Dissertation

Role of RNA Polymerase I in Maintaining The Chromatin State of rRNA Genes

Submitted by

Scott Fields

Department of Biochemistry and Molecular Biology

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SCOTT FIELDS ENTITLED ROLE OF RNA POLYMERASE I IN MAINTAINING THE CHROMATIN STATE OF rRNA GENES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Paul Laybourn

U. LUGER

ERICA Selkman

Laurie Stargell

Advisor Marvin R. Paule

Department Head Norm Cartwright

Abstract of the Dissertation

Role of RNA Polymerase I in Maintaining the Chromatin State of Ribosomal RNA Genes

Eukaryotic cells have between 150 and 25,000 copies of rRNA genes that are expressed by RNA polymerase I (pol I), which is its only role in the cell. Psoralen cross-linking showed there are two populations of rRNA genes: nucleosomal and nucleosome free, and cross-linking of pulse-labeled nuclei demonstrated only the latter are actively transcribed. Following DNA replication, all rDNA is packaged by nucleosomes, but about half subsequently get stripped. Pol I has been implicated in nucleosome stripping in yeast, because rRNA genes become nucleosomal in the absence of active pol I. This contrasts with genes transcribed by pol II, which remain nucleosomal during transcription. We found, *Acanthameoba castellanii* Pol I initiated at the rRNA promoter displaces nucleosomes from DNA *in vitro*. A nucleosome slows the elongation rate of pol I by approximately two-fold, but elongation eventually is completed without the aid of other factors or increased ionic strength. Similarly, *in vivo*, psoralen cross-linking of *Saccharomyces cerevisiae* rRNA genes that are anomalously transcribed by pol II in a polymerase switch (*PSW*) mutant

revealed the pol I specific nature of pol I stripping as these genes are not stripped of nucleosomes. The ability of pol I to strip nucleosomes plays a key role in maintaining the correct chromatin structure observed for rRNA genes.

Scott Fields
Department of Biochemistry and Molecular Biology
Colorado State University
Fort Collins, CO
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Chapter 1

BACKGROUND AND SIGNIFICANCE

1.1 DNA DEPENDENT RNA POLYMERASES

In eukaryotes, RNA is transcribed from DNA by three types of DNA dependent RNA polymerases. RNA polymerase I transcribes the 18S, 5.8S and 28S rRNA genes as a single transcript that gets processed into the individual RNAs that are incorporated into the ribosome. RNA polymerase II transcribes mRNAs that are translated into proteins. RNA polymerase III transcribes the 5S rRNA gene, transfer RNA genes, and some small RNAs. Each polymerase has its own method for initiating and terminating transcription as well as their own means of regulating transcriptional activity (Paule, 1998).

1.2 ORGANIZATION OF RIBOSOMAL RNA GENES

Ribosomal RNA (rRNA) genes are transcribed by RNA polymerase I (pol I). In an actively growing cell, 75% of the total RNA produced is rRNA. This is required to meet the demands of the cell for ribosomes used in mRNA

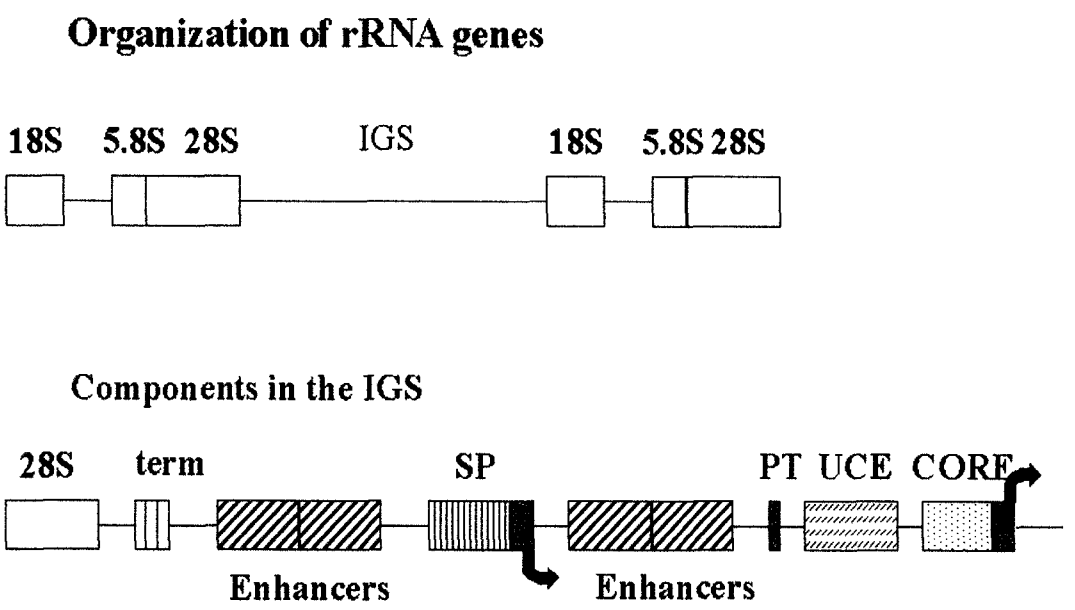
translation. To meet this demand, the rRNA genes are multiple copy. rRNA genes are organized into head to tail repeats. The number of copies is species dependent, with *Saccharomyces cerevisiae* having 150 copies and *Acanthamoeba castellanii* having 600, but *Xenopus laevis* oocytes can have up to 500,000 copies following amplification. Between each copy is the intergenic spacer that contains all of the promoter elements including the upstream promoter element, core promoter, terminators, and enhancer regions (Figure 1.1) (Paule, 1998). While rRNA promoter element sequences are not well conserved between species, the spatial organization of these elements is similar (Pape et al., 1990; Xie and Rothblum, 1992), and it contains several subelements.

The core promoter is located just upstream of the transcription initiation site (tis) and extends upstream to about –40bp. The core promoter can be broken down into two separate regions. A region surrounding the tis and the upstream element (Iida et al., 1985; Kownin et al., 1985). The tis is very well conserved between species and extends from –10 to +10 and is AT rich (Perna et al., 1992), and contains an element called the initiator element (Inr) that can weakly initiate transcription on its own, but requires upstream elements for high levels of transcription (Radebaugh et al., 1997). Upstream portions of the core contribute to factor binding (Kownin et al., 1985).

The upstream promoter element is located about –150bp relative to the tis (Skinner et al., 1984; Windle and Sollner-Webb, 1986; Windle and Sollner-Webb, 1986). Spacing between the upstream promoter element and the core is critical for function in rRNA transcription (Pape et al., 1990; Xie and Rothblum, 1992).

A. castellanii transcription factors:

- **TIF-IB**
- **TIF-IE**
- **RNA polymerase I**



S. cerevisiae transcription factors:

- **Upstream Activation Factor**
- **Core Factor**
- **RRN 3p**
- **RNA polymerase I**

Figure 1.1. Organization of rRNA genes. rRNA genes are multiple copy and arranged in head to tail repeats separated by the intergenic spacer. All of the regulatory elements are in the IGS. Listed are the primary transcription factors found in *Acanthamoeba* and yeast.

The importance of the upstream promoter element is species specific, and may not be required at all for some species, such as *A. castellanii.*

The proximal terminator (PT) is located 200bp upstream of the tis (Grummt et al., 1986; Henderson and Sollner-Webb, 1986; McStay and Reeder, 1986). The proximal terminator plays several key roles in rRNA transcription. The proximal terminator ensures pol I transcription of an upstream gene is terminated prior to reaching the promoter of a downstream gene (Bateman and Paule, 1988). The PT also plays a role in remodeling chromatin by recruiting remodeling factors to the DNA via termination factors (Langst et al., 1998; Langst et al., 1997; Strohner et al., 2001). In some species, factors that bind the PT loop the promoter DNA in such a way that places the recently terminated polymerase I molecule in close proximity to the start site of another promoter (Kulkens et al., 1992).

1.3 TRANSCRIPTION FACTORS USED IN POL I TRANSCRIPTION

1.3.1 RNA POLYMERASE I

Pol I is a multisubunit complex consisting of 14 subunits and has a molecular weight of approximately 650 KDa. It has five subunits that also exist in the other two RNA polymerases (e.g. ABC 23), two subunits that exist in pol III and seven unique subunits not found in either of the other two polymerases (D'Alessio et al., 1979). When recruited to the tis, pol I can melt the DNA (without the need for ATP hydrolysis) (Perna et al., 1992) melting approximately

9bp DNA. After the first phosphodiester bond forms, and the polymerase clears the promoter, the transcription bubble enlarges to approximately 20bp (Kahl et al., 2000). The size of the transcription bubble remains steady during transcription (Kahl et al., 2000). The RNA:DNA hybrid formed during transcription remains at a steady state of 9bp during transcription (Kahl et al., 2000).

1.3.2 TRANSCRIPTION INITIATION FACTOR-IB

In all organisms, RNA pol I is recruited via its interaction with the core transcription factor **T**ranscription **I**nitiation **F**actor **IB** (TIF-IB). This transcription factor (also known as SL-1 in humans and Core Factor in yeast) binds to the core promoter and recruits pol I to the tis (Iida et al., 1985; Kownin et al., 1987; Schnapp and Grummt, 1991; Steffan et al., 1996). TIF-IB consists of TBP and three TBP associated factors called TAFs (Paule and White, 2000). TBP itself does not bind the rRNA promoter directly, but instead binds via the TAFs (Steffan et al., 1996). TIF-IB has been shown to directly interact with pol I during recruitment, and is necessary for spatial positioning of pol I over the transcription initiation site (tis). In *A. castellanii*, bound TIF-IB cannot be competed from DNA when challenged by another pol I promoter (Radebaugh et al., 1998; Windle and Sollner-Webb, 1986)

1.3.3 UPSTREAM ACTIVATION FACTORS

Upstream of the core promoter is the upstream promoter element. Upstream Activation Factors bind this element and aid in recruitment or stabilize

the binding of the fundamental transcription factor to the promoter. The importance of this element, and the transcription factors that bind it, varies from species to species. In humans and *Xenopus*, **U**pstream **B**inding **F**actor (UBF) is necessary for transcription (Bell et al., 1990; Stefanovsky et al., 2001). It binds DNA as a homo-dimer and has an architectural role by bending the DNA (Bell et al., 1990; Pikaard et al., 1990; Smith et al., 1990). This bending of DNA aligns the upstream promoter element and the core promoter elements near each other, which contributes to transcriptional efficiency (Bazett-Jones et al., 1994; Putnam et al., 1994; Stefanovsky et al., 2001). In other mammals, basal specific transcription can occur in the absence of UBF. However, UBF is required for activated transcription (Smith et al., 1990). In yeast, **U**pstream **A**ctivation **F**actor (UAF) binds the UPE and recruits core factor and pol I to the core promoter. UAF is necessary for activated transcription, but is not required for basal transcription (Keys et al., 1996), and unlike what is observed in UBF, there is no evidence UAF binds the UPE as a dimer and there is no evidence for DNA bending for UAF.

1.3.4 TRANSCRIPTION FACTOR RRN3P

In all systems, a transcription factor called Rrn3p is also required for promoter driven transcription. In the cell, there are two forms of RNA polymerase I. One population is found associated with Rrn3p and is active for specific transcription, the other, is inactive for specific transcription and not associated with Rrn3p (Bodem et al., 2000; Fath et al., 2001; Hirschler-Laszkiewicz et al., 2003; Miller et al., 2001; Yamamoto et al., 1996). The regulation of Rrn3p

association with pol I appears to be an important step in regulating pol I it is growth regulated (Bodem et al., 2000). In yeast, inactive Rrn3p is phosphorylated while active/pol I bound Rrn3p is not (Fath et al., 2001). In mouse however, it is the unphosphorylated form of Rrn3p that is associated with pol I (Cavanaugh et al., 2002). In fact, bacterial expressed Rrn3p that is presumably unphosphorylated cannot activate mouse rRNA transcription with pol I lacking Rrn3p (Cavanaugh et al., 2002). In *A. castellanii*, phosphorylation of either Rrn3p or pol I subunits interacting with Rrn3p is important as treatment of these proteins with phosphatase inhibits transcription (Joe Gogain unpublished results). The role of Rrn3p in pol I transcription varies depending on species. In humans, Rrn3p is required for recruitment of pol I (Miller et al., 2001). In yeast and mouse, however, pol I can get recruited to the promoter but it cannot initiate transcription (Aprikian et al., 2001; Schnapp and Grummt, 1991), raising the question of the role RRN3p plays in pol I promoter clearance or formation of the first phosphodiester bond.

1.3.5 ACANTHAMOEBA CASTELLANII TRANSCRIPTION INITIATION FACTOR-IE.

In *Acanthamoeba castellanii*, there appears to be no upstream element or transcription factor required for activated transcription, but another factor, TIF-IE, stabilizes TIF-IB binding (Figure 1.2) (Al-Khouri and Paule, 2002). TIF-IB lacking TIF-IE does not stably bind to promoter DNA. TIF-IE does not bind DNA during TIF-IB stabilization, but is associated with the complex along with TIF-IB, possibly eliciting a conformational change that stailized the TIF-IB complex with

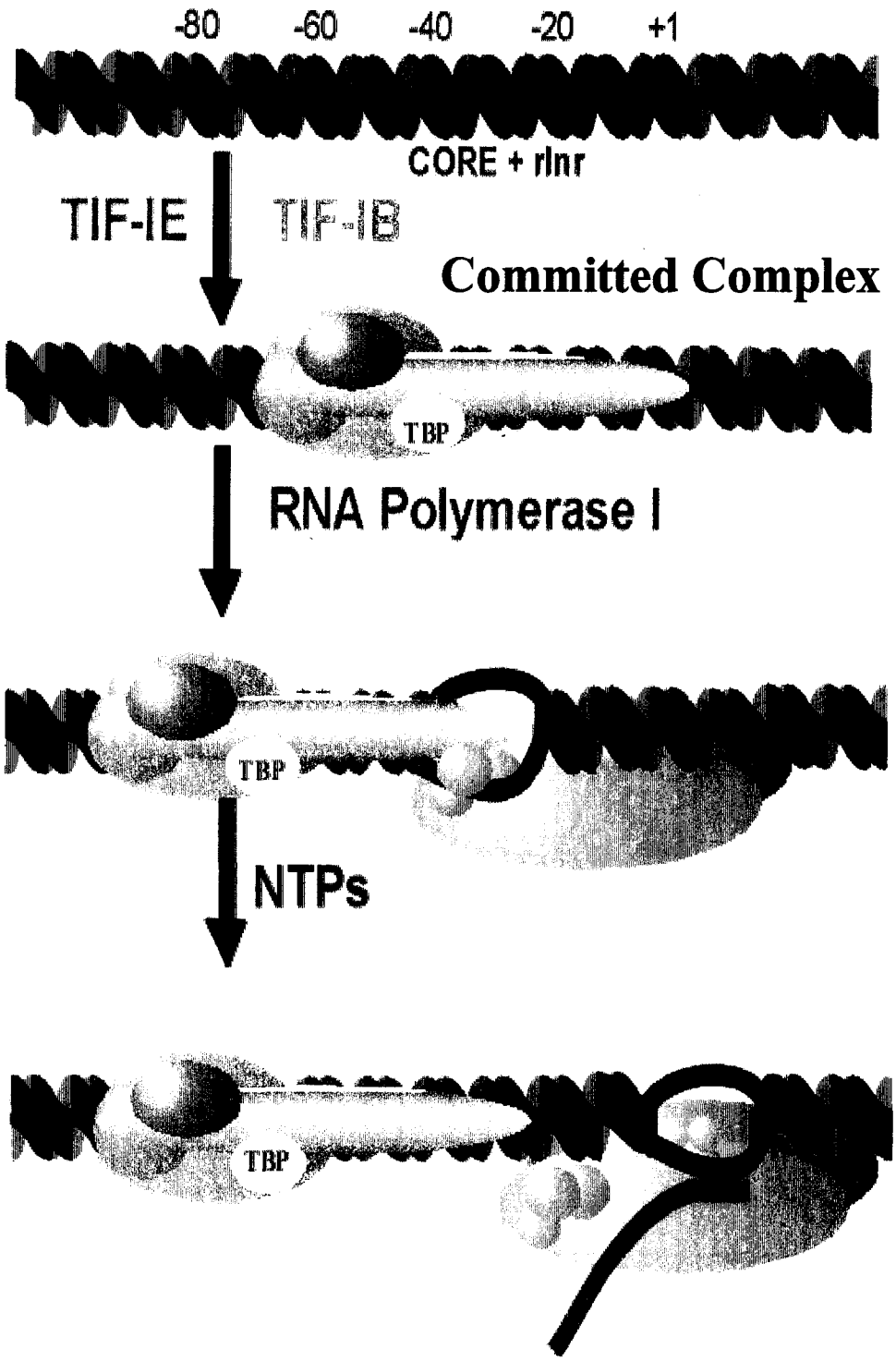


Fig. 1.2 Mechanism of transcription initiation in *Acanthamoeba castellanii.* TIF-IB binds to the promoter and recruits pol I via protein-protein interaction. TIF-IE stabilizes TIF-IB binding, but does not bind to the DNA.

the promoter. This factor is associated with both TIF-IB and RNA polymerase I during purification, but it is unknown if it plays a role in pol I recruitment.

1.4 NUCLEOSOME COMPONENTS AND STRUCTURE

In eukaryotes, DNA is packaged into chromatin, the fundamental unit of which is the nucleosome. The nucleosome consists of four core histones (H2A, H2B, H3, and H4), with two of each forming a core histone octamer (Johns, 1967; Kornberg, 1974; Kornberg and Thomas, 1974). About 146bp of DNA winds around the core histone octamer forming what is called the nucleosome core particle. Histone H1 then binds the NCP where the DNA enters and leaves the core particle to form the chromatosome, which, in total, contains approximately 166bp of DNA depending on the species (McGhee and Felsenfeld, 1980; Simpson, 1978; Varshavsky et al., 1976). The complete nucleosome unit consists of the chromatosome and linker DNA (which spans the distance between chromatosomes) with a typical length of 195bp (McGhee and Felsenfeld, 1980; Simpson, 1978; Varshavsky et al., 1976). Chromatosomes can form structures such as the "beads on a string" form seen in the 11nm fiber, or can compact giving the 30nm and higher order fibers (Thoma, 1992).

1.5 CHROMATIN PROFILE OF RIBOSOMAL RNA GENES

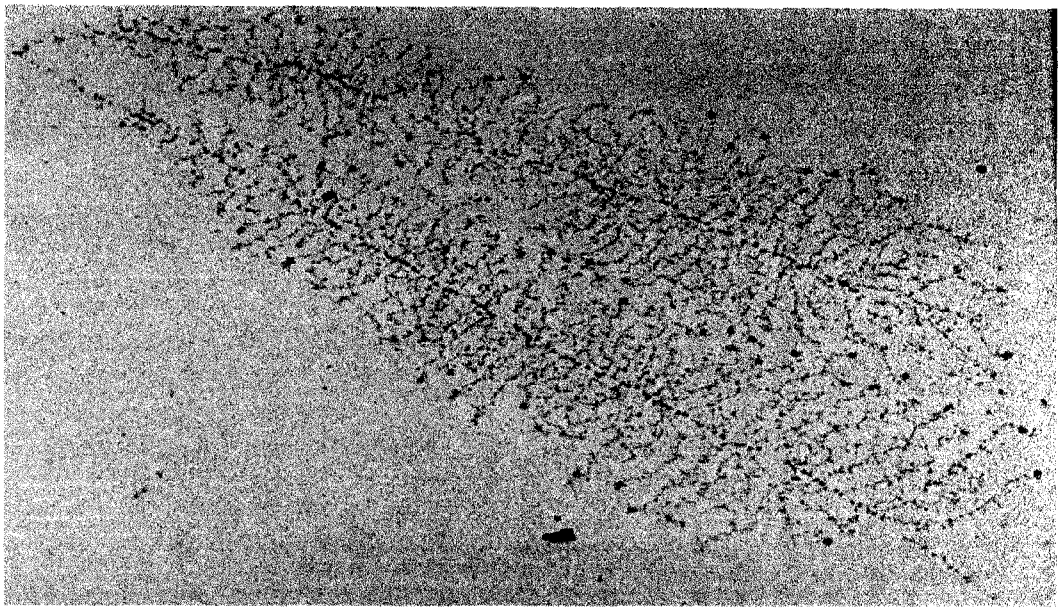
1.5.1 ELECTRON MICROSCOPY

Miller spreads (electron micrographs) of nucleolar genes show that active rRNA genes are devoid of nucleosomes (Miller and Beatty, 1969). In fact, the compaction ratio of the active genes is 1.6 to 1 versus 6-7 to 1 for typical "beads

on a string" chromatin (Figure 1.3). Active rRNA genes are packed with transcribing polymerase I molecules (as many as 100 per gene) prohibiting deposition of nucleosomes on the DNA. However, in *Xenopus* oocytes, active genes remain non-nucleosomal even when transcriptional activity is reduced (Figure 1.4) (Scheer, 1978). In this case, the number of polymerases on the gene is greatly reduced, allowing space for the deposition of nucleosomes, but they remain non-nucleosomal. Inactive genes demonstrate a typical "beads on a string" chromatin structure (Miller and Beatty, 1969). Learning how the cell maintains this nucleosome free state of rRNA genes is one of the objectives of this project.

1.5.2 PSORALEN CROSS-LINKING

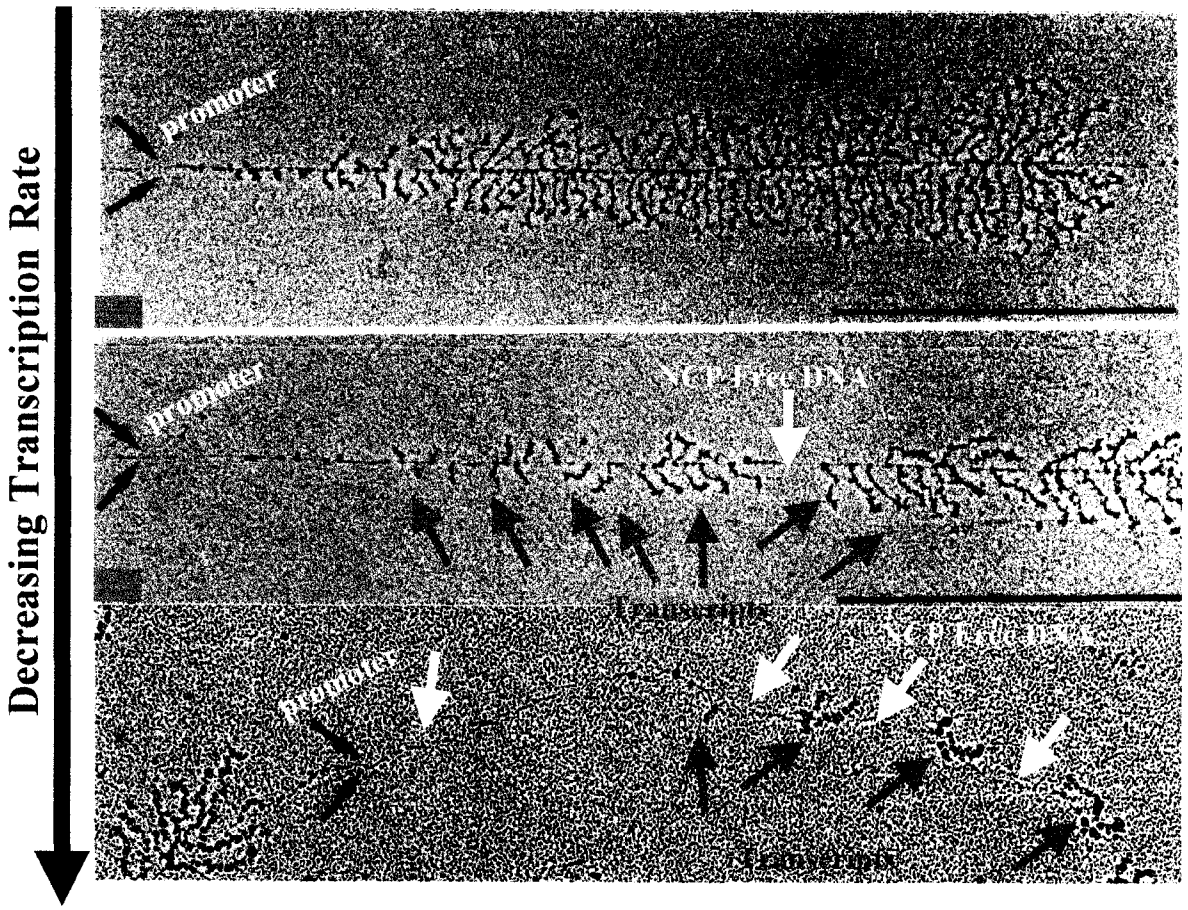
Psoralen is a DNA intercalating agent that cross-links thymine residues on opposite strands of DNA when exposed to UV light (Cimino et al., 1985) (Figure 1.5). Psoralen can be used to probe chromatin structure because nucleosomes block psoralen intercalation. Psoralen cross-linking of rRNA genes show there are two populations of rRNA genes: nucleosomal and non-nucleosomal (Amero et al., 1988; Colavito-Shepanski and Gorovsky, 1983; Conconi et al., 1989; Dammann et al., 1993; Dammann et al., 1995; Lucchini and Sogo, 1992; Lucchini and Sogo, 1995; Widmer et al., 1988). This was elucidated because heavily cross-linked DNA (which is free of protein- e.g. nucleosomes) migrates slower during gel electrophoresis than less frequently cross-linked DNA (e.g. nucleosomal) (Conconi et al., 1989; Sinden et al., 1980). After treating nuclei or cells with psoralen, genomic DNA was isolated followed by digestion with EcoRI.



The ratio of the known length of the gene to its measured length yields compression ratio

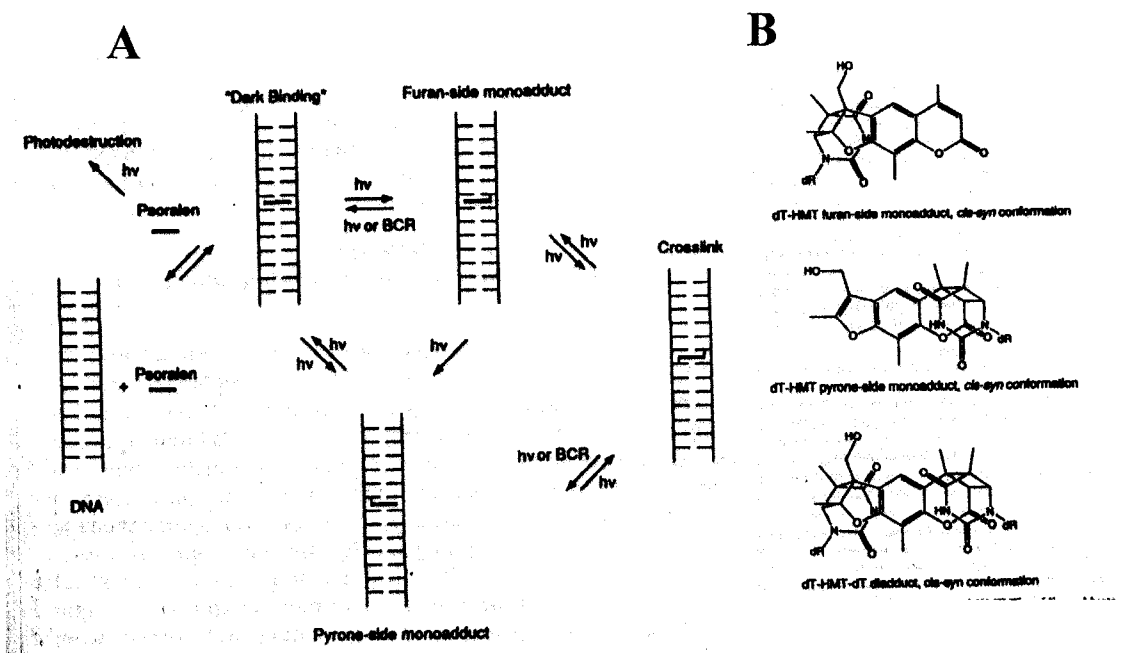
Miller. Science (1969)

Figure 1.3 Electron micrograph of rRNA genes shows a compression ratio of 1.6:1 as compared to 6:1 for "beads on a string" chromatin. On an actively transcribing gene, there are as many as 100 RNA polymerase I molecules transcribing the gene at the same time yielding the typical Christmas tree appearance.



Scheer. Cell (1978).

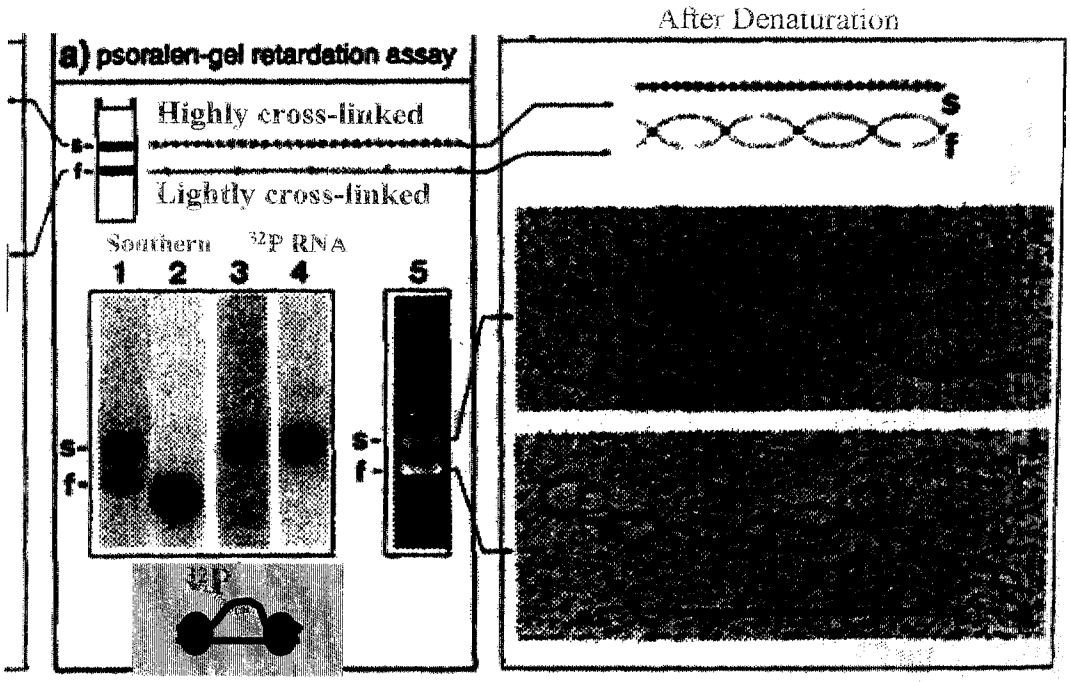
Figure 1.4. Electron micrograph of *Xenopus* oocyte rDNA. Notice when transcription rate decreases the nucleosome content remains the same even though enough space is available for nucleosome deposition.



Cimino et al., 1985

Figure 1.5. Mechanism of psoralen action. Psoralen is a DNA intercalating agent that cross-links thymine residues on opposite strands when exposed to UV light (A). (B) shows the mono and diadducts formed during cross-linking.

Digested DNA was resolved on an agarose gel and Southern blotted. The blot was probed with a specific rDNA probe, and two populations of rRNA genes were observed: a fast (nucleosomal) and a slow (non-nucleosomal) (Figure 1.6). Pulse-labeling of nuclei with radioactive precursors, followed by psoralen cross-linking, revealed only the slower migrating, non-nucleosomal fraction was active for transcription. Since psoralen will cross-link RNA:DNA hybrids, genes that are actively transcribed become labeled. (Figure 1.6) (Conconi et al., 1989). This ratio of nucleosome free to nucleosomal DNA was found to be invariant to growth conditions in mice and plants, but this ratio does change to some degree in humans and yeast. In stationary phase yeast, this ratio becomes 80:20 nucleosomal to non-nucleosomal, but never becomes entirely nucleosomal even when rRNA transcription is shut off (Sandmeier et al., 2002). Although actively transcribing rRNA genes are devoid of nucleosomes, newly synthesized rDNA is completely nucleosomal (Lucchini and Sogo, 1995). Psoralen cross-linking of *S. cerevisiae* that had just replicated their genomes showed this to be the case (Lucchini and Sogo, 1995). This suggests nucleosomes are displaced by the process of transcription. In yeast, the cell requires polymerase I transcription to strip nucleosomes because a temperature sensitive mutation of pol I abolished nucleosome stripping when pol I is inactivated by switching to the non-permissive temperature (Dammann et al., 1995). However, it was not clear if this effect was strictly a result of pol I transcription, or if other factors recruited to the rRNA genes (that require a transcribing polymerase) were responsible for nucleosome stripping.



Dammann *et. al,* Nucl Acids Res 1993

Figure 1.6. Psoralen cross-linking assay. Southern blot of psoralen cross-linked rDNA reveals two migrating species: a nucleosome free, slow migrating species and a nucleosomal, fast migrating species (lane 1 is cross-linked DNA and lane 2 is the non-cross-linked control). The southern blot was probed using a labeled fragment of the rDNA coding region. The two fragments were excised from the gel (lane 5) and examined using electron microscopy (right panel). Using nuclear run-on experiments coupled with psoralen cross-linking, demonstrated the nucleosome free species is transcriptionally active (lanes 3 and 4). The inset shows psoralen cross-linking RNA to DNA

1.6 CHROMATIN'S ROLE IN REGULATING RIBOSOMAL RNA TRANSCRIPTION

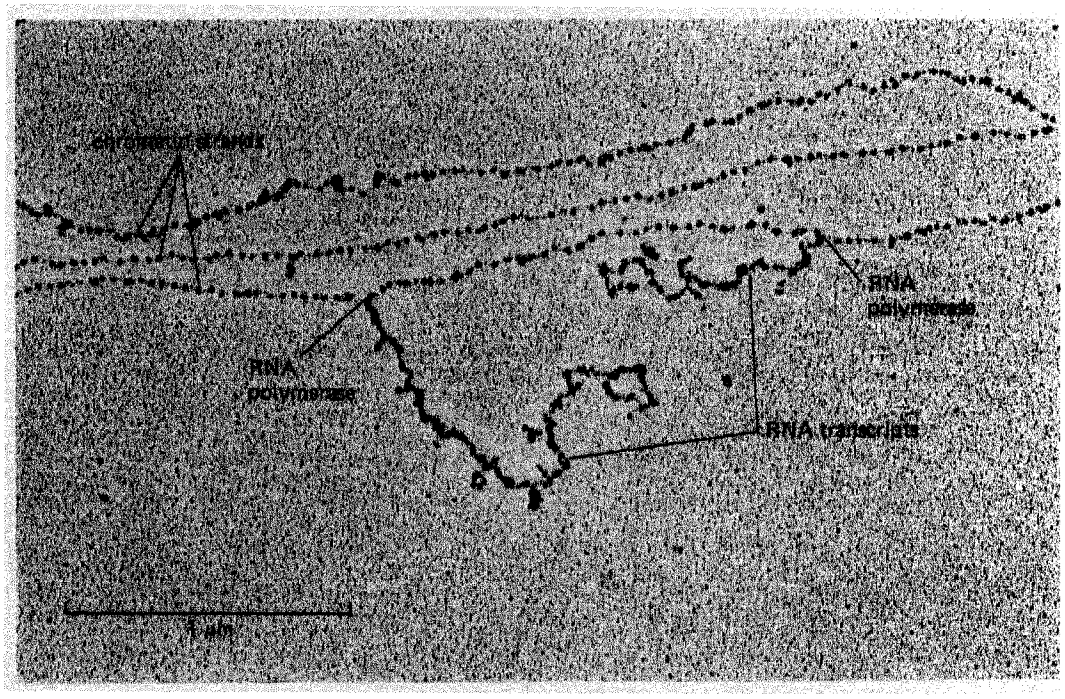
Given that actively growing cells typically transcribe only half of their ribosomal RNA genes, and that the other half are inactive and packaged into chromatin, it stands to reason that chromatin may play a role in the regulation of rRNA transcription. Early work by the Pikaard laboratory showed an epigenetic means of activating rRNA genes inherited from only one parent in interspecific hybrids of *Xenopus, Drosophila* and plants. rRNA transcription of the rRNA genes from the other parent was repressed, a concept called nucleolar dominance (Chen and Pikaard, 1997). It is thought that the silenced parent's DNA is packaged by nucleosomes. In general, silenced rDNA is heavily methylated and nucleosomes that package it are hypo-acetylated. This type of silencing could be regulatory and reversible. In fact, treatment of cells with 5-aza-2'-deoxyctidine, which results in the loss of methylated CpGs in DNA, activates rRNA transcription well beyond normal levels (Chen and Pikaard, 1997; Santoro et al., 2002; Zhou et al., 2002). Treatment of cells with sodium butyrate or trichostatin A, (known inhibitors of histone deacetylases) has the same affect (Chen and Pikaard, 1997; Santoro et al., 2002; Zhou et al., 2002). In yeast, the amount of nucleosomal rDNA increases in response to slow growth, coinciding with down regulation of rRNA transcription (Dammann et al., 1993). Given these lines of evidence, it might seem chromatin regulates rRNA transcription. However in higher eukaryotes, the fraction of nucleosomal rDNA remains constant even with reduction in growth rate (Conconi et al., 1989; Scheer, 1978), and recent

studies in yeast showed that while there is an increase in nucleosomal rDNA when pol I transcription is down regulated, this change is not primarily responsible for regulating rRNA transcription (French et al., 2003; Sandmeier et al., 2002). Yeast cells containing a knockout mutation in the rDNA-specific histone deacetylase (RPD3) do not alter their chromatin state upon entering stationary phase. However, rRNA transcription was shut-off normally, indicating that the primary regulation of rRNA transcription in yeast is unrelated to chromatin (Sandmeier et al., 2002), as in mammals and plants. Additionally, yeast that contain only 42 of their original 150 copies of rRNA genes are found to have all 42 copies completely non-nucleosomal. These cells survive as well as their wild-type counterparts indicating nucleosomes do not play a key role in regulating rRNA transcription (French et al., 2003) since there is not a requirement for nucleosomal rRNA genes for cell viability. While nucleosomes do not play a role in regulating rRNA transcription initiation, a definitive study examining how nucleosomes affect pol I elongation rates has not been conducted.

1.7 NUCLEOSOMES AND TRANSCRIPTION.

In general, nucleosomes inhibit transcription initiation by blocking transcription factor binding sites, and present a strong barrier to polymerase elongation. Nucleosomes inhibit initiation of transcription and elongation of RNA polymerase III when positioned over the internal control region of *Xenopus*

oocyte 5S RNA genes (Morse 1989). However, pol III genes are relatively short (400bp) and the necessity for elongation through nucleosomes may not be as relevant as their effect on transcription factor binding. Nucleosomes also inhibit initiation and elongation by prokaryotic T7 RNA polymerase (O'Neill et al., 1992). In contrast, while nucleosomes inhibit pol II transcription initiation, RNA polymerase II genes have been shown to transcribe their nucleosomal templates *in vivo* (Figure 1.7) (Foe et al., 1976; McKnight et al., 1978; Scheer, 1978). While chromatin generally represses transcription initiation, Pol II genes have evolved to recruit nucleosome remodeling factors (e.g. histone acetlytransferases, swi/snf, and nurf) to allow transcription factor access to promoter DNA by modifying nucleosomes via acetlyation or by sliding nucleosomes (in an ATP dependent manner) exposing promoter elements to transcription factors (Becker and Horz, 2002). However, once transcription begins, pol II requires the aid of a protein called FACT (**fa**cilitates **c**hromatin **t**ranscription) to elongate through nucleosomes in its path (LeRoy et al., 1998; Orphanides et al., 1998; Orphanides and Reinberg, 2000; Wada et al., 2000). FACT is a two-subunit protein complex that is known to bind histone proteins in the nucleosome core particle. Currently, FACT is believed to act as a histone protein chaperon that destabilizes nucleosome core particles during transcription by pol II allowing for RNA chain elongation. Without this protein complex, pol II cannot transcribe through DNA packaged by nucleosomes under physiological conditions (Belotserkovskaya et al., 2003; Chang and Luse, 1997; Izban and Luse, 1992; Kireeva et al., 2002; LeRoy et al., 1998; Orphanides et al., 1998;



Foe, V.E. , Wilkinson, L.E. & Laird, C.D. Ceil (1976).

Figure 1.7. Electron micrograph of RNA polymerase II transcribing a nucleosomal template. Notice that the template remains nucleosomal during transcription.

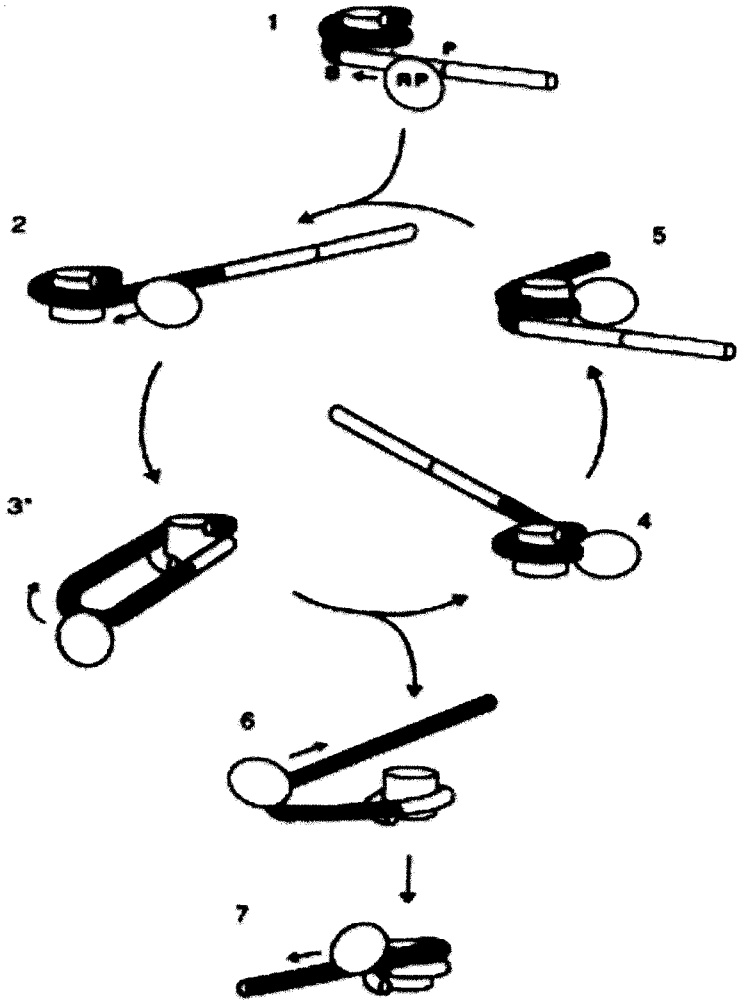
Orphanides and Reinberg, 2000; Wada et al., 2000). In contrast, SP6 RNA polymerase (initiated from the SP6 promoter) and yeast RNA polymerase III (initiated from a tailed template) could transcribe a short sequence of mono-nucleosomal DNA, but nucleosomes reduced the rate of transcription by slowing elongation rate and induced strong pausing for both polymerases (Studitsky et al., 1994; Studitsky et al., 1997).

1.8 NUCLEOSOME DISPLACEMENT BY RNA POLYMERASES.

The fate of nucleosomes during transcription are is unclear despite several studies. Transcription of nucleosomal arrays *in vitro* showed nucleosomes are disrupted by bacteriophage T7 RNA polymerase when visualized by electron microscopy (ten Heggeler-Bordier et al., 1995). However, when the yeast HSP82 gene (engineered to be transcribed only by T7 pol) was transcribed *in vivo*, nucleosomes remained intact (Sathyanarayana et al., 1999). Nucleosomes were also displaced *in vitro* from DNA via RNA polymerase II (Lorch et al., 1987). However, in these experiments pol II was initiated from a tailed template, which might affect the interpretation of results (discussed below), especially when electron micrographs show pol II genes remain nucleosomal during transcription *in vivo* (Foe et al., 1976). In contrast, in *in vitro* studies a nucleosome is transferred out of the path of a transcribing SP6 RNA polymerase molecule and deposited upstream of its original position (Studitsky et al., 1994). The nucleosomes "stepped around" the transcribing SP6 RNA polymerase,

shifting to a site 80 base pairs upstream of its original position. The nucleosome never leaves its template when the polymerase transcribes through it because (at low transcription rate) this cis translocation was resistant to challenge with excess competitor DNA. In the model proposed, because the newly transcribed tail wraps around the nucleosome as polymerase transcribes (Figure 1.8) (Studitsky et al., 1994). In fact, removal of the upstream tail after initiation of transcription, abolishes this "step around" effect. The same phenomenon occurs during yeast RNA polymerase III transcription (Studitsky et al., 1997) when transcription was initiated from a tailed template. However, tailed templates used to initiate transcription may not re-anneal, but form DNA/RNA hybrids that might interfere with nucleosome translocation (Campbell and Setzer, 1992). This may explain why pol II initiated from a tailed template appeared to displace nucleosomes (Lorch et al., 1987). However, the pol III study claimed there is no evidence of RNA/DNA hybrids during transcription. The question of RNA/DNA hybrids makes the interpretation of this "step around" effect difficult. In addition, critical controls seem to be missing for the pol III experiments, such as an examination of pol III stability on template DNA in the presence of competitor DNA (recall that "step around" should occur in the presence of competitor DNA). With pol I initiated on a tailed template, we find the polymerase dissociates from the template upon challenge with competitor DNA (See chapter 4). However, this control is missing in the examination of nucleosome translocation during pol III transcription making it difficult to interpret the results.

This "step around" phenomenon was also examined with RNA pol II.



Bednar J, Studitsky VM, Grigoryev SA,
Felsenfeld G, Woodcock CL, Mol Cell 1999.

Figure 1.8. Model of nucleosome "wrap-around" put forth for SP6 polymerase (initiated from the SP6 promoter (P)) and RNA polymerase III (initiated from the tail of the template). As the polymerase approaches the nucleosome the previously transcribed tail wraps around and binds the nucleosome. The nucleosome is translocated upstream of its original position allowing the polymerase to finish transcription. It is important to note that the nucleosome never leaves the template during this process.

Transcription using an immobilized polymerase II molecule initiated on a synthetic melted-bubble template showed a different effect. The nucleosome does not translocate upstream of the original position, nor is it displaced. The nucleosome remains in its original location, but has an altered structure resulting form a loss of one of its H2A/H2B dimers, forming a nucleosome hexamer (Kireeva et al., 2002). Addition of exogenous H2A/H2B dimers following transcription by pol II could reconstitute the intact nucleosome. It should be noted, however, that template usage was low (20-50%) in these experiments and transcriptions were conducted at 300mM KCl because elongation through nucleosomes was inefficient at physiological ionic strength (~100 mM KCl). At 300mM KCl, however, the nucleosome may have an altered structure allowing for easy loss of the H2A/H2B dimer. Differences in how individual experiments were conducted could account for the conflicting conclusions drawn for pol II (Lorch et al., 1987).

1.9 NUCLEOSOMES AND TRANSCRIPTION BY POL I.

In vitro, activated RNA polymerase I transcription is repressed on a chromatin template compared to a naked template. Repression was alleviated when transcription termination factor (TTF-I) was added prior to transcription initiation. TTF-1, which binds the promoter proximal terminator adjacent to the promoter, is thought to be responsible for recruiting nucleosome remodeling factors to the core promoter (Strohner et al., 2001). These factors remodeled

chromatin in the IGS, causing a nucleosome to be positioned directly over the core promoter (Langst et al., 1998; Langst et al., 1997). This sliding of nucleosomes allowed efficient transcription initiation by pol I, and in these studies, pol I elongation seemed to be unaffected by the presence of nucleosomes. This is contrary to what is found in RNA polymerase II transcription, where transcription is repressed when binding sites for the primary transcription factors are blocked by a nucleosome (Owen-Hughes and Workman, 1994), and elongation through nucleosomes is inefficient. Recently, the remodeling factors recruited by TTF-1 were identified in mouse. NoRC (nucleolar remodeling complex) consists of TIP5 (TTF-1 interacting protein 5) (which shares similarity to the homologues of human remodeling factors hCHRAC and WCRF) and SNF2h. NoRC was found to associate with TTF-1, UBF and the rRNA promoter. However, it was later discovered *in vivo* that NoRC plays a role in silencing rDNA transcription. The differences observed *in vitro* versus *in vivo* may be due to differences in chromatin assembly *in vitro*. It remains a mystery whether rRNA transcriptional activation in a chromatin context *in vivo* requires nucleosome remodeling factors. However, evidence for the presence of nucleosome remodeling factors associated with RNA polymerase I comes from the Pikaard laboratory. They found evidence of histone acetyltransferase activity associated with the pol I holoenzyme isolated from *Xenopus laevis* (Albert et al., 1999). It is unclear, however, what role this HAT may play in rRNA transcription.

1.10 NUCLEOSOMES AND THE *A. CASTELLANII* POL I SYSTEM.

The *A. castellanii* promoter competed for positioning of nucleosomes when assembling nucleosomal arrays (Georgel et al., 1993). The promoter's affinity for positioned nucleosomes prevented correct positioning of nucleosomes on long arrays of *Lytechinus variegatus* 208bp 5S RNA gene fragments located downstream. However, pre-incubation with the fundamental transcription factor TIF-IB and RNA polymerase I prior to nucleosome deposition restored correct positioning (Georgel et al., 1993). We could not reproduce these positioning results (see chapter 4). However, Chris Terpenning, a former postdoctoral investigator in our laboratory, was able to demonstrate with incorrectly positioned nucleosomes, *A. castellanii* pol I could transcribe through an array of four nucleosomes in its path (unpublished data) (see chapter 4), which agrees with *in vitro* data (discussed above) showing nucleosomes do not inhibit transcription initiation or elongation by pol I.

1.11 POLYMERASE SWITCH AND RIBOSOMAL DNA EXPANSION IN *S. CEREVISIAE* CONTAINING A UAF KNOCKOUT MUTATION

The yeast pol I transcription factor UAF (upstream activation factor) binds the upstream promoter element and recruits core factor and pol I to the promoter (Figure 1.9). UAF is a multisubunit complex consisting of Rrn5, Rrn9, and Rrn10, UAF30p and histones H3 and H4. What role UAF30p, H3 and H4 play in UAF

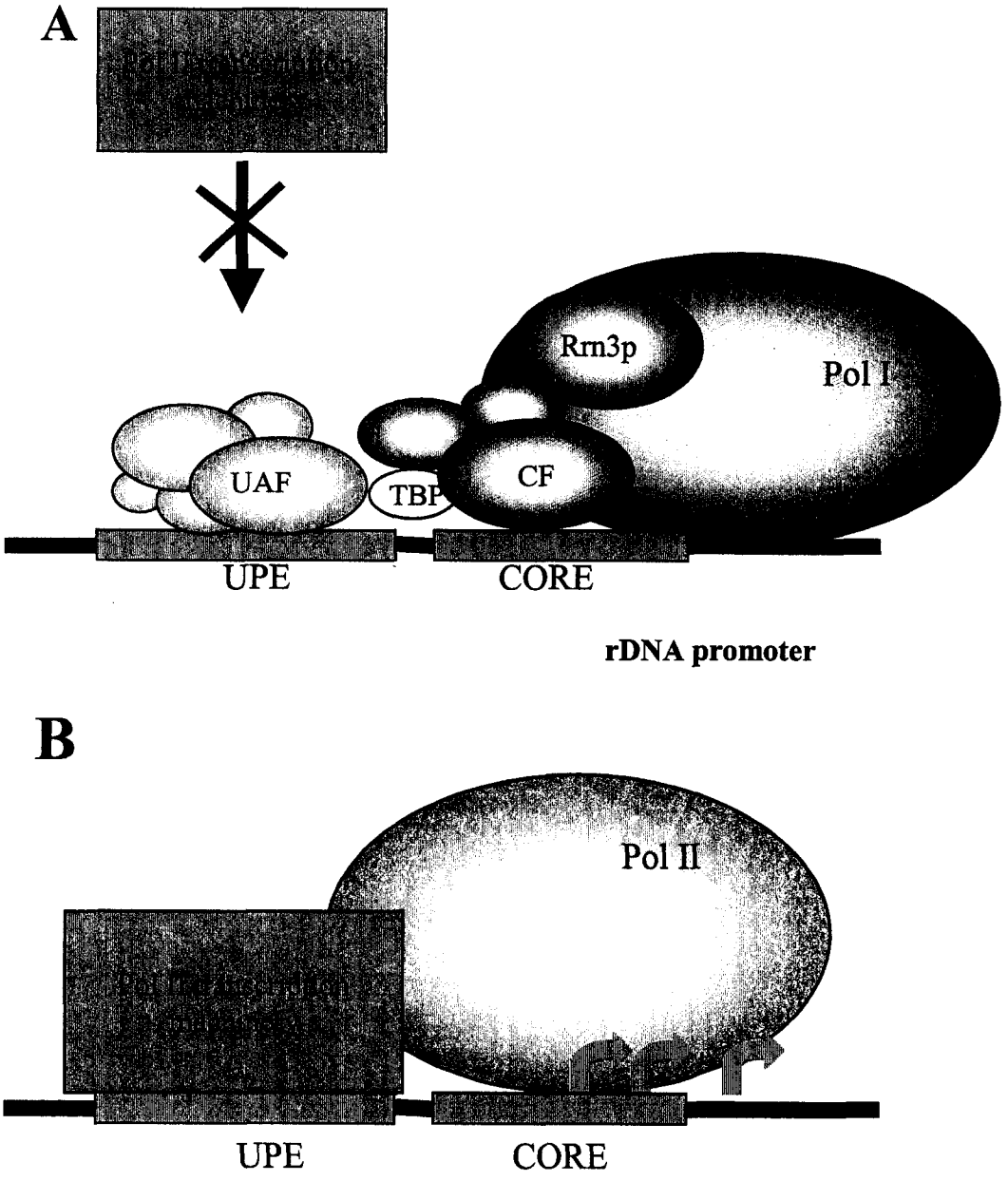


Figure 1.9 UAF blocks a cryptic pol II promoter in yeast. **A.** UAF binds to the UPE in yeast and recruits CF to the core promoter which then recruits pol I to the transcription start site. UAF that is bound to the promoter prevents pol II transcription factors from binding in the UPE **B.** Pol II Transcription machinery gains access to the promoter in UAF knockout cells, so the rRNA genes are transcribed by pol II. Transcription by Pol II is initiated from several start sites (grey arrows).

function is unclear. The histone proteins are found substochiometricly and might play a structural role for the promoter (Keys et al., 1996). UAF commits the promoter for transcription and remains bound to the promoter during multiple rounds of transcription. When the RRN5 gene for UAF was knocked out in *S. cerevisiae*, primer extension analysis of rRNA transcripts reveled rRNA genes were transcribed by pol II instead of pol I (Oakes et al., 1999) (Figure 1.9). It was determined there is a cryptic pol II promoter in the upstream promoter element of yeast rRNA genes (Oakes et al., 1999). This polymerase switch was accompanied by the eventual three-fold expansion in the number of copies of the rRNA genes. Given the relative weakness of pol II transcription, the cell increases the number of copies of the rRNA genes to meet the high demands of the cell for intact ribosomes. Cells grow slower under polymerase switch conditions than wild-type (generation time of 9hrs instead of 1.7hrs), but will allow a definitive comparison of the chromatin state rRNA genes transcribed by pol II versus pol I. Psoralen cross-linking of rRNA genes transcribed by pol II will reveal what changes in chromatin structure occur during this process, and whether stripping of nucleosomes is a pol I specific phenomenon or if another polymerase can substitute for specific nucleosome stripping *in vivo.*

1.12 OBJECTIVES AND SIGNIFICANCE OF THE DISSERTATION

My goal is to determine if nucleosomes are displaced from DNA during transcription by RNA polymerase I, or whether nucleosomes undergo the

"stepping around" phenomenon observed for SP6 polymerase and RNA polymerase III. I will examine this question, *in vitro* and *in vivo*, by using the *A. castellanii* and *S. cerevisiae* systems. Given the prokaryotic nature of SP6 polymerase, and the poor template usage of pols II and III used so far in nucleosome displacement experiments, we feel the highly defined pol I systems of *A. castellanii* and *S. cerevisiae* provide an excellent means to examine displacement by RNA polymerase I. *A. castellanii* TIF-IB can commit virtually all the template DNA for transcription *in vitro*. In addition, template usage by RNA polymerase I is 75% or more. By examining >75% of usable template we can draw a more accurate picture of how RNA polymerase I interacts with nucleosomes in its path compared to other systems. Another advantage of using the pol I system is the ease by which pol I elongates through chromatin *in vitro* under physiological conditions. This contrasts with pol II, where elongation through nucleosomes requires 300mM KCl. We also will take advantage of yeast UAF knockout strains and the psoralen cross-linking technique to compare displacement *in vivo* by pol I and pol II. This work will be the first direct examination of the role RNA polymerase I plays in maintaining the correct chromatin structure of RNA genes during transcription.

Chapter 2

MATERIALS AND METHODS.

2.1 NUCLEOSOME DISPLACEMENT EXPERIMENTAL DESIGN

The overall design for *in vitro* nucleosome displacement assays is outlined in figures 2.1 and 2.2. Purified nucleosome core particles were ligated to the *A. castellanii* pol I promoter containing a fragment of linker DNA (120bp) (figure 2.1). Linker DNA provides room for nucleosome translocation during transcription. Sufficient amounts of TIF-IB and pol I were incubated with the ligated DNA to transcribe >75% of all template. Transcription stalls at +30 when only three of the four NTPs, omitting UTP , are added to the protein-template complex. When pUC19 plasmid DNA (50μg/ml final) and UTP were added a single round of transcription occurred. The nucleosome's position was mapped before and after transcription by restriction enzyme protection assays as described previously (Kireeva et al., 2002; Studitsky et al., 1994; Studitsky et al., 1997) (Figure 2.2).

2.2 NUCLEOSOME CORE PARTICLE RECONSTITUTION

Recombinant *Xenopus laevis* core histones and PST5S plasmid containing a 146bp fragment of *Lytechinus variegatus* 5S DNA were provided by the Karolin Luger laboratory. PST5S DNA was digested with Sal I and Hind III

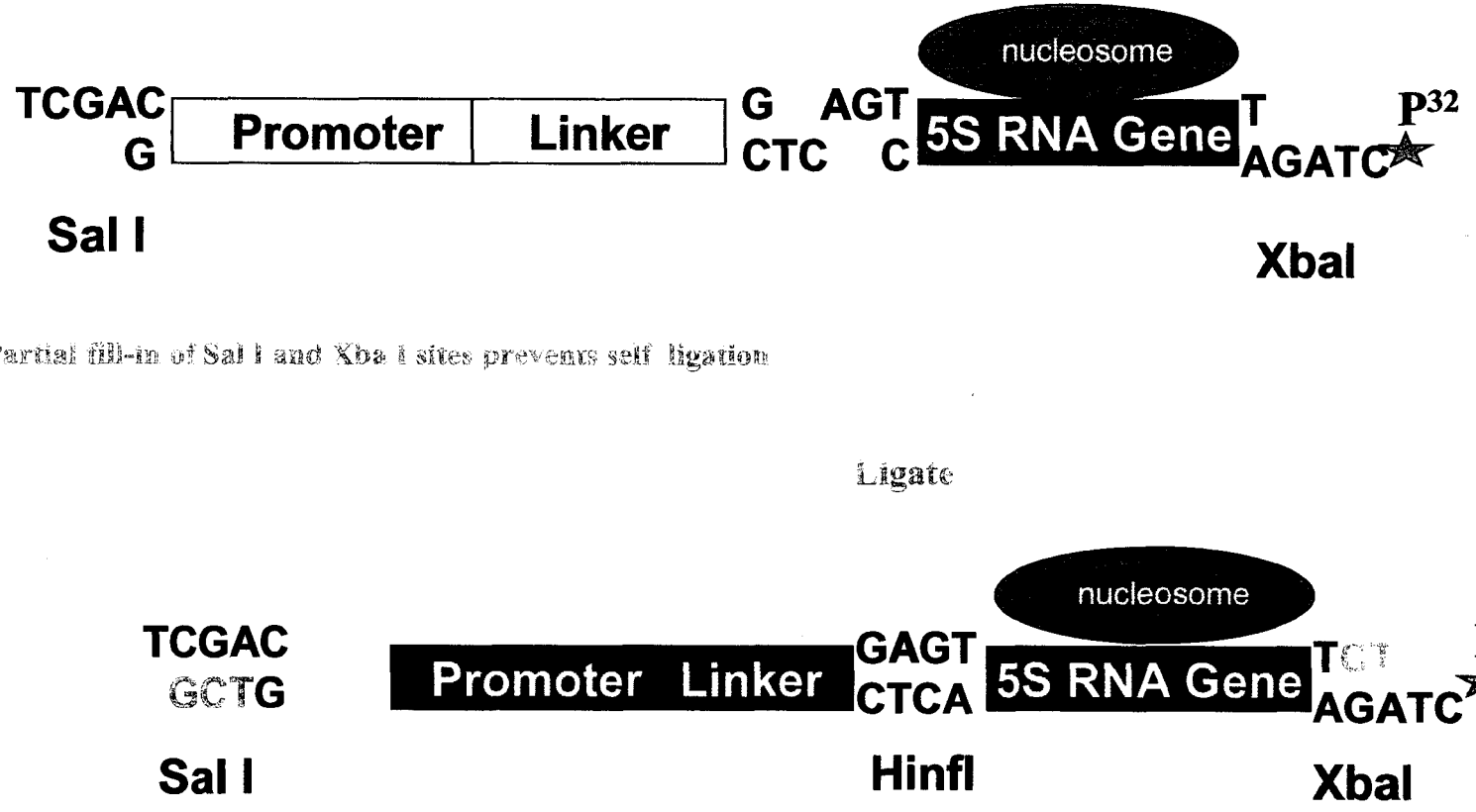


Figure 2.1. Generating the template for nucleosome displacement assays. Mononucleosomes are ligated to promoter/linker DNA via the Hinf I site. Partial fill-in of the Xba I and Sal I sites prevents self-ligation of P/L and 5S DNA.

•Stall polymerase at +30 by starving the transcription reaction of UTP
•Add 200 fold excess base pairs competitor DNA followed by the addition of UTP
•Allow transcription to proceed for 2 minutes
•Digest with restriction enzyme for 30 minutes

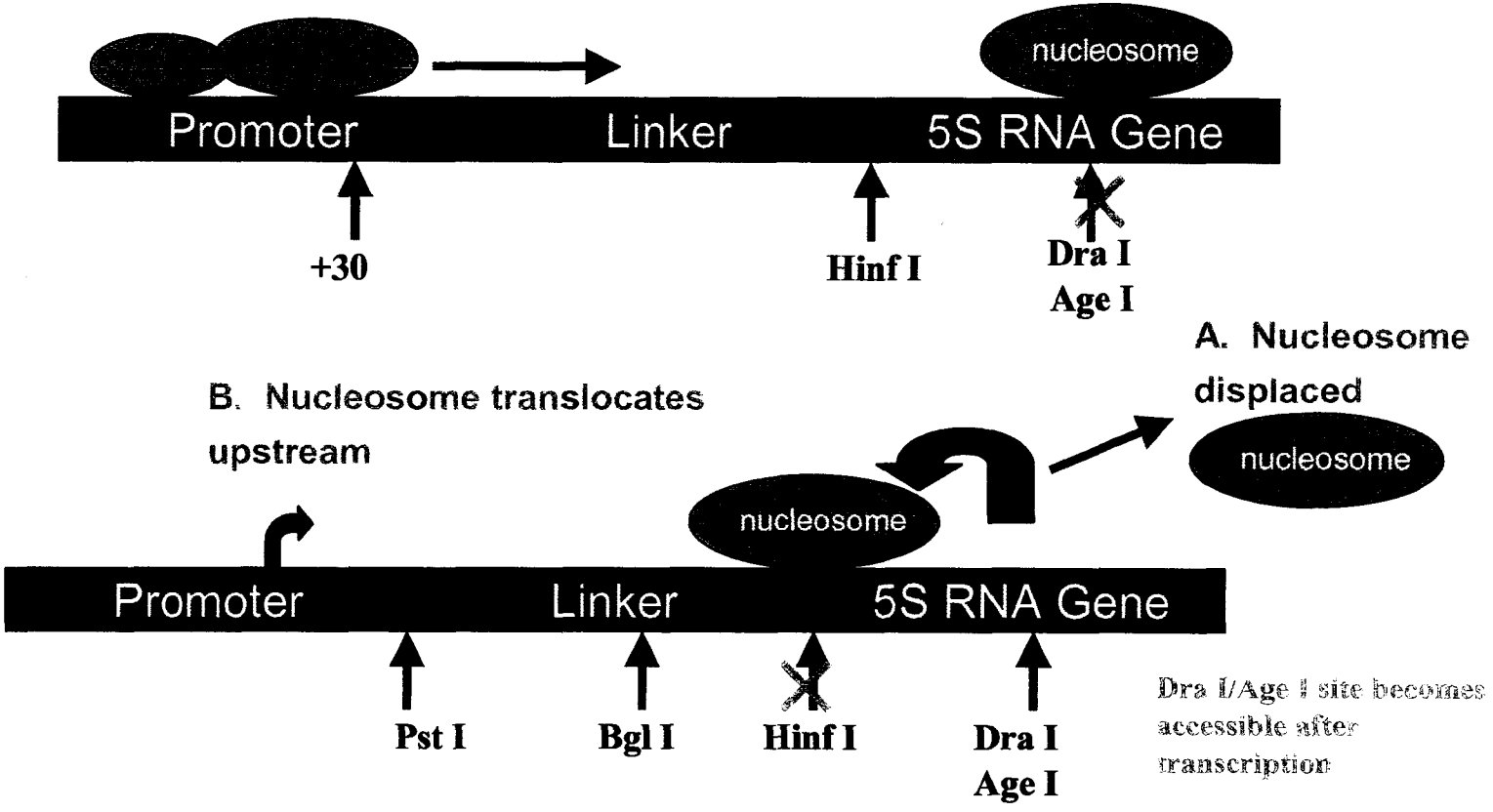
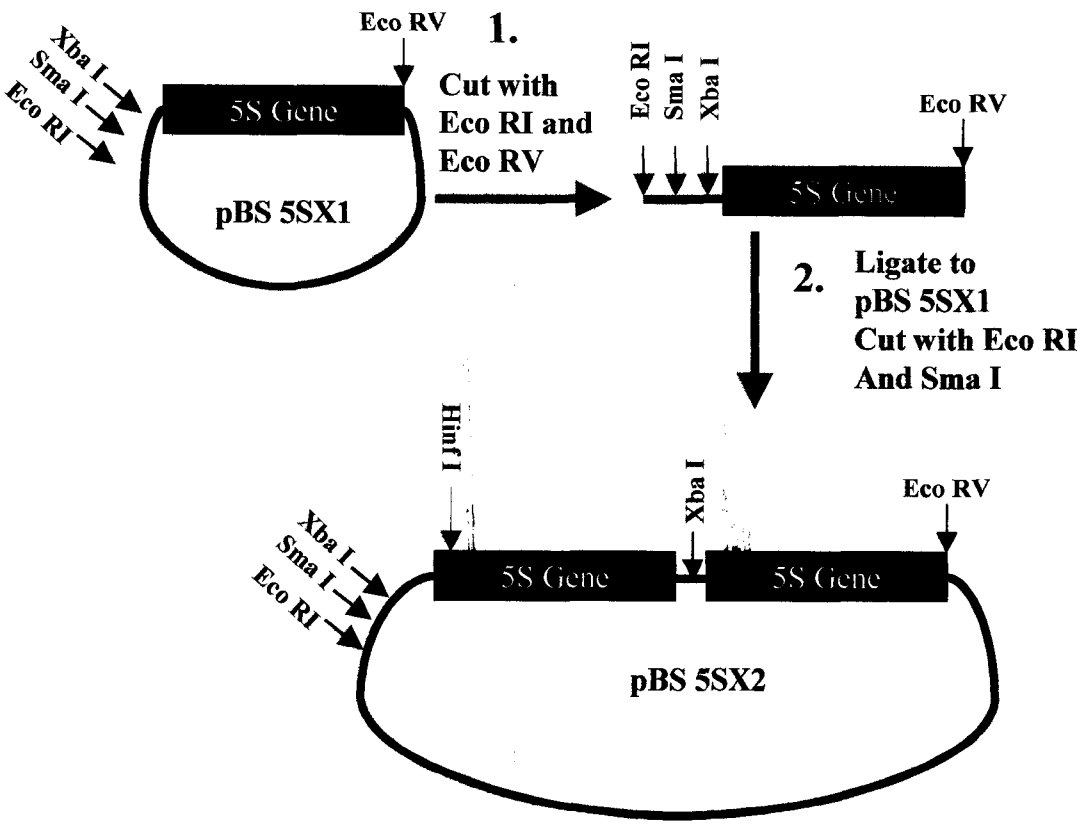


Figure 2.2. Design for nucleosome displacement assays.

and the 5S DNA was ligated into PBS(-) plasmid digested with the same restriction enzymes yielding the PBS(-)5S construct. PBS(-)5S was digested with EcoRV (located on each side of the 5S DNA fragment) and cloned into PBS(-)5S digested with EcoRV and Hinc II by blunt-end ligation. This destroys one EcoRV site. The 5S DNA was then made multiple-copy in the plasmid by digesting the plasmid with EcoRV and EcoRI, isolating the DNA fragment containing the 5S DNA, and ligating it to PBS (-)5S DNA digested with EcoRI and Sma I (Figure 2.3). This was repeated generating the PBS(-)5SX8 DNA construct. Digestion of PBS(-)5SX8 DNA with Xba I removes seven fragments of DNA 160bp in length with each containing the 146bp 5S DNA fragment plus 14bp of plasmid DNA. Ten μg of purified, Xba I digested 5S DNA was partially filled-in by Klenow fragment lacking exonuclease activity, digested with Hinf I, ^{32}P end-labeled, and purified with Qiaex II resin (Qiagen). Partial fill-in of overhangs prevents self-ligation of the DNA. 5 μg of core histone octamer was incubated with 5S DNA at 2M NaCl in 10mM TRIS- HCl pH 7.5, and step-wise diluted to 1M, 0.8M, 0.67M, 0.2M, and 0.1M NaCl in 10mM TRIS- HCl pH 7.5 to form nucleosome core particles (NCP). Efficiency of reconstitution was determined by native 5% PAGE. NCPs were separated from naked DNA via 5-30% sucrose gradient sedimentation (containing 50mM TRIS-HCL pH 7.5, 1mM EDTA, and 100 μg/ml BSA) by cetrifugation at 35,000 RPM in a Beckman SW-41 rotor for sixteen hours at 4°C, dialyzed overnight at 4°C in 10mM TRIS-HCl pH 7.5, and concentrated to approximately 25 ng/μl using a Centricon 100 concentrator.

Generating Multiple Copy 5S RNA Gene Fragments



Repeat steps 1 and 2 until the pBS 5SX8 construct is complete. To excise 5S DNA fragments, digest with Xba I.

Figure 2.3. Generating the 5SX8 construct

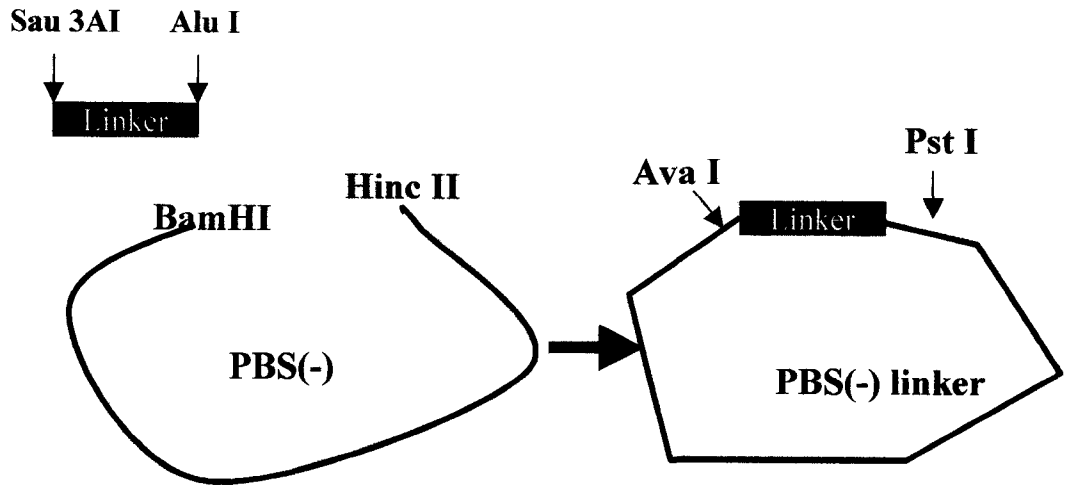
2.3 PREPARATION OF PROMOTER/LINKER DNA

Linker DNA consists of the 97bp Sau 3AI/Alu I fragment from PBS (-) plasmid cloned into the multiple cloning site of new PBS (-) at the BamHI/Hinc II sites (Sau 3AI/BamHI are compatible cohesive ends and Alu I/Hinc II generate blunt ends). This DNA was selected because it contained several restriction enzyme sites not located in either the 5S DNA or the promoter DNA. Linker DNA was excised with Pst I/Ava I (120bp) and ligated to the *A. castellanii* rDNA promoter (-120 to +80) containing EcoRI/Ava I ends (227bp total length). Ligated promoter/linker DNA (347bp total length) was then cloned into PBS (-) at the EcoRI/Ava I sites (Figure 2.4). Promoter/linker DNA was excised with Sal I, and 367ng of purified promoter/linker DNA was partially filled-in by Klenow fragment lacking exonuclease activity with dCTP, dTTP, and dGTP. The DNA was phosphatased, digested with Hinf I, extracted with phenol-chloroform, precipitated, and resuspended in 20µl of TRIS-HCl pH 8.0.

2.4 LIGATION OF PROMOTER/LINKER DNA TO 5S/NCP DNA

Ligations were optimized by titrating increasing amounts of promoter/linker DNA against a constant amount of 5S DNA or NCP. 10µl ligation reactions (in 1X ligation buffer (MBI) were conducted by mixing 18.38ng promoter/linker DNA and 10ng 5S DNA or NCP DNA, and heating the reaction for 5min at 37°C followed by the addition of 2.5 units of T4 DNA ligase (MBI)). The mixture was ligated overnight at 4°C. Efficiency of ligation to the 486 bp product was verified by 5% native PAGE and visualized using a phosphorimager. Greater than 94%

Linker DNA Cloning



Promoter/linkier DNA cloning

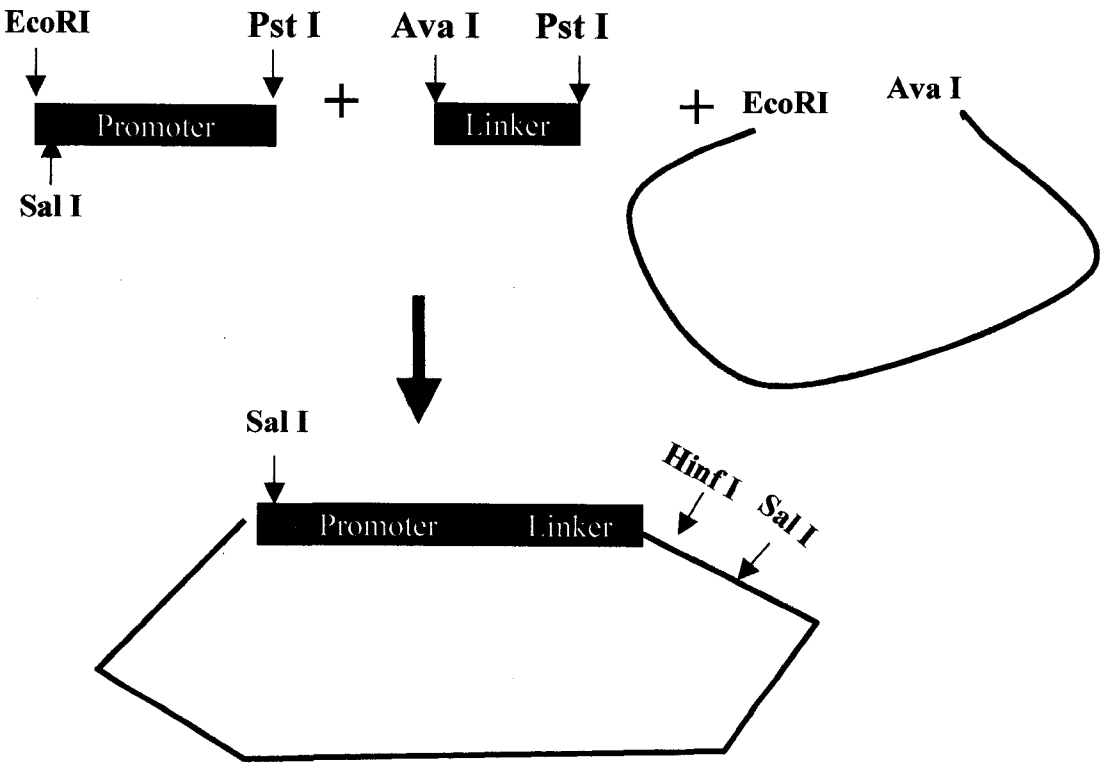


Figure 2.4. Generating the promoter/linker construct

efficiency of ligation was obtained in all experiments.

2.5 PREPARATION OF PROTEINS

TIF-IB was purified as described (Radebaugh et al., 1998). Crude nuclear extract was subjected to DEAE-Fast Flow, BioRex 70 and oligo promoter affinity chromatography (Radebaugh et al., 1998) generating highly purified TIF-IB. TIF-IB purified to this stage has sufficient TIF-IE for stable binding to promoter DNA. Pol I was provided by Anna Al-Kouri, John Anderson, and Joe Gogain and was purified by the method of Ida and Paule 1992.

2.6 EMSA OF TIF-IB AND TRANSCRIPTION OF TEMPLATE DNA

Increasing amounts of TIF-IB were added to 3.48 ng of ligated DNA in an electrophoretic mobility shift assay (EMSA) to determine the optimal amount of transcription factor needed to commit all labeled template to transcription. All binding reactions were conducted in 20mM TRIS-HCl pH 7.5, 10mM $MgCl_2$, 1mM BME, 100 mM KCL, and 0.1% NONIDET p40, and electrophoresed on a 5% native polyacrylamide gel in 1X TRIS-glycine-EDTA buffer. The amount of TIF-IB that bound >95% of the template was used for all experiments.

Run-off transcription assays were conducted as described previously (Iida et al., 1985), except that TRIS-HCl pH 7.5 was used instead of HEPES pH 7.9. For single-round transcription assays, 8 µl of highly purified *A. castellanii* TIF-IB and RNA polymerase I (75 mU non-specific activity) were pre-incubated in a 50 µl reaction with 3.48 ng of template DNA for 15min in the presence of 600 mM

ATP, CTP, and GTP, allowing the polymerase to elongate and then stall at +30. 50 μg/ml pUC19 plasmid DNA was added to prevent re-initiation of transcription. Concurrently, UTP was added to allow the polymerase to transcribe the remaining template DNA. Transcription was allowed to proceed for 2min followed by a 5min chase with excess cold UTP (to elongate plasmid DNA transcripts to a lenth that did not interfere with analysis of the specific transcript) and stopped by the addition of 50μl stop mix (0.1% SDS and 1 mg/ml proteinase K) followed by incubation at 50°C for 1hr. The RNA was precipitated with 95% ethanol and resuspended in 5λ loading buffer containing 98% formamide and loading dye. All transcripts were resolved on a 6% denaturing polyacrylamide gel, the gel was exposed to phosphorimager screens and read on a Molecular Dynamics Storm phosphoimager. ^{32}P labeled, Msp I digested PBR322 DNA was used a marker for all transcription assays.

2.7 NUCLEOSOME DISPLACEMENT ASSAYS

2.7.1 RESTRICTION ENZYME PROTECTION ASSAY

3.48 ng of ligated, free or NCP promoter/linker/5S DNA that was labeled on the 5' end of the 5S DNA with ^{32}P was transcribed under single-round conditions as described above, except the transcription reactions did not include labeled NTPs, contained 600 μM UTP, the reactions were not chased by UTP, and transcription was for 5min. 10U of restriction endonucleases were added after transcription to test for nucleosome position. Digests were conducted at

37°C for 30 minutes followed by either proteinase K/SDS treatment or phenol/chloroform extraction and ethanol precipitation. Digested DNA was resolved on a native 5% polyacrylamide gel and analyzed as described above.

2.7.2 PSORALEN CROSS-LINKING ASSAY

Wild-type BJ926 and UAF knockout NOY577 *S. cerevisiae* were provided by the Nomura laboratory (Department of Biological Chemistry-University of California, Irvine). All rRNA transcription in NOY577 cells is carried out by RNA pol II Oakes et al., 1999). To select for polymerase switched/expanded copy number mutants, YPGal streaks of NOY577 were grown for 16hrs in 5 mls of YPGal medium at 30°C and 100 µl was plated on YPD plates. The plates develop small and large colonies when grown at 30°C. Large colonies selected for all psoralen experiments have an expanded rDNA copy number.

Wild-type BJ926 cells were grown in 250 mls YPD at 30°C or YPRaffanose at 15°C and mutant cells were grown in YPD at 30°C to an OD_{600} of 0.5-1.0. Cell suspensions were pelleted and resuspended to an OD_{600} of 20 in buffer containing 1.1 M sorbitol, 5mM Pipes pH 7.0, and 25mM EDTA and placed in a 30ml beaker with a stir bar. 11.2 µg of psoralen (Sigma) was added and the mixture was exposed to a 365nm Specroline model SB 100P high intensity UV lamp with constant mixing for 2min, followed by a second addition of 11.2 µg of psoralen, and UV light exposure for another 2min. Cross-linked cells were pelleted, washed with nano-pure water and stored at -70°C. Cells were thawed, and 30 µl were added to 420 µl sorbitol buffer containing 200U lyticase enzyme

(Sigma) followed by incubation at 30°C for 30min. The mixture was centrifuged in an Eppindorf microfuge at 5200 RPM for 20min and the pellet was resuspended in 500 μl 10 mM TRIS-Cl/0.1 mM EDTA (TE) buffer pH 8.0, phenol/chloroform extracted, precipitated and resuspended in 500 μl TE. 50 μg of RNase A was added followed by incubation at 37°C for 10min followed by phenol/chloroform extraction and precipitation. The isolated yeast genomic DNA was resuspended in 100 μl TE.

1.95 μg of wild type DNA and 0.39 μg of mutant DNA were digested with 5U of EcoRI for 16hrs at 37°C and resolved on a submarine 1.5% agarose, 1% TAE gel at 80V for 20hrs. The gel was exposed to 266nm UV light for 3hrs to reverse DNA cross-linking and blotted to a Gene Screen Plus (NEN) membrane by capillary transfer using the high salt transfer method (NEN). The blot was probed with the 3600bp Xba I fragment encoding 18S and 25S rRNA purified from the pNOY 103 plasmid (Nomura laboratory), and labeled by the Ambion random-primed labeling kit and anaylized as described above (Figure 2.5). In a separate experiment, the digested DNA was end-labeled and electrophoresed on a vertical 1.5%, 1%TAE agarose gel at 80V for 1hr, dried, and phosphorimaged. Because rRNA genes are multiple-copy, end-labeled rDNA restriction enzyme fragments can be resolved from the background end-labeled genomic DNA (Figure 2.5).

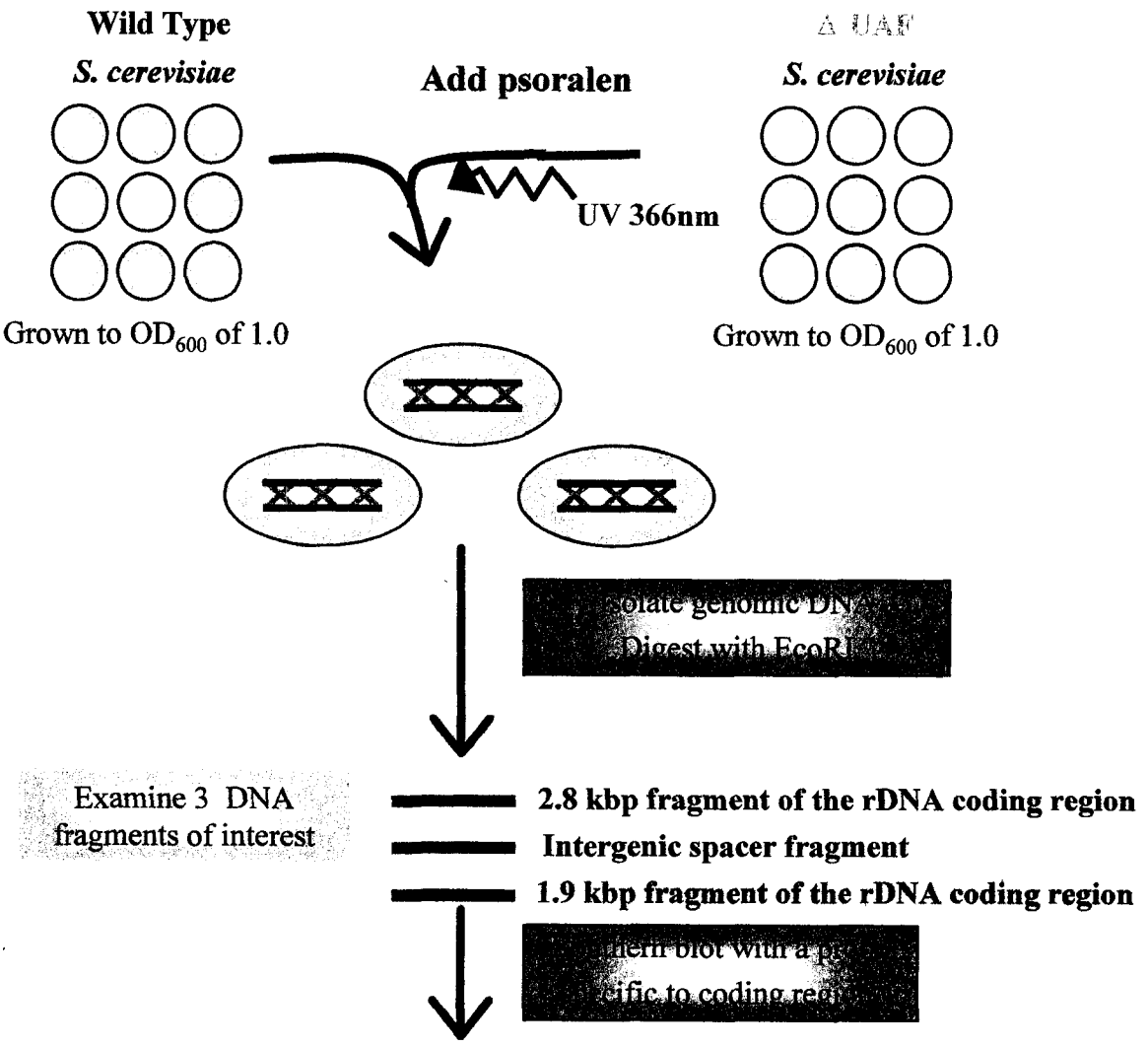


Figure 2.5. Experimental design of yeast psoralen cross-linking experiments.

Chapter 3

NUCLEOSOMES ARE DISPLACE BY RNA POLYMERASE I

The work presented here is shown in the same form as the journal Nature Structural and Molecular Biology with the following exceptions: 1) all references are in the same format as the rest of the dissertation, but are not included at the end of this section; they are in the reference list at the end of the thesis. 2) one figure is included here that was not included in the paper proper but was included in online supplementary information.

The psoralen cross-linking experiments were initiated by Sarah Fox Roberts (an undergraduate research assistant) and were completed by Amy Chotvacs (an undergraduate Hughes research scholar) who worked under my supervision.

NUCLEOSOMES ARE DISPLACED BY RNA POLYMERASE I

3.1 ABSTRACT

Eukaryotic cells have between 150 and 25,000 copies of rRNA genes (Paule, 1998) that are expressed by RNA polymerase I (pol i), its only role in the cell. Psoralen cross-linking showed there are two populations of rRNA genes: nucleosomal and nucleosome free, and cross-linking of pulse-labeled nuclei demonstrated only the latter are actively transcribed (Conconi et al., 1989; Dammann et al., 1993; Dammann et al., 1995). Following DNA replication, all rDNA is packaged by nucleosomes, but about half subsequently get stripped (Lucchini and Sogo, 1995). Pol I has been implicated in nucleosome stripping in yeast, because rRNA genes become nucleosomal in the absence of active pol I (Dammann et al., 1995). This contrasts with genes transcribed by pol II, which remain nucleosomal during transcription (Foe et al., 1976). Here we report *Acanthameoba castellanii* Pol I initiated at the rRNA promoter displaces nucleosomes from DNA *in vitro*. Similarly, *in vivo*, psoralen cross-linking of *Saccharomyces cerevisiae* rRNA genes that are anomalously transcribed by pol II in a polymerase switch (*PSW*) mutant revealed the specific nature of pol I stripping as these genes are not stripped of nucleosomes.

The fate of nucleosomes during transcription by RNA polymerases varies depending on the type of polymerase and conditions used. Studies of SP6 polymerase (initiated from the SP6 promoter) and RNA polymerase III (initiated

from a tailed template) revealed a nucleosome steps around a transcribing polymerase, translocating upstream of its original position (Studitsky et al., 1994; Studitsky et al., 1997). The nucleosome never leaves the template DNA because challenge with competitor DNA fails to capture nucleosomes during transcription. Kornberg and Lorch originally found RNA polymerase II (initiated from the ends of DNA) displaced nucleosomes during transcription *in vitro* (Lorch et al., 1987). Recently, however, a different story has emerged for RNA polymerase II. When transcription was initiated from a synthetic melted-bubble template, nucleosomes were not displaced from DNA, but instead remained in their original location. This correlates with electron micrograph data showing pol II genes remain nucleosomeal during transcription (Foe et al., 1976). However, these nucleosomes lose one H2A/H2B dimer during *in vitro* transcription (Kireeva et al., 2002). We are examining the fate of nucleosomes during transcription by RNA polymerase I in order to understand the role pol I plays in maintaining the correct chromatin structure observed for active rRNA genes.

To test for nucleosome displacement *in vitro*, a pre-assembled nucleosome core particle (NCP) was ligated to the *A. castellanii* rRNA promoter located upstream of a piece of linker DNA (figure 3.1). The template has a sequence that allows pol I to stall at +30 when depleted of UTP during transcription (Kahl et al., 2000). This promoter is very efficient for initiating RNA polymerase I transcription, yielding a template usage of 75% or more (Iida and Paule, 1992; Kahl et al., 2000). Linker DNA provides room to accommodate nucleosomes in the event they are translocated upstream during transcription. Ligated DNA was

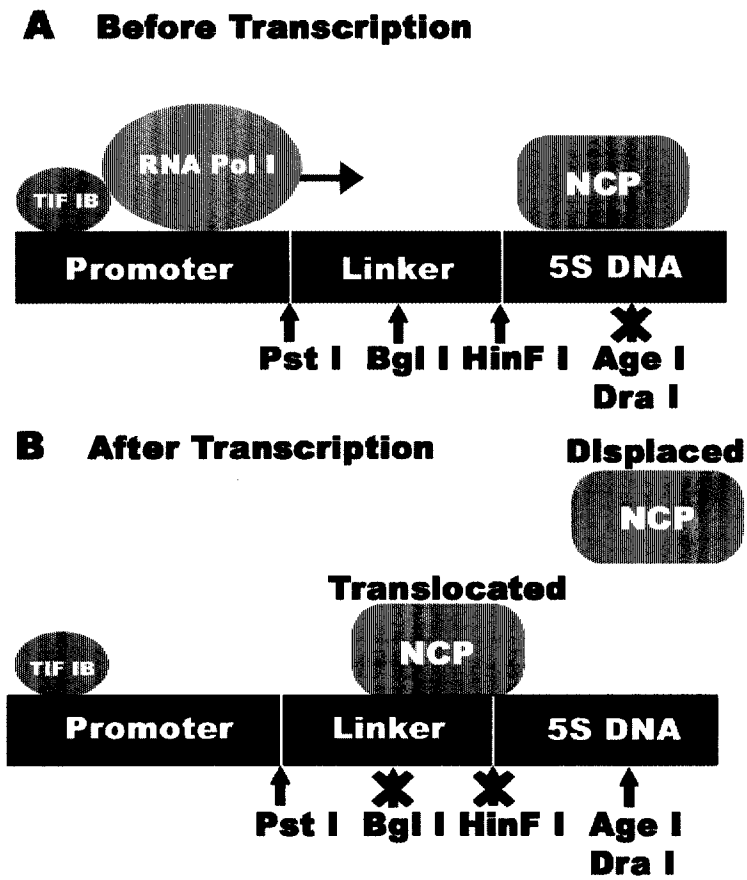


Figure 3.1. Design for nucleosome displacement assays. A pre-assembled nucleosome core particle is ligated to the *A. castellanii* rRNA promoter containing a piece of linker DNA. After stalling pol I at +30, pUC19 competitor DNA and UTP are added, and transcription proceeds for 5min, followed by restriction enzyme digestion for 30min. Restriction enzyme protection will allow mapping of nucleosome position before and after transcription.

supplied with highly purified *A. castellanii* Transcription Initiation Factor-IB (TIF-IB), RNA polymerase I (pol I), and all NTPs necessary for transcription except UTP, which stalls the polymerase at +30. Plasmid pUC19 DNA (50ug/ml final) was added to act as a sink for displaced nucleosomes and to limit pol I transcription to single round, followed by addition of UTP. After allowing transcription to proceed for 5min, DNA was digested by various restriction endonucleases to map NCP position. One of two scenarios were anticipated: 1) nucleosomes are displaced from template DNA and are transferred to pUC19 or 2) nucleosomes will remain on the DNA, translocating upstream or remaining in the original position. Looking for restriction enzyme sites that become protected after transcription provides an excellent means for determining which scenario occurs. If nucleosomes are completely displaced after transcription, none of the indicated restriction enzyme sites will be protected (figure 3.1).

Purified, recombinant *Xenopus laevis* core histone octamers were deposited on 160bp fragment of *Lytechinus variegatus* 5S DNA via step-wise salt dilution(Gottesfeld et al., 2002) (figure 3.2a) to form nucleosome core particles (NCPs). NCPs were purified from free DNA by sucrose gradient sedimentation (figure 3.2b) and ligated to promoter/linker (P/L) DNA with T4 DNA ligase (MBI) overnight at 4°C. Ligations were proteinase K/SDS extracted, ethanol precipitated, and resolved by native PAGE (figure 3.2c). Ligation efficiency was high (95%) and NCP DNA ligated with the same efficiency as free 5S DNA (compare lanes 2 and 4).

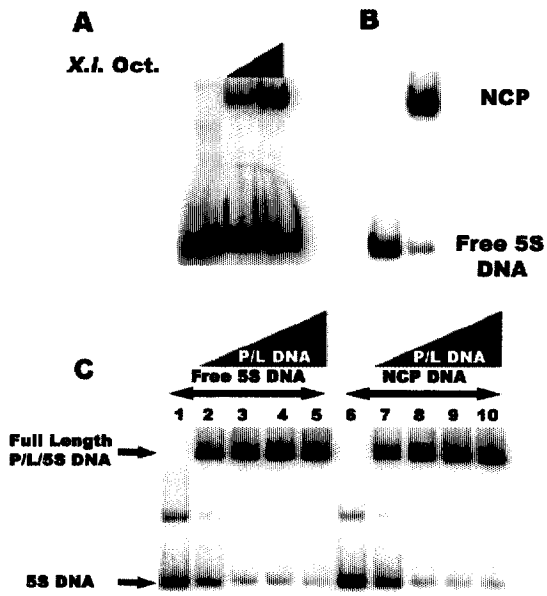


Figure 3.2. Nucleosome assembly, purification of NCP, and ligation of 5S DNA (Free) or NCP DNA (NCP) to promoter/linker (P/L) DNA. **a,** *Zenopus laevis* core histone octamer was mixed with ^{32}P end-labeled sea urchin 5S DNA at 2M NaCl and step-wise diluted to 100mM NaCl allowing for nucleosome core particle reconstitution. **b**, NCPs were purified by 5-30% sucrose gradient sedimentation. Shown are the purified free 5S DNA and purified NCPs. **c**, Increasing amounts of P/L DNA is titrated against a constant amount of free 5S DNA (lanes 1-5) or NCP DNA (lanes 6-10) in the presence of T4 DNA ligase.

The *A. castellanii* primary transcription factor TIF-IB was incubated with ligated promoter/linker/5S (Free, F) or promoter/linker/NCP (NCP, N) DNA in an electrophoretic mobility shift assay (Geiss et al., 1997) and was found to complex with 90% of DNA in the reaction (figure 3.3a). This is critical, because in *A. castellanii*, TIF-IB is solely responsible for recruiting pol I to the promoter and commits the DNA for transcription (Radebaugh et al., 1998). NCPs do not affect TIF-IB binding (figure 3.3b), and run-off transcription assays (Iida and Paule, 1992) reveal the efficiency of transcription is equal for free (F) and NCP (N) DNA (figure 3.3c compare lanes 1 and 2). Competitor DNA acts as an exceptional sink for pol I, as transcription from the promoter was single-round up to 30 min, matching results with Sarcosyl (Kahl et al., 2000) (data not shown). NCPs present a modest obstacle to pol I transcription; elongation rate was slowed approximately 2 fold during transcription of NCP DNA when compared to free DNA (figure 3.3d). Full-length free DNA (F) transcripts begin to appear by 30sec (figure 3.3d lanes 1and 2), but full-length NCP (N) transcripts do not appear until 60sec (figure 3.3d lanes 3 and 4). Single-round transcription is complete by 90sec for both templates (Data not shown). Complete pol I elongation through nucleosomes does not require high ionic strength (transcriptions were conducted at 100mM KCl), and because highly purified transcription components were used, it appears full-length transcription does not require additional components like FACT (Facilitates Chromatin Transcription), unlike what has been reported for pol II transcription (Belotserkovskaya et al., 2003; LeRoy et al., 1998;

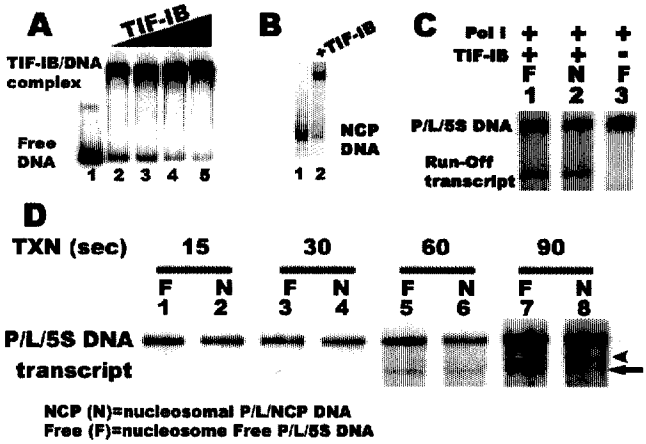


Figure 3.3. Assembly of committed transcription complexes, single-round transcription assays, and analysis of pol I elongation rate through nucleosomes. **a**, EMSA of TIF-IB bound to ligated P/L/5S (Free) DNA reveals 90% of template is complexed to the primary transcription factor TIF-IB. This means 90% of all template to be used in displacement assays is committed for transcription. **b**, TIF-IB binds P/L/NCP (NCP) DNA as efficiently as Free DNA. The same amount of TIF-IB used in lane 5 of (**a**) binds the same amount of NCP DNA (lane 2). **c**, After stalling the polymerase I molecule at +30, competitor DNA and α–^{32}P UTP were added and transcription proceeded for 2min, followed by chasing the transcription reaction with cold UTP for 5min. Chasing with cold UTP elongates paused plasmid transcripts from the field of view. **c**, Single-round transcription at shorter time points (15, 30, 60, and 90 sec) revealed NCP slow elongation rate approximately 2 fold. By 30sec, full-length transcripts (arrows) from nucleosome free (F) P/L/5S DNA begins to accumulate with little accumulation of full-length transcripts from NCP (N) P/L/5S DNA. However, by 60sec, NCP transcription begins to catch up to Free transcription, and at 90sec complete full-length transcription of both DNAs is observed. Since these transcriptions were not chased with cold UTP, a transcript (arrowhead) from plasmid DNA is observed.

Orphanides et al., 1998; Orphanides and Reinberg, 2000; Orphanides et al., 1999; Wada et al., 2000; Walter and Studitsky, 2001).

Following single-round transcription, the position of the nucleosome was determined by restriction enzyme protection assays (Kireeva et al., 2002; Studitsky et al., 1994; Studitsky et al., 1997). Free and NCP DNA was transcribed as described above, followed by digestion with 10U of the indicated restriction endonuclease (figure 3.1) for 30 minutes at 37°C. The reaction was proteinase K/SDS treated and ethanol precipitated prior to native PAGE. Before transcription, the nucleosome is positioned over the 5S DNA, as demonstrated by protection of the Age I restriction enzyme site from digestion (figure 3.4 compare lanes 3 and 4). After transcription, the Age I site becomes accessible to digestion, indicating the nucleosome was displaced from this site (compare lanes 4 and 5). The nucleosome was 65-70% displaced from the Age I site in these assays as determined by measuring the amount of undigested NCP DNA (full length P/L/5S DNA) remaining after transcription, and by the appearance of faster migrating, Age I digested fragments. Reduction in overall fragment band intensity in lane 5 compared to lane 4 is due to some Age I-dependent aggregation of the fragments (ca, 15-20%) that migrated near the top of the gel (data not shown). The question becomes where did the nucleosome go? None of the unique, upstream restriction enzyme sites were protected before or after transcription, in distinct contrast to Age I, which required nucleosome displacement by transcription to uncover its site (figure 3.4). If any of these upstream sites were protected, full-length P/L/5S DNA would be recovered.

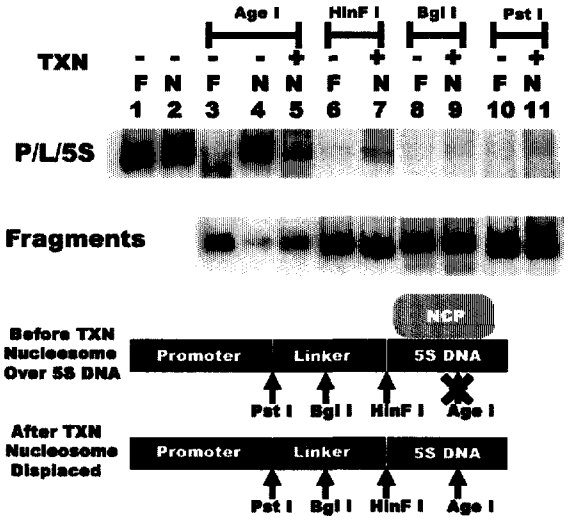


Figure 3.4. Pol I transcription leads to displacement of nucleosomes. **a**, NCP position was mapped by restriction enzyme protection assays before transcription (lanes 4, 6, 8, and 10) and after transcription (lanes 5, 7, 9, and 11) Transcriptions were conducted as described in figure 2b except transcriptions were cold and transcription was for 5 min. Following transcription, reactions were digested with the indicated restriction endonucleases for 30 min at 37°C. Digestions were stopped by incubation with proteinase K/SDS at 50°C for 1 hour followed by ethanol precipitation, resuspension in 10mM Tris pH 8.0 and resolved by native 5% PAGE. The Age I site is protected prior to transcription (compare lanes 3 and 4) but accessible after transcription (compare lanes 4 and 5), indicating the nucleosome has been removed from the 5S DNA fragment. Reduction in total counts between lanes 1 and 3 (Free DNA) and lanes 4 and 5 (NCP DNA) is due to aggregation of Age I fragments that migrate near the top of the gel (data not shown). To test for upstream translocation, other restriction endonuclease sites were tested as indicated. None of the upstream sites were protected (failed to recover full-length P/L/5S DNA), indicating the nucleosome does not remain associated with DNA after transcription, but is instead transfered to competitor DNA.

Accessibility of upstream restriction enzyme sites indicates the nucleosome has not translocated upstream of its original position after transcription by pol I (compare lanes 6 to 7, 8 to 9, and 10 to 11). The extent of displacement is directly proportional to the fraction of template assembling active transcription complexes prior to transcription initiation (figure 3.5). We conclude the nucleosome is displaced and captured in an unknown form by competitor pUC19 DNA during transcription.

In *S. cerevisiae*, the pol I transcription factor upstream activation factor (UAF) binds the upstream promoter element and recruits TBP, core factor and pol I to the promoter driving transcription initiation (Keys et al., 1996; Serebriiskii et al., 2001). Bound UAF blocks a cryptic pol II promoter upstream of the pol I initiation site (Kayahara et al., 1999). In yeast containing a UAF knockout mutation, rRNA genes increase in copy number (from 150 to 450) and are transcribed solely by RNA polymerase II (Oakes et al., 1999). This allows a unique opportunity to examine whether another polymerase can strip nucleosomes from rRNA genes *in vivo*. Psoralen cross-linking of pol II transcribed rRNA genes will reveal if stripping is indeed pol I specific, or whether other factor(s) (associated with rRNA genes during transcription) are responsible.

S. cerevisiae wild-type strain BJ926 and UAF knockout strain NOY 577 (Δuaf) were grown to an OD_{600} between 0.5 and 1.0, harvested, and DNA was psoralen cross-linked (Dammann et al., 1993). Genomic DNA was isolated, digested with EcoRI, and either electrophoresed on a 1.5% agarose gel and

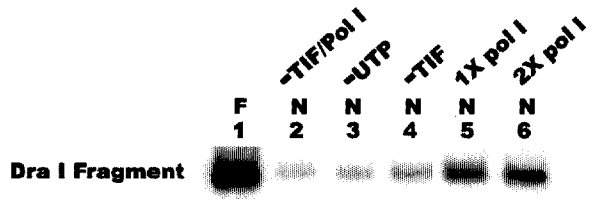


Figure 3.5. The extent of displacement is directly proportional to the fraction of template assembling active transcription complexes prior to transcription initiation. With 20% template usage on nucleosomal DNA (N), 20% of nucleosomes are displaced as determined by measuring the amount of fast migrating Dra I fragment recovered after transcription (compare lane 5 to lane 2). The amount of fast migrating Dra I fragment recovered from Free DNA (F) is shown in lane 1. The stalled complex (lane 3) does not lead to nucleosome displacement, and pol I alone without TIF-IB (lane 4) displaces a small amount of nucleosomes presumably to a small amount of end-to-end transcription.

Southern blotted, or end-labeled with ^{32}P, electrophoresed and viewed directly by fluorescence imaging. Because rRNA genes are multiple-copy, end-labeled rDNA can be distinguished from the bulk end-labeled genomic DNA (figure 3.6a). The 2.8kbp and1.9kbp coding region fragments, as well as the intergenic spacer (IGS) fragment were analyzed (figure 3.6a lanes 1 and 3). When wild-type rDNA is cross-linked by psoralen, the coding regions resolve into two populations-the slow migrating, actively transcribed nucleosome free (Slow) and the fast migrating, transcriptionally silent nucleosomal (Fast) (compare the coding regions in lanes 1 and 2). The intergenic spacer, on the other hand, is nucleosomal in both active and silenced genes. This coincides with earlier studies (Dammann et al., 1993). When the Δuaf strain's rDNA is psoralen cross-linked, only the nucleosomal population was observed, indicating pol II cannot substitute for pol I in stripping of nucleosomes from rDNA. However, the Δuaf strain grows slowly (generation time of 9hrs), and in yeast the ratio of nucleosomal to non-nucleosomal rRNA genes shifts to more nucleosomal with decreased growth rate(Dammann et al., 1993). The Δuaf strain's rDNA could appear to be completely nucleosomal because this strain grows slower than wild-type. To account for this, we slowed the growth rate of wild-type strain BJ926 by growing them with a different sugar (YP-raffinose instead of YP-dextrose) and dropping the temperature to 15°C instead of 30°C. This increased the generation time of the wild-type strain from 1.7 to 8hrs (data not shown). rDNA from the wild-type strain growing under these two conditions was compared to the Δuaf strain by psoralen cross-linking in a Southern blot that was probed for the 2.8 kbp rRNA

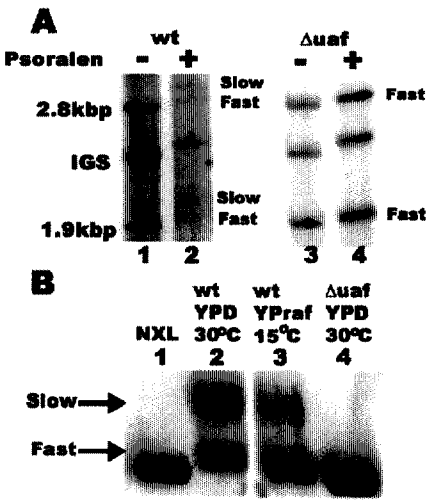


Figure 3.6. Pol II cannot substitute for Pol I specific displacement *in vivo*. **a**, Psoralen cross-linking of rDNA from yeast wild-type strain BJ926 and Δuaf strain NOY577 reveals nucleosome stripping in pol I transcribed rRNA genes (lane 2-Slow) but not pol II transcribed rRNA genes (lane 4). Following isolation of psoralen cross-linked rDNA, DNA was digested by EcoRI and end-labeled with ^{32}P. Shown are the 2.8kbp coding region, intergenic spacer (IGS) and 1.9kbp coding region fragments. The coding regions of wild-type cells when psoralen cross-linked resolve into two populations of rDNA the non-nucleosomal (Slow) and nucleosomal (Fast). The IGS remains nucleosomal. However, the Δuaf strain contains only nucleosomal rDNA (Fast) indicating pol II cannot substitute for pol I stripping. **b**, Southern blot comparison of psoralen cross-linked rDNA from wild-type BJ926 cells grown in YPD at 30°C (lane 2), wild-type BJ926 cells grown in YPraffanose at 15°C (lane 3), and Δuaf cells grown in YPD at 30°C (lane 4). Due to slower growth rate by wild-type cells grown in YPraffanose at 15°C, rDNA shifts to a more nucleosomal population. However, the slow migrating, non-nucleosomal rDNA population is undetectable in the Δuaf strain (compare (Fast) population in lanes 3 and 4).

gene fragment (figure 3.6b). Cross-linked rDNA isolated from the slower growing, wild-type strain still resolved into two populations, but, as expected, there was a shift to a higher proportion of nucleosomal, but the nucleosome-free fraction is still clearly visible (lane 3). In contrast, nucleosome-free rDNA was not observed in the Δuaf strain (lane 4), reinforcing the conclusion that even *in vivo* pol II cannot strip nucleosomes from rRNA genes, and that stripping is indeed a pol I specific phenomenon.

In this study, we have demonstrated nucleosomes interact with RNA polymerase I in a novel and distinctly different manor than observed for RNA polymerases II and III. Nucleosomes are specifically displaced from DNA transcribed by pol I *in vitro* and *in vivo*, and pol II cannot substitute for this pol I driven displacement *in vivo*. Given that a TIF-IB molecule, which is bound to DNA with a Kd of 56 pM (Radebaugh et al., 1998), is displaced by a transcribing RNA polymerase I molecule (Bateman and Paule, 1988), it is not unexpected that pol I would displace nucleosomes in its path. Additionally, we have discovered other important differences between how pol I and pol II elongate through nucleosomes. Pol II transcription is slowed five times more *in vitro* (Belotserkovskaya et al., 2003) than what we observed for pol I, and it appears pol I does not require additional components like FACT or increased ionic strength to elongate efficiently through nucleosomes *in vitro*. These differences highlight the dynamic interplay between nucleosomes and pol I, and demonstrate the importance of RNA polymerase I in maintaining the nucleosome-free chromatin structure observed for rRNA genes *in vivo*. In a rapidly growing cell,

the actively-transcribed nucleosome-free rRNA genes are loaded with transcribing RNA polymerase I molecules (French et al., 2003; Scheer, 1978) which precludes the possibility of nucleosome deposition. However, even under slow growth rates (when there are several times fewer polymerase I molecules associated with rDNA), some population of rRNA genes remain stripped even though enough space is provided for nucleosome deposition (Sandmeier et al., 2002; Scheer, 1978). Nucleosomes are assembled on rRNA genes after DNA replication (Lucchini and Sogo, 1995) and attainment of the nucleosome-free state *in vivo* requires transcription (Dammann et al., 1995). We have demonstrated *in vitro* that transcribing RNA polymerase I alone is sufficient for displacing nucleosomes in its path, and therefore we propose that RNA polymerase I alone establishes the nucleosome-free state of active rRNA genes *in vivo*.

Chapter 4

OTHER DATA NOT PRESENTED IN THE PAPER

4.1 INTRODUCTION

Chapter 4 includes some experiments that were not included in the paper (chapter 3). Included here are nucleosome array experiments using yeast native core histones and nucleosome assembly protein 1 (NAP-1) reconstituted on the plasmid pPolI 208-4, and tailed template experiments as described by Studitsky *et al.* 1997. These were side projects that could not be completed because of due to technical obstacles (see below).

4.2 INTRODUCTION-ARRAY EXPERIMENTS

Our laboratory decided to examine nucleosome read-through *in vitro*. We collaborated with the Ken VanHolde laboratory (department of Biochemistry and Biophysics, Oregon State University) to reconstitute nucleosomes on a plasmid that contained the rRNA promoter from *A. castellanii* and four tandem copies of the 208bp sea urchin 5S RNA gene fragment (Figure 4.1). When nucleosomes were deposited via salt dialysis in the presence of polyglutamic acid, evenly spaced nucleosomes were assembled. However, nucleosomes were not positioned correctly over the 5S RNA gene fragments. Nucleosome positions were shifted upstream towards the promoter as demonstrated by protection of EcoRI and Ava I sites that linked individual 5S RNA gene fragments (Figure 4.2). It appeared the promoter contained a dominant positioning sequence that determined the position of the downstream nucleosomes. However, pre-incubation with TIF-IB and RNA polymerase I prior to nucleosome reconstitution restored 5S sequence-directed positioning (figure 4.3) (Georgel et al., 1993). The Paule laboratory used this chromatin template to determine if pol I could transcribe a nucleosomal array. However, Chris Terpening (a former postdoctoral fellow in the laboratory) could not reproduce the positioning results, even in the presence of bound TIF-IB and polymerase I. Several methods of nucleosome assembly were attempted including yNAP-1 mediated assembly. When yeast core histones are incubated with yNAP-1 followed by incubation with DNA, correctly spaced nucleosomes assemble (Pilon et al., 1997). However,

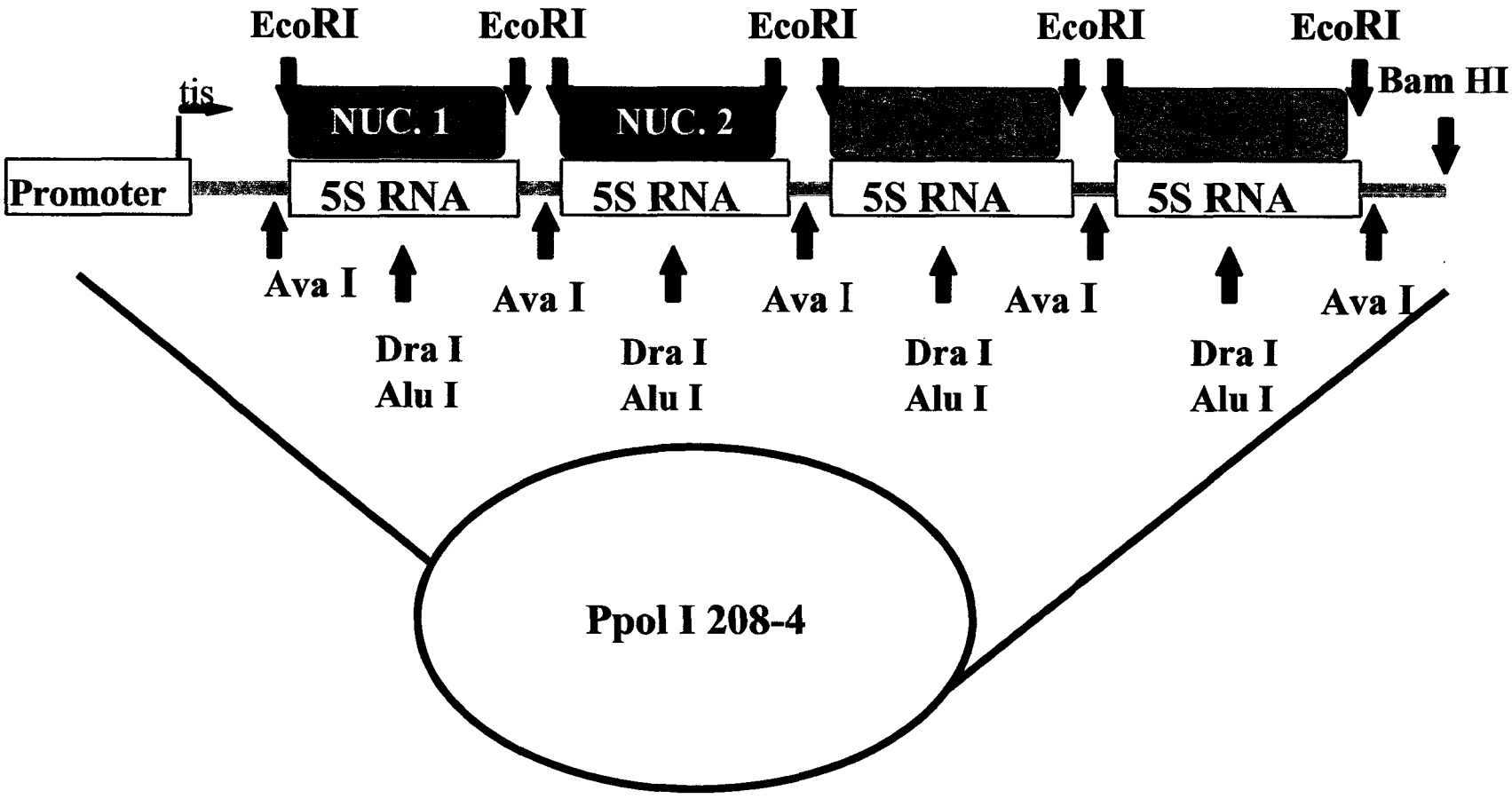


Figure 4.1. Ppol I 208-4 construct assembled with correctly spaced nucleosomsomes. When nucleosomes are positioned correctly the Ava I and EcoRI sites should be accessible for digestion while the Dra I and Alu I sites will be protected. To generate run-off transcripts, plasmid DNA will be digested with Bam HI prior to reconstitution.

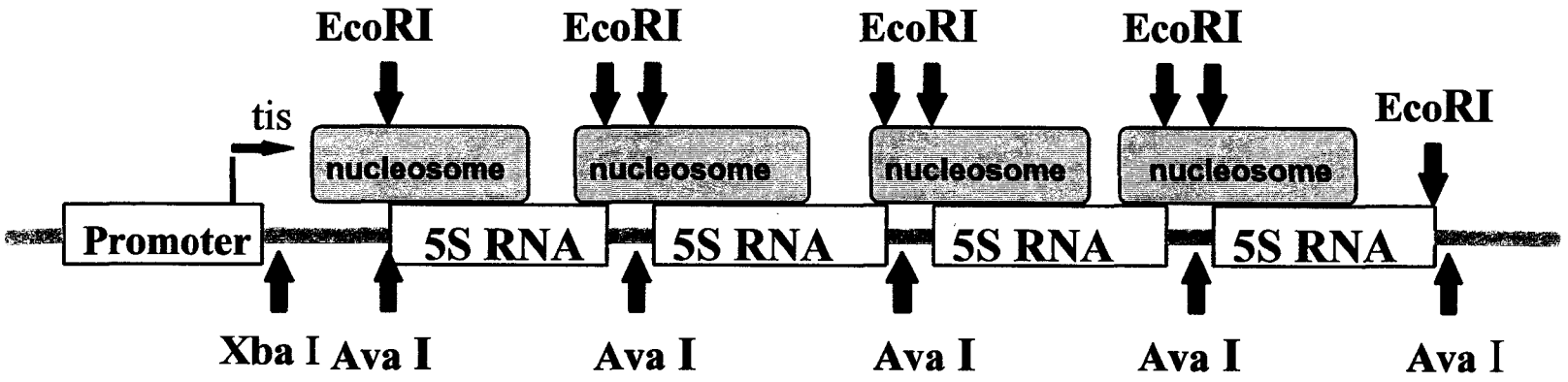


Figure 4.2. Incorrect positioning of nucleosomes observed by the our laboratory and the VanHolde laboratory. The EcoRI and Ava I sites are protected due to a shift in nucleosome position.

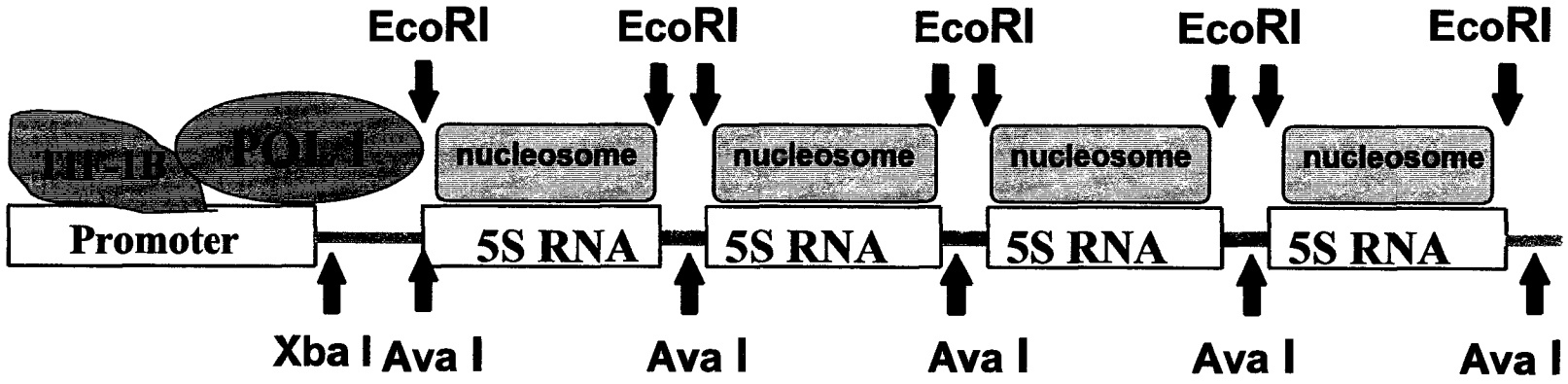
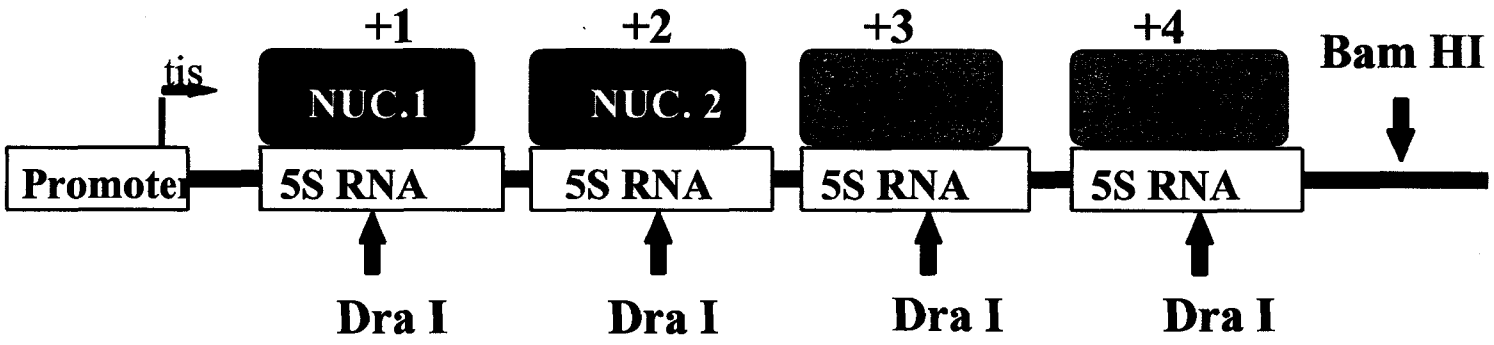


Figure 4.3. The VanHolde laboratory found that preincubation with TIF-IB and RNA polymerase I, before reconstitution, restored correct positioning of nucleosomes.

every method he tried failed to fix the positioning problem. At the time, it was decided that even with nucleosomes incorrectly positioned on the DNA, we could still examine pol I read-through of a multi-nucleosomal template. BamHI linearized plasmid DNA was under-assembled by reducing the nucleosome:DNA ratio and the DNA was digested with Dra I. The Dra I site was completely protected when nucleosomes were fully assembled, but when under-assembled, some of the Dra I sites become accessible giving rise to a ladder of DNA fragments depending on how many nucleosomes were assembled on any given plasmid DNA molecule (Figure 4.4). This generates templates on which pol I must transcribe through one to four nucleosomes to reach the Dra I DNA runoff end. Pol I was able to transcribe though one, two, three, and four nucleosomes, indicating nucleosomes do not block pol I transcriptional elongation (Figure 4.5). The next goal was to map the fate of an array of nucleosomes during pol I transcription. However, due to the variable positions of nucleosomes, mapping them would be difficult. Therefore, I tried to correct the positioning problem (methods and results for these experiments are discussed below).

4.3 INTRODUCTION: TAILED TEMPLATE EXPERIMENTS

In addition to examining nucleosome displacement by RNA polymerase I initiated from the *A. castellanii* rRNA promoter, an examination of nucleosome displacement by pol I initiated from a tailed template (similar to Studitsky's pol III experiments) was also carried out. Pol III initiated on a tailed template does not



Expected size of run-off transcripts:

150nt--No Nulceosomes present or RNAP I cannot transcribe through nucleosomes.

358nt--One nucleosome present or RNAP I cannot transcribe through multiple nucleosomes.

566nt

774nt

982nt

Figure 4.4. Under reconstituting pPol I 208-4 followed by complete digestion by Dra I generates various sizes of DNA leading to various length of RNA transcripts. Depending on how many nucleosomes are assembled on a given template, pol I would transcribe one through four nucleosomes.

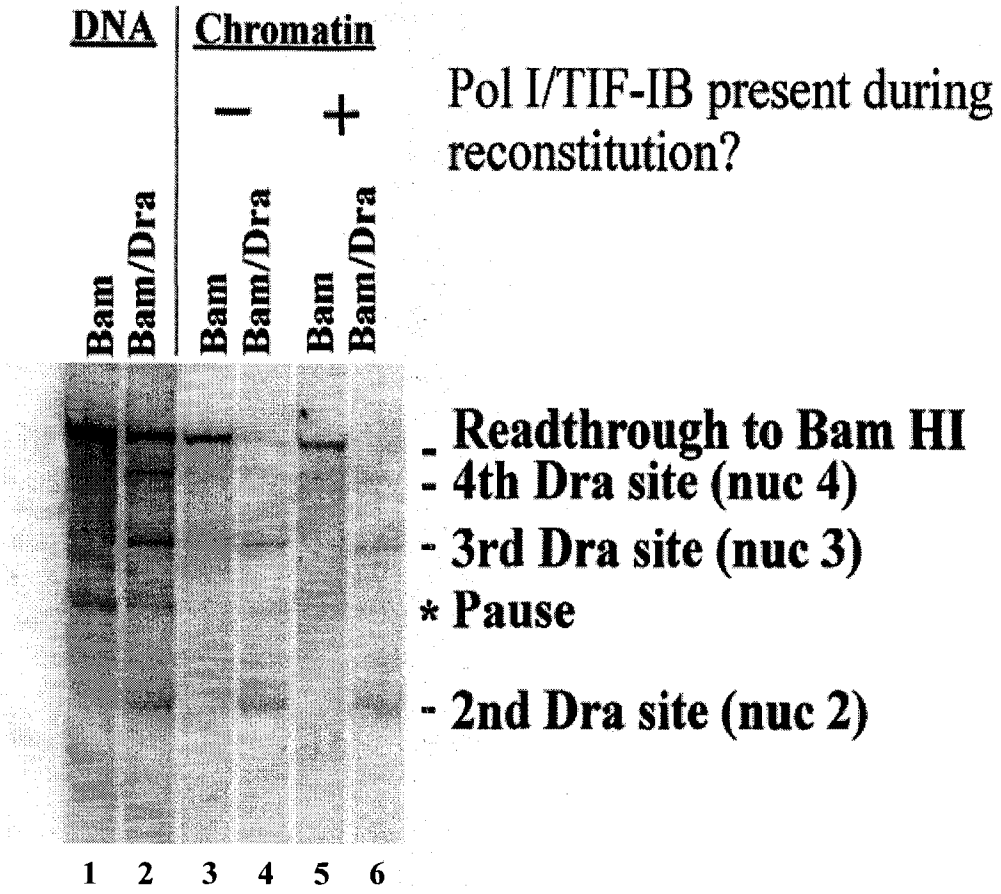


Figure 4.5. Run-off transcription assay of under-assembled nucleosomal (lanes 3-6) or naked (lanes 1 and 2) pPolI 208-4. The plasmid DNA was digested with Bam HI (lanes 3-6) and preincubated with TIF-IB and RNA polymerase I (lanes 5 and 6) prior to nucleosome reconstitution. Following reconstitution, DNA was completely digested with Dra I (lanes 4 and 6). Lane 2 is naked DNA partially digested with Dra I. Run-off transcription assays were conducted as described in Idia and Paule (1992).

displace nucleosomes from DNA, but a nucleosome translocates similar to SP6 polymerase (Studitsky et al., 1994). This translocation occurs in the presence of competitor DNA indicating the nucleosome never leaves the template DNA during translocation. However, template usage was low (about 30%) and while, in this instance, RNA:DNA hybrids did not form during transcription, they are common for all RNA polymerases. In addition, elongation complexes initiated from a tailed template can be very unstable (see below). We wanted to try these experiments with pol I to determine whether the fate of nucleosomes was when transcription is initiated from a tailed template.

4.4 MATERIALS AND METHODS

4.4.1 ARRAY NUCLEOSOME ASSEMBLY

Native yeast core histones were combined in a 1:1 (w/w) ratio with recombinant yNAP1p (provided by the Laybourn laboratory) to a final concentration of 0.25 mg/ml each in 10mM TRIS-HCl, pH8.0, 1mM EDTA, 150mM NaCl, 100μg/ml acetylated bovine serum albumen (reconstitution buffer). Histone:NAP1p was incubated at 37°C for 15 minutes, followed by incubation with 1 μg pPolI208-4 plasmid DNA for one hour at 37°C under the same buffer conditions. To test for TIF-IB/pol I effects on reconstitution, DNA was linearized by BamHI, and incubated with 2 μl TIF-IB and RNA polymerase I (30 mU nonspecific activity) for 30min at 25°C prior to reconstitution.

4.4.2 TOPOLOGICAL ASSAYS

Prior to nucleosome deposition, supercoiled pPolI 208-4 DNA was relaxed by incubation with topoisomerase I (MBI) (0.6 U/μg DNA) in reconstitution buffer at 37°C for 40min, followed by the addition of more topoisomerase I (0.6 U/μg DNA) and incubation for another 40min. Various amounts of histone:NAP1p complex was incubated with relaxed DNA for one hour at 37°C followed by the addition of 200 μl stop mix (20mm EDTA, pH 8.0, 200mM NaCl, 1% SDS (w/v). Five μl 2.5mg/ml proteinase K was added and incubated at 37°C 15min followed by phenol/chloroform extraction and sodium acetate-ethanol precipitation. The DNA was resuspended in 10mM TRIS-HCl pH 8.0 with loading dye and electrophoresed in a 10cm 1% agarose gel in 1X TBE buffer until the bromophenol blue dye ran to the bottom of the gel. The gel was stained with ethidium bromide and photographed.

4.4.3 MICROCOCCAL NUCLEASE DIGESTION OF NUCLEOSOMAL TEMPLATES

1.5 μg of reconstituted plasmid DNA was incubated in 100 μl at 37°C for 2min with 0.2, 0.4, 0.6 and 0.8U micrococcal nuclease in reconstitution buffer supplemented to 1mM $CaCl_2$. The digestion was stopped by adding 6 μl 0.5M EDTA followed by proteinase K digestion, incubated at 37°C for 15min, phenol/chloroform extracted, ethanol precipitated, and resuspended in 9 μl 10mM TRIS-HCl pH 8.0 with loading dye. The samples were electrophoresed on a 1.5% agarose, 1X TBE gel at 80V until the bromophenol blue dye migrated

approximately 8cm down the gel. The gel was stained with ethidium bromide to visualize the DNA.

4.4.4 MAPPING OF NUCLEOSOMES BY RESTRICTION ENDONUCLEASE DIGESTION

Prior to reconstitution, pPolI 208-4 was digested with BamHI in order to generate run-off transcripts, and incubated with TIF-IB and pol I for 30min at 25°C. DNA was reconstituted with nucleosomes as described. TIF-IB/pol I bound, BamHI linearized, reconstituted pPolI 208-4 (500 ng) was digested with 10U of EcoRI, AvaI, or Alu I for 30 min at 37°C. Digested DNA was phenol/chloroform extracted, precipitated, and resuspended in 10mM TRIS-HCl pH 8.0 with loading dye, and electrophoresed on a 10cm 1X TAE, 1% agarose gel until the bromophenol blue dye migrated 5cm. The gel was blotted to a gene screen plus membrane (NEN) via the salt transfer method (NEN). The blot was hybridized with a labeled probe to the sea urchin 208bp 5S RNA gene fragment made by random priming over-night at 60°C in hybridization buffer. The blot was washed two times for 5min at room temperature, washed twice at 60°C for 30 min in 1XSSC/0.1%SDS buffer, and washed once at room temperature with 0.1XSSC buffer. The probed blot was visualized by a phospoimager as described in chapter 2.

4.5 MATERIALS AND METHODS-TAILED TEMPLATE EXPERIMENTS

4.5.1 DESIGN OF SP6 5S DNA

Two oligonucleotides, TATCATACACATACGATTTAGGTGACACTATA-GAATTAAT and TCGAGATAGTATGTGTATGCTAAATCCACTGTGATATCTTA-ATTA were synthesized (IDT) and annealed to otain the minimal SP6 RNA polymerase promoter. The promoter DNA was inserted into PBS(-) plasmid containing the sea urchin 160bp 5S DNA fragment at the Sma I site to generate the PBS(-)SP6 5S construct. The PBS(-)SP6 5S DNA was digested with Sac I/Hind III to yield the 235bp tailed-template and gel purified. The SP6 start site is located 33bp from the Sac I end. This construct is designed to stall a polymerase at +15 when initiated from the Sac I end and depleted of CTP.

4.5.2 RECONSTITUTION OF SP6 5S DNA

1 μg of purified SP6 5S DNA was labeled by filling-in the Hind III overhang with Klenow fragment (MBI), dGTP, dCTP, dTTP, and α-P^{32} dATP. This was combined with 9 μg of cold filled-in SP6 5S DNA. The SP6 5S DNA was assembled into nucleosome core particles as described previously (Chapter 2). Reconstituted NCPs on this template were purified via 5-30% sucrose gradient sedimentation, dialyzed in 10mM TRIS-HCL over-night at 4°C, and concentrated to approximately 26 ng/μl. Purified NCPs were stored at 4°C.

4.5.3 ASSEMBLY OF TRANSCRIPTION COMPLEXES

5 ng of NCP SP6 5S DNA was added to a 50 μl transcription reaction at

25°C for 15min containing 40mM TRIS-HCl pH 8.0, 6mM $MgCl_2$, 100mM NaCl, 3mM DTT, 100 μg/ml BSA, 5% glycerol, and 400μM CTP, 400μM ATP, 10μM UTP, and 10mU (non-specific activity) RNA polymerase I. This stalls the polymerase at +15. 400μM GTP, and α-P^{32} UTP were added and transcription proceeded for 5min. The reactions were proteinase K/SDS digested, precipitated, resuspended in 98% formamide buffer with loading dye, and electrophoresed in a 6% polyacrylamide, 7M urea denaturing gel. To test for polymerase stability, the paused transcription complexes were challenged with 50 μg/ml pUC19 competitor DNA for 5min followed by the addition of 6 μl 50% glycerol and resolved by 5% native PAGE.

4.4.4 RNA:DNA HYBRID ANALYSIS

To test for RNA:DNA hybrids in the tailed template experiments, transcriptions were conducted as described, except competitor DNA was not added. RNA transcripts were digested with either RNase A (digests single stranded RNA) or RNase H (digests RNA:DNA hybrids) at 37°C for 10min followed by proteinase K/SDS extraction, precipitation, and electrophoresis on a denaturing gel.

4.6 RESULTS

4.6.1 ASSEMBLY OF NUCLEOSOME ARRAYS ON PPOLI 208-4

Topological assays of reconstituted pPolI 208-4 revealed reconstitution

was dependent on the ratio of histones/NAP1p to DNA (Figure 4.6). Relaxed, closed-circular DNA became completely supercoiled at a ratio of histones to DNA (w/w) of one to one. Samples electrophoresed in the presence of 1.8 μg/ml chloroquine revealed the DNA became relaxed at the same ratio, indicating reconstitution of the DNA was complete. Micrococcal nuclease digestion of reconstituted pPolI 208-4 showed that nucleosomes were assembled with spaced nucleosomes, as mono, di, and tri-nucleosomes are visible after digestion (Figure 4.7).

If the nucleosomes were positioned correctly on pPol I 208-4, only the 5S DNA fragment should be detected in a Southern blot when digested with EcoRI or Ava I and probed with 5S DNA, and the Alu I site should be completely protected (Figure 4.3). However, nucleosomes were reconstituted on BamHI linearized pPolI 208-4 the Ava I and EcoRI restriction enzyme sites were partially blocked (Figure 4.8 lanes 3, and 5) indicating nucleosomes were positioned incorrectly on the 5S RNA genes. This incorrect positioning was not relieved when TIF-IB and pol I were preincubated prior to reconstitution (lanes 4 and 6). A ladder of fragments representing one, two, or three nucleosomes, or the full-length plasmid with all four nucleosomes present on the DNA is observed. This indicates positioning is random on these reconstituted templates, which causes some restriction sites to be accessible while others remain blocked (Figure 4.2). Since we could not reproduce correct positioning, this phase of the project was discontinued (discussed below).

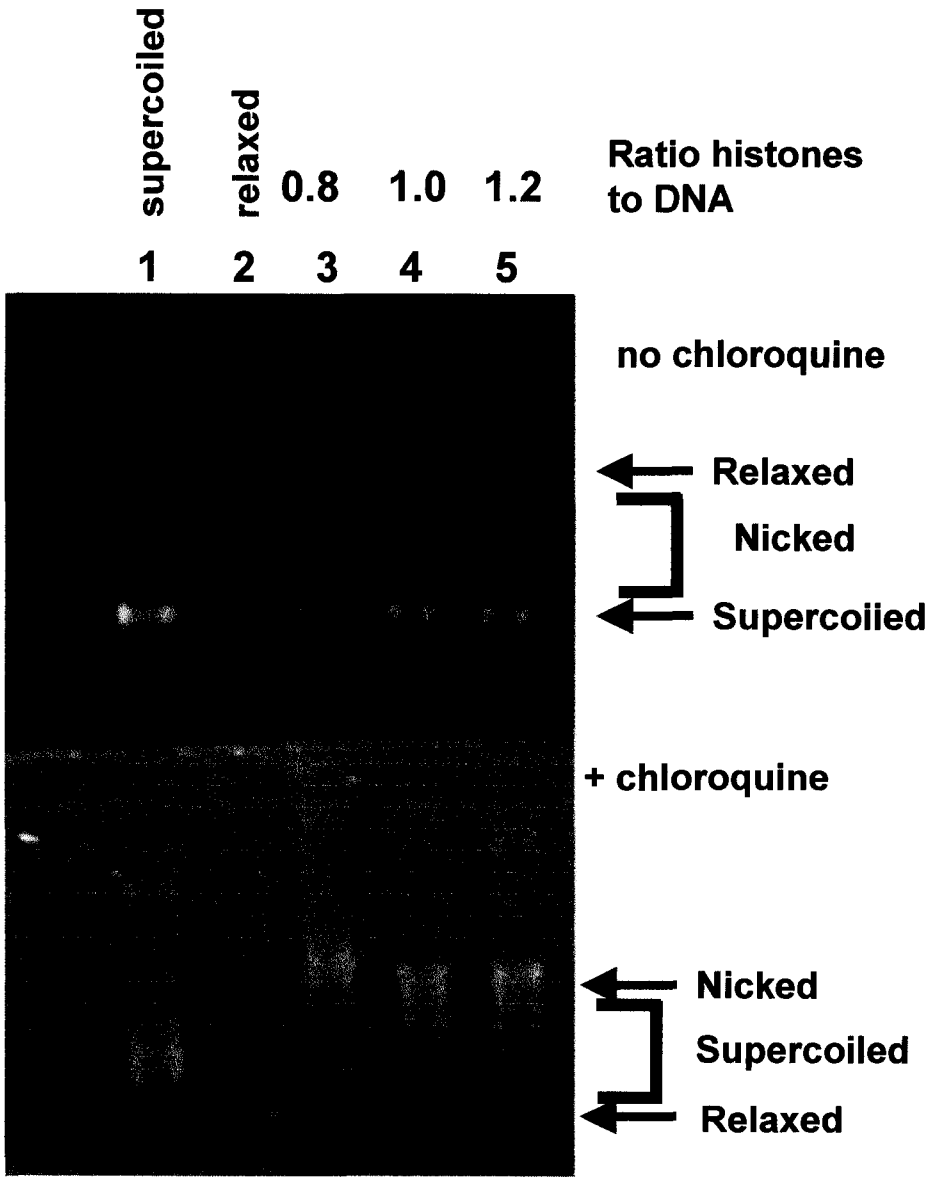


Figure 4.6. Topological assay of reconstituted pPolI 208-4. The bottom gel contains chloroquine. Lanes 3,4, and 5 contain increasing amounts of histones to DNA.

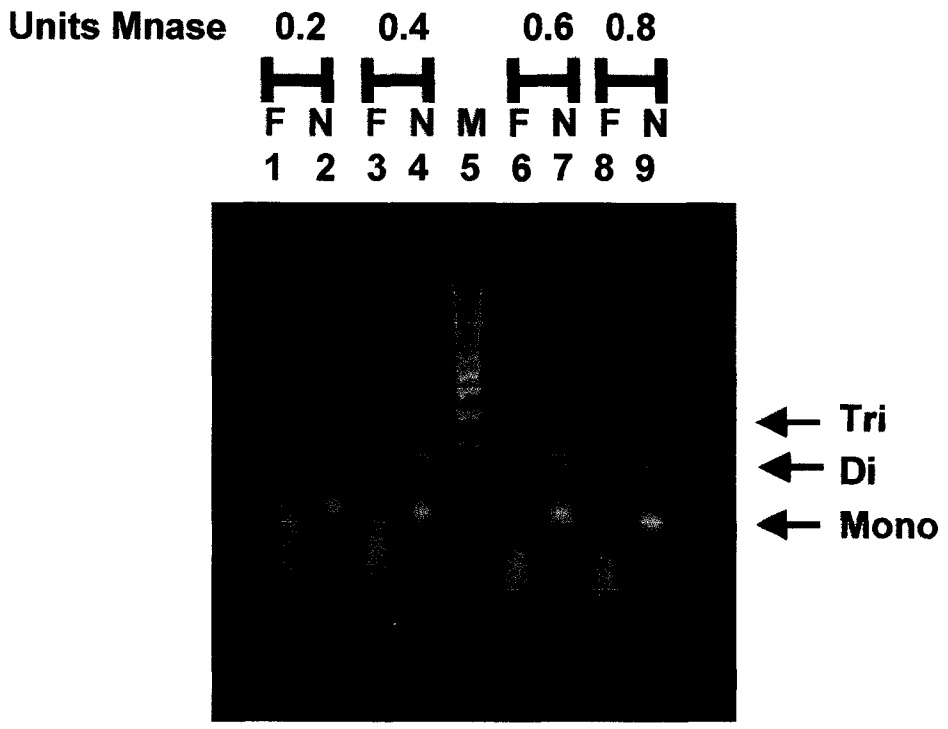


Figure 4.7. Micrococcal nuclease digestion of reconstituted pPol I 208-4. Naked DNA (lanes 1,3,6, and 8) and NCP DNA (lanes 2,4, 7, and 9) were digested with increasing amounts of Micrococcal nuclease.

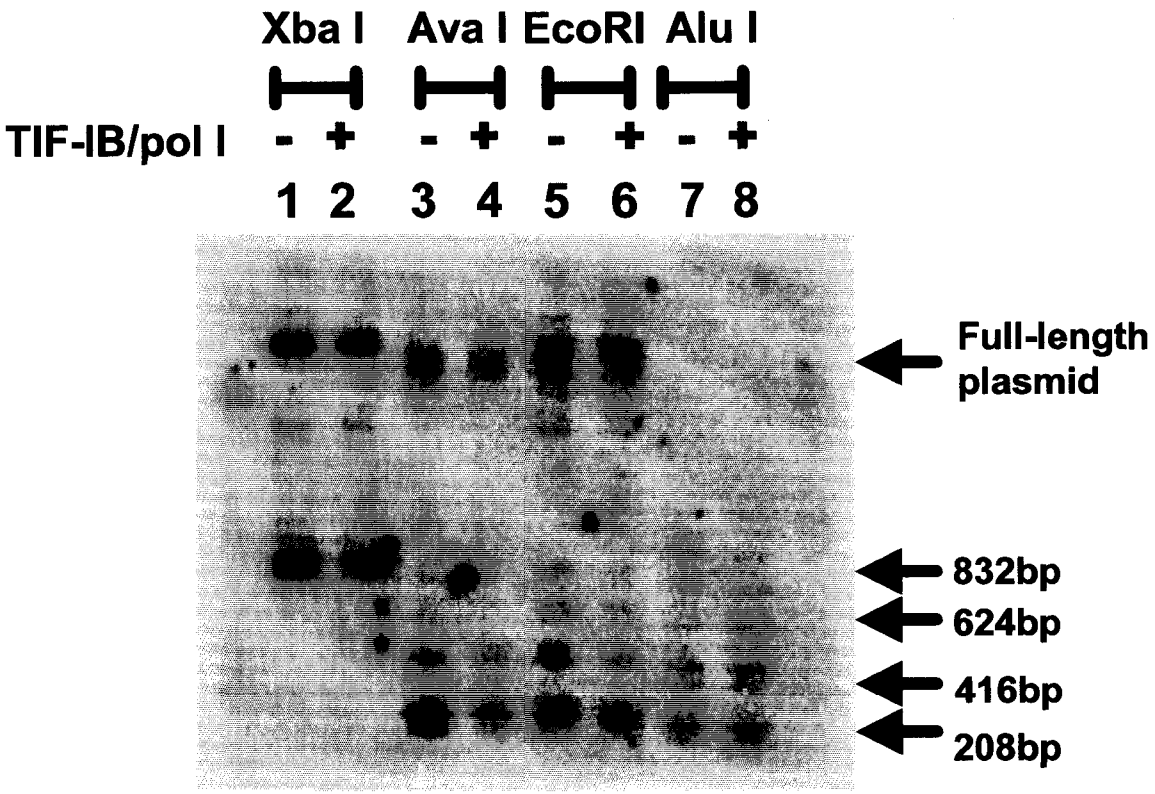


Figure 4.8. Nucleosomes are incorrectly positioned on Bam HI linearized, reconstituted pPolI 208-4. Nucleosomal DNA was digested with Xba I (lanes 1and 2), Ava I (lanes 3 and 4), EcoRI (lanes 5 and 6), or Alu I (lanes 7 and 8). DNA was preincubated with TIF-IB and pol I (lanes 2, 4, 6, and 8) prior to reconstitution.

4.6.2 TAILED TEMPLATE EXPERIMENTS

When nucleosomes were deposited on the SP6 5S DNA, two different positioned nucleosomes were observed (Figure 4.9). One major positioned nucleosome was located over the 5S DNA and one minor location over the SP6 promoter. This agrees with what has been observed previously (Studitsky et al., 1994). To test for efficiency of transcription complex formation, transcription complexes were formed by incubation with polymerase I and stalled at +15 (Figure 4.10). Nearly all the SP6 5S DNA in the reaction was bound by pol I in an NTP specific manor, indicating efficient assembly of transcription complexes. Transcription of SP6 5S DNA by RNA polymerase I (initiated from the Sac I end) yielded the correct size transcript (235nt). However, when the stalled complex was challenged with competitor DNA, the transcription complex dissociated, indicating the transcription complex initiated on the tailed template was not stable to challenge with competitor DNA (Figure 4.10 compare lanes 4 and 5). This means we could not test for nucleosome displacement in a single round of transcription because the polymerase was removed from the template DNA in the presence of competitor DNA. Thus, examination of nucleosome fate was not possible with this system. In addition, when the DNA was transcribed in the absence of competitor DNA, a large proportion of transcripts were in a RNA:DNA hybrid. RNA was found to be susceptible to RNase H and resistant to RNase A digestion (Figure 4.11). The size of the hybrid equaled the length of DNA, indicating the entire transcript formed a hybrid. The polymerase was not stable to competitor DNA and nucleosomes have trouble assembling on RNA:DNA

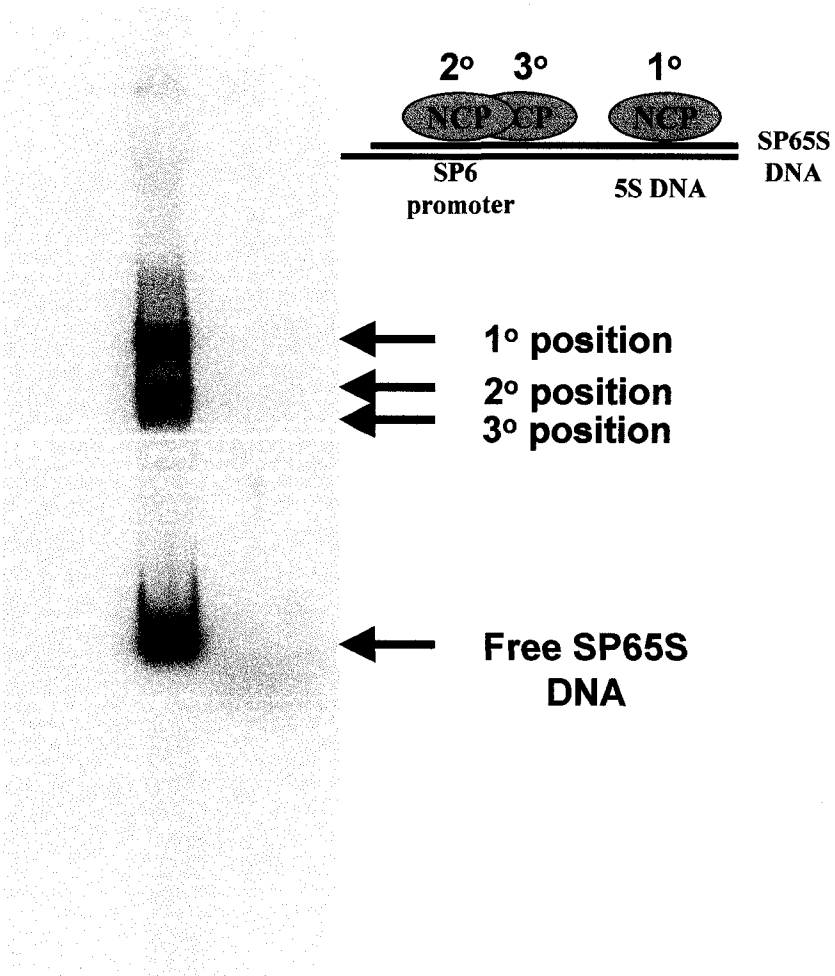


Figure 4.9. Nucleosomes assembled on SP65S DNA three dominant positions. The primary position is over the 5S DNA and the secondary and tertiary positions are located over the SP6 promoter.

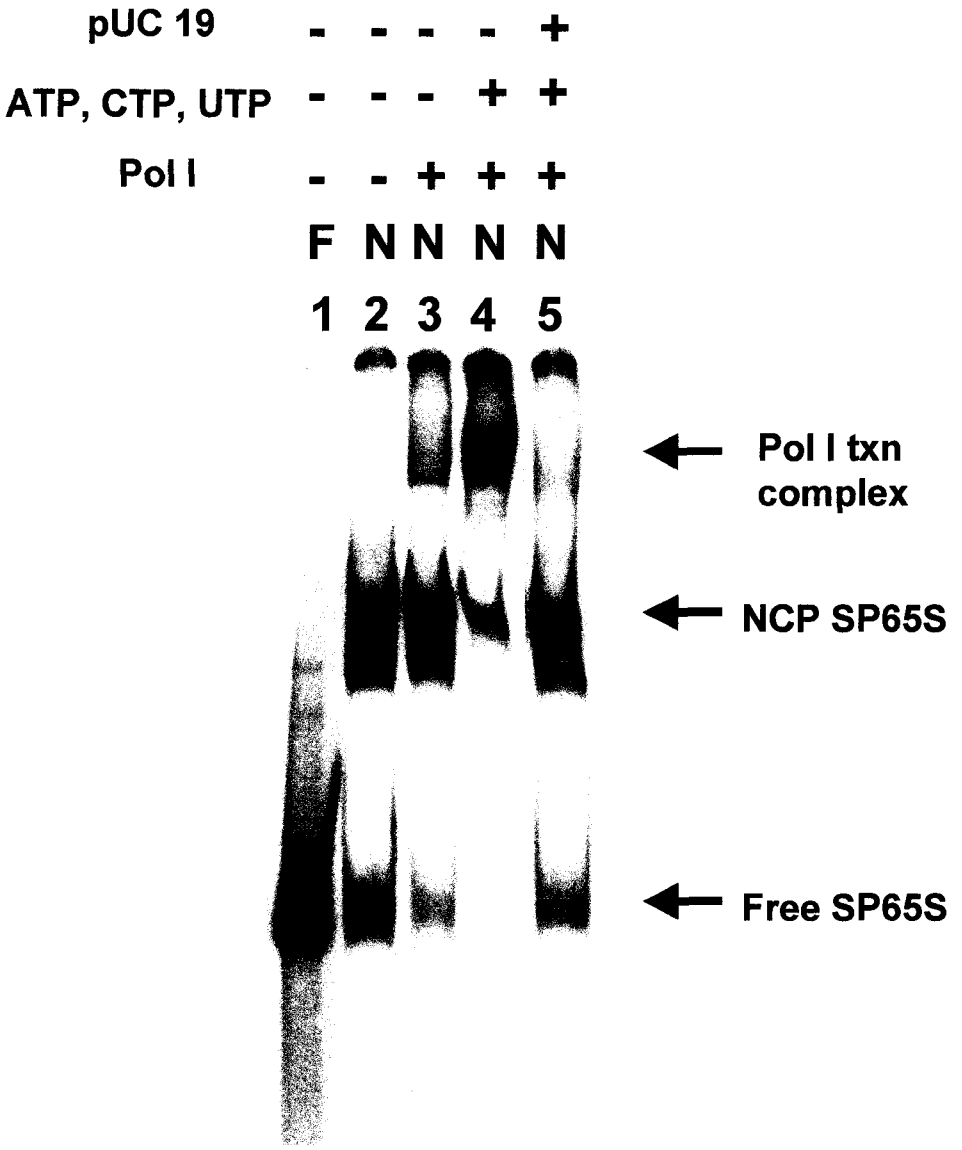


Figure 4.10. Pol I transcription complexes are unstable in the presence of competitor DNA. Shown are the free DNA (lane 1), NCP DNA with out transcription factors (lane 2), NCP incubated with pol I in the absence of NTPs or competitor DNA (lane 3), NCP DNA incubated with pol I, all NTPs except GTP but without competitor DNA (lane 4), and NCP under the same conditions as lane 4 except competitor DNA has been added (lane 5).

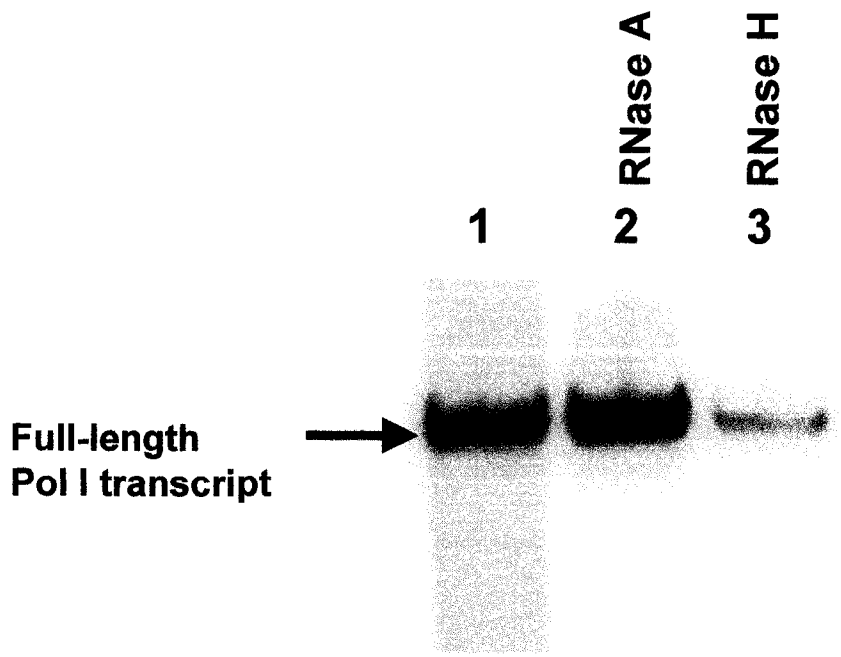


Figure 4.11. RNA produced during pol I transcription forms an RNA/DNA hybrid with SP65S DNA. Run-off transcripts were treated with RNase A (lane 2) or RNase H (lane 3) prior to electrophoresis.

hybrids. Therefore, we could not examine nucleosome displacement with pol I initiated from a tailed template.

4.7 DISCUSSION

4.7.1 ARRAY EXPERIMENTS

The promoter appears to contain a dominant nucleosome positioning element compared to sea urchin 5S DNA. The nucleosome that presumably assembles on the promoter competes with the 5S RNA genes for positioning the array of nucleosomes on the DNA such that positioning becomes random in the population. The proximity of the promoter is important for this phenomenon, because when pPolI 208-4 is linearized with Xba I prior to nucleosome assembly, which puts the promoter on the other end of the linearized plasmid (Figure 4.3), correct positioning was restored (Georgel et al., 1993). However, pre-incubation with TIF-IB and pol I when using the circular nucleosomal template seemed to correct the positioning problem (Georgel et al., 1993). Neither Dr. Terpening nor I could duplicate his result with our histone preparations, and because having precisely positioned nucleosomes was critical for the displacement assays, we decided to abandon the array experiments. Since this data was collected, a new recombinant assembly system has been developed using ACF1/NAP1 to assemble nucleosomes on arrays. Perhaps conducting nucleosome reconstitution in this manor could fix the positioning problem. These arrays were originally to be used to test if multiple polymerases were required to displace nucleosomes. However, since one polymerase seemed to displace nucleosomes efficiently (see chapter 3) we decided it was not crucial to use the

arrays.

4.7.2 TAILED-TEMPLATE EXPERIMENTS

It was disappointing to see the RNA polymerase I transcription complex was not stable to challenge with competitor DNA when initiated from a tailed-template. We wanted to determine whether nucleosomes were displaced from DNA in the presence of competitor DNA when using a tailed-template. Recall that nucleosomes are not displaced from tailed-template DNA when transcribed by pol III (Studitsky et al., 1997). In those experiments, in the absence of competitor DNA, nucleosomes translocated upstream of their original position as shown by restriction enzyme digestion. However, when the authors conducted experiments with competitor DNA and pol III, a test of elongation complex stability was not conducted. The DNA remained nucleosomal after the transcription reaction, but the position of the nucleosome after transcription was not determined. Transcription may not have occurred because the pol III was displaced from the template by competitor DNA, throwing into doubt the conclusion that nucleosomes translocate even in the presence of competitor DNA. In addition, it seemed when pol I transcription is initiated from a tailed-template, a large proportion of transcripts formed stable RNA:DNA hybrids with template strand DNA. Nucleosomes have difficulty forming on RNA:DNA hybrids (Campbell and Setzer, 1992) making interpretation of any results questionable at best. We would have liked to see the tailed-template experiments corroborate the promoter driven displacement experiments. However, because in our system promoter-directed transcription is more efficient, tailed-template experiments are

not necessary to support our finding that nucleosomes are displaced by RNA polymerase I. When studying pol II-dependent displacement, the Studitsky laboratory had the same problem with RNA:DNA hybrids. They fixed the problem by initiating transcription from a synthetic melted-bubble template. Perhaps we could do the same, but when initiating transcription this way, template usage was reduced (50%), and since we were able to get efficient displacement from the promoter, initiation in this manner not required.

Chapter 5

FUTURE DIRECTIONS

Now that we have determined that nucleosomes are displaced by RNA polymerase I, we would like to examine how nucleosomes affect initiation of rRNA transcription. In mouse, transcription initiation was inhibited in the presence of nucleosomes (Langst et al., 1998), but our laboratory discovered transcription initiation was not affected by the nucleosomal pPolI 208-4 template when using *Acanthameoba* transcription factors (Chris Terpening unpublished results). However, the nucleosomes were not correctly positioned over the 5S DNA fragments in the pPolI 208-4 template. Use of the recombinant Nap1p/ACF1 system (See discussion in chapter 4) may fix the incorrect positioning of nucleosomes on our template. However, there are other experiments that could determine if nucleosomes affect pol I transcription initiation. For example, assembling a mono-nucleosome directly over the *A. castellanii* promoter may block TIF-IB binding. We could monitor blockage by EMSA or footprinting analysis. If TIF-IB binding is blocked, we could test nucleosome remodeling factors for their ability to relieve blockage and stimulate transcription.

We know the *A. castellanii* rRNA promoter competes for positioning of nucleosomes on downstream 5S DNA genes (Georgelet al., 1993). It would be

interesting to examine whether the promoter contains a strong nucleosome positioning sequence. Mapping the nucleosome's position could provide valuable insight into how nucleosomes might block or aid in transcription initiation. This would be of particular interest in yeast because histones H3 and H4 are known components of the pol I transcription factor UAF. Perhaps UAF interacts with nucleosomes present in the upstream promoter element to bind DNA. *In vitro*, transcription efficiency is weaker in yeast systems than for *A. castellanii* (Joe Gogain unpublished results), and it could be that yeast may require a nucleosomal template for high levels of transcription.

Finally, we would like to try psoralen cross-linking of rDNA in our Δuaf yeast strain containing a temperature sensitive mutation of Spt6 (*spt6-1004*). Spt6 is another chromatin transcription elongation factor that interacts with histones and plays a role in maintaining the chromatin structure of a wide variety of genes *in vivo* (Bortvin and Winston, 1996; Kaplan et al., 2003). Yeast containing the *spt6-1004* mutation have severe defects in chromatin structure following transcription by RNA pol II (Kaplan et al., 2003). Genes transcribed by pol II become extremely susceptible to micrococcal nuclease, presumably because nucleosomes are not re-assembled. It is currently believed Spt6 reforms nucleosomes following pol II transcription, and chromatin immunoprecipitation assays, using antibodies against H4, revealed nucleosomes are missing from these genes *in vivo*. This contradicts what was observed when a mono-nucleosomal template was transcribed by pol II *in vitro* where only one H2A/H2B dimer was displaced when transcription was initiated from a synthetic

melted-bubble template (Kireeva et al., 2002). We would like to use the psoralen cross-linking technique to see if pol II transcribed rRNA genes become stripped of histones in an *spt6-1004* background. If histones become stripped during pol II transcription in this new strain, it would be another line of evidence contradicting what has been observed during pol II transcription *in vitro* (Kireeva et al., 2002).

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