RNA is flanked on both its 5' and 3' sides by arrays of positioned nucleosomes. Prevention of TFIIIC binding by a 2 bp deletion in the box B region not only debilitated transcription, but also resulted in a loss of positioned nucleosomes in the flanking regions. Thus, the chromatin structure of the U6 gene in vivo was found to correlate with its functional state. Further, in vivo MNase and DNase 1 footprinting experiments showed that when TATA box was substituted or the distance between boxes A and B was reduced by 42 bp, there was a loss of protection over the upstream region making the TATA box hypersensitive to MNase. The in vivo footprinting analysis also suggested that the productive binding of TFIIIC to SNR6 is sensitive to A and B box spacing and directly implicated the existence of structured chromatin between these two boxes.

The unusually large separation between box A and box B, its contrasting requirements for TFIIIC in vitro and in vivo suggest that in order to allow TFIIIC binding in vivo, foreshortening of spacer DNA probably mediated by chromatin maybe required to bring the two TFIIIC binding sites closer together.

In this context, the present study aims to understand the TFIIIC-mediated mechanism of de-repression of transcription on SNR6 gene as a chromatin template. The following questions are asked in this study:

(a) Is the de-repression of transcription by TFIIIC on chromatin templates accompanied by a positioning of a nucleosome between the two boxes?
(b) How does TFIIIC access its binding sites in chromatin?
(c) What are the steps involved in making TATA box accessible for pre-initiation complex assembly?

In order to answer these questions, we used Drosophila embryonic assembly extract to assemble regularly spaced nucleosomes on a plasmid containing the SNR6 gene.
We carried out *in vitro* transcription assays using purified proteins in the continued presence of chromatin remodelers present in the S-190 extract. Structure analysis was done using high and low resolution footprinting techniques to look for chromatin remodeling accompanying the binding of TFIIIC. The results from this thesis work suggest that TFIIIC, which is a basal transcription factor for pol III genes, also plays a direct role in chromatin remodeling and transcriptional activation on the yeast SNR6 gene.