DISSERTATION

MECHANISM AND REGULATION OF RIBOSOMAL RNA TRANSCRIPTION

Submitted by

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In partial fulfillment of the requirements for

the degree of Doctor of Philosophy

Colorado State University

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ABSTRACT OF THE DISSERTATION

MECHANISM AND REGULATION OF RIBOSOMAL RNA TRANSCRIPTION

The sole responsibility of RNA polymerase I (pol I) is the transcription of the ribosomal RNA (rRNA) genes. This transcription can account for up to 60% of the total cellular RNA that is being transcribed in an actively dividing cell. Due to the large amounts of energy this transcription requires, it is of utmost importance for the cell to be able to regulate rRNA transcription efficiently. The rate at which pol I transcribes rRNA, closely follows cellular growth rate. Any change in cell proliferation results in immediate down regulation of rRNA transcription. The mechanisms responsible for mediating this rapid change in transcription rate are not completely understood. In order to fully comprehend the process by which rRNA is being regulated, it is important to characterize the functions of each of the pol I transcriptional components. The work presented herein focuses on identifying the important interactions involved in transcription factor binding, to both other factors within the pol I transcriptional machinery, and to the rRNA promoter. We report here the institution of a reconstituted transcription system for *Saccharomyces cerevisiae*, and the initial characterization of transcription factor DNA-binding activities using electrophoretic mobility shift and photo-cross-linking assays. Furthermore, we identify an interaction between a pol I specific subunit and a subunit of the fundamental pol I transcription factor. An interaction that may be important for the recruitment of pol I to the rRNA promoter. Additionally, we report the identification of the *Acanthamoeba castellanii* homologue to the yeast pol I transcription factor Rrn3p.

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We have characterized the activity of this factor, TIF-IA, in the *A. castellanii* system and show that it is required for the recruitment of pol I to the rRNA promoter.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 INTRODUCTION

Eukaryotic cells contain three DNA-dependent RNA polymerases. Each of these polymerases play a role in the elaboration of the ribosome. The sole responsibility of RNA polymerase I (pol I) is transcription of the 35S ribosomal RNA gene, with the only exception being in the African trypanosomes where coat protein genes are also transcribed (reviewed in Paule, 1998a). Following transcription, the 35S precursor rRNA is processed into the 28S, 18S, and 5.8S rRNAs, which are required for construction of the ribosome. Pol II transcribes the protein genes that encode for approximately 80 proteins used in a functional ribosome. Pol III is responsible for transcription of the 5S RNA. The 18S RNA along with approximately 33 ribosomal proteins form the small subunit of the ribosome, while the 28S and 5.8S RNAs come together with the pol III transcribed 5S RNA and approximately 49 ribosomal proteins to form the large ribosomal subunit.

The transcription of rRNA can account for up to 60% of all RNA being transcribed in an actively dividing cell. Additionally, it has been estimated that 50% of all pol II initiation events are for the ribosomal proteins (reviewed in Warner, 1999). Therefore,

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due to the large amounts of energy both transcriptional and processing events require for the construction of the ribosome, it is of utmost importance for the cell to efficiently regulate ribosomal production. Understanding how this regulation is coordinated will provide a general understanding of cellular growth control.

1.2 ORGANIZATION OF THE RIBOSOMAL RNA TRANSCRIPTION UNIT

In most species, the ribosomal RNA genes are organized in a head-to-tail repeated structure (Figure 1.1A). The size of each repeat varies between species and can range from 9 kb in lower eukaryotes up to 40 kb in mammals (Paule, 1998a). The head-to-tail repeats are present in multiple copies within the cell. The number of copies that each species contain is quite variable. For example the polyploid organism *Acanthamoeba castellanii* contains 24 copies per haploid genome, haploid *Saccharomyces cerevisiae* has approximately 150 copies, and mammalian cells contain 300 or more copies per haploid genome. Separating each rRNA gene is the intergenic spacer (IGS). The IGS generally exhibits poor sequence conservation between species, although there is conservation of both structural features (Marilley and Pasero, 1996) and sequence element organization (reviewed in Grummt, 2003; Paule and White, 2000; Paule, 1998a). Alteration of the conserved structure has actually been shown to produce detrimental effects on transcription factor binding (Marilley et al., 2002). The sequence elements required for transcriptional regulation include: the transcription initiation site (*tis*), the core promoter, the upstream promoter element, the proximal terminator, enhancer elements, spacer promoters, and transcription terminators (Figure 1.1B).

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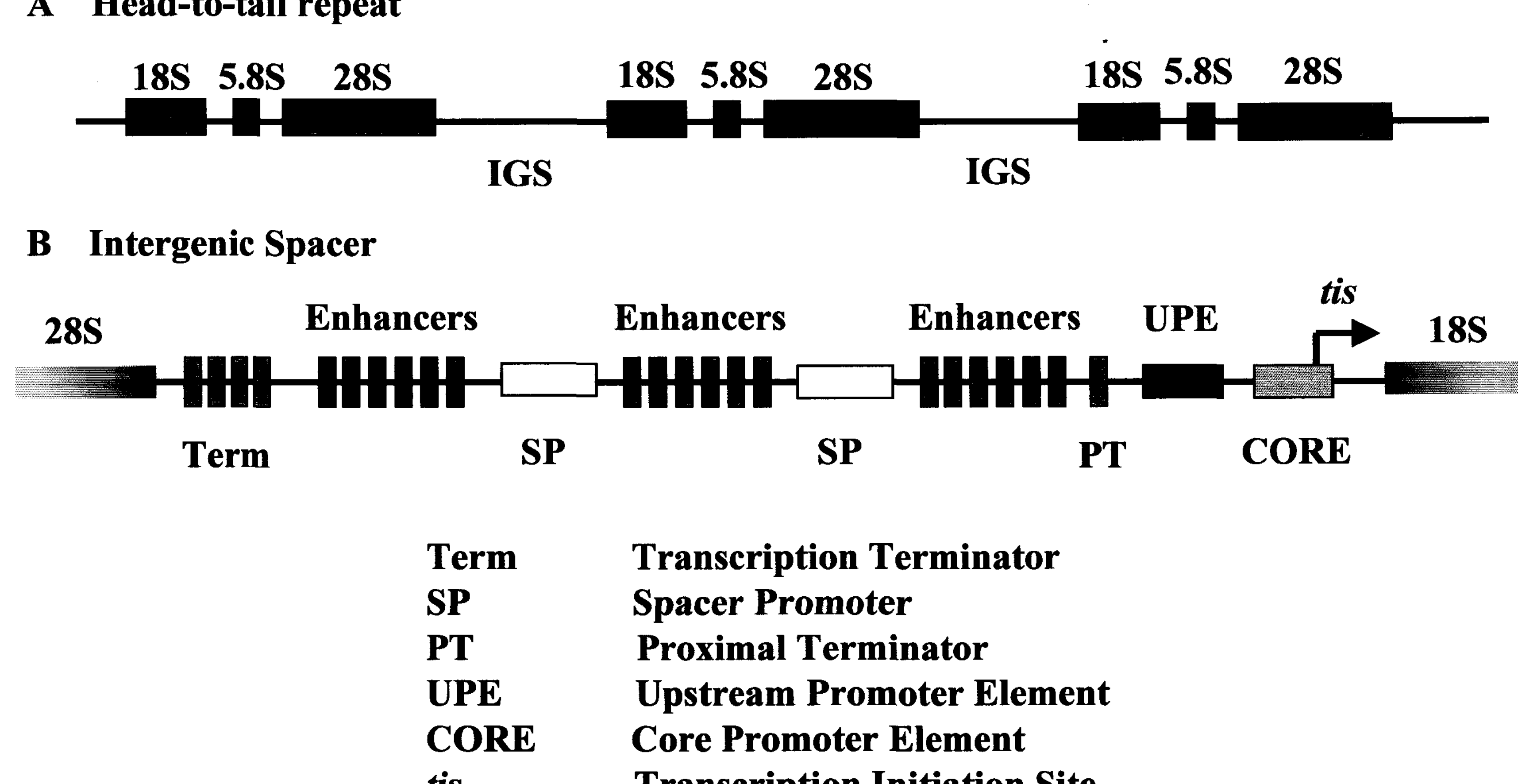

Figure 1.1. Organization of the head-to-tail repeats and the intergenic spacer. A) Shown is the general layout of the repeated heat-to-tail rRNA genes. **B)** Shown are the elements found within the intergenic spacer.

The core promoter is responsible for binding a fundamental transcription factor (see below) required for transcription initiation. Together, the core promoter and the transcription factor are necessary and sufficient for basal levels of transcription in most species. *In vitro*, the core promoter is the only sequence element absolutely required for correct initiation of rRNA transcription. *In vivo*, the core promoter generally requires additional elements to accommodate the levels of rRNA transcription needed by the cell. The core promoter extends from approximately 40 bp upstream to a few base pairs downstream of the *tis* (Keener et al., 1998). Two functional elements make up the core promoter, the *tis* and an upstream element (Iida et al., 1985; Kownin et al., 1985). Template commitment assays have shown the upstream regions of the core promoter are responsible for the majority of transcription factor binding (Kownin et al., 1985), while the *tis* may aid in transcription factor binding (Doelling and Pikaard, 1995; Radebaugh et al., 1997) and function as a site for strand separation upon transcription initiation (Bateman and Paule, 1988). The region surrounding the *tis* is the only well conserved element present in the rRNA IGS (Perna et al., 1992). This AT-rich conserved region functions in a manner that allows the DNA to melt more efficiently upon transcription initiation (Kahl et al., 2000). This region was termed the ribosomal initiator (rInr) due to its ability to direct transcription on its own from a reconstituted *in vitro A. castellanii* transcription system (Radebaugh et al., 1997). Subunits of the fundamental transcription factor have been shown to photo-cross-link to the rInr (Radebaugh et al., 1997).

The upstream promoter element (UPE) is responsible for binding transcriptional activators that enhance rRNA transcription over basal levels. These transcription factors aid in the recruitment of the general transcription factors to the rDNA promoter and

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provide overall stability to the complex. Studies from several species indicate the UPE can extend upstream of the *tis* approximately 150 bp (Haltiner et al., 1986; Kulkens et al., 1991; Musters et al., 1989; Windle and Sollner-Webb, 1986). Both the spacing between the UPE and the core promoter, and their helical alignment are important for transcriptional initiation (Pape et al., 1990; Xie et al., 1992). The only pol I transcription system that has not been shown to possess a UPE is *A. castellanii*. Transcription from the *A. castellanii* core promoter is believed to function so efficiency *in vitro* that the requirement of an UPE cannot be detected.

The proximal terminator (PT) is found approximately 200 bp upstream of the *tis* (Grummt et al., 1986; Henderson et al., 1989; McStay and Reeder, 1986). The PT is responsible for stopping any rogue polymerase molecules that have transcribed through the transcription terminator (see below) before they are able to disrupt the transcriptional machinery bound to the UPE and core promoter elements. The PT has also been shown to interact directly with the downstream promoter elements functioning to stimulate transcription of the rRNA gene (McStay and Reeder, 1990). Additionally, the PT has been implicated in chromatin remodeling at the promoter (Langst et al., 1997), and may function by bringing together the end of one transcription unit and the beginning of the next by oligomerization (Sander and Grummt, 1997).

The IGS also contains enhancers for ribosomal RNA transcription. These elements generally are able to function independent of distance, in a forward or reverse orientation, and in a *cis* or *trans* manner. Although ribosomal enhancers have been identified in such organisms as *Xenopus laevis*, mouse, *Drosophila melanogaster*, *A. castellanii*, rat, and yeast, their function in pol I transcription is poorly defined. In fact, it has recently been

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shown in *S. cerevisiae*, that deletion of the rDNA enhancer had no effect on rRNA synthesis or cell growth (Wai et al., 2001).

Spacer promoters are found within the IGS and contain functional pol I promoters. The spacer promoters stimulate rRNA transcription by producing a short transcript terminated at the proximal terminator (see below) (Grimaldi et al., 1990; Paule, 1998a). The mechanisms by which this stimulation of the downstream pre-rRNA promoter occurs is not completely understood.

The transcription terminator is the site for binding the mammalian termination factor, TTF-1, or the yeast termination factor Reb1p (Evers et al., 1995; Morrow et al., 1993). The binding of these factors present a physical block for the elongating pol I molecule and transcription is halted, followed by the release of the polymerase. The release of the pol I molecule is facilitated by protein factors such as the pol I transcript release factor (PTRF) (Jansa et al., 2001). In *S. cerevisiae*, the transcription terminator is located 93 bp downstream of the 28S ribosomal gene (Reeder et al., 1999). In mammalian cells, eight repeated Sal box elements make up the transcription terminator (Mason et al., 1998). In nearly every species examined, the primary transcription termination site provides leaky transcriptional termination (Reeder et al., 1999). Therefore, the presence of backup termination sites are necessary, such as the proximal terminator. Interestingly, both TTF-1 and Reb1p have been shown to play a role in relieving transcriptional repression from chromatin effects (Angermayr et al., 2003; Langst et al., 1997).

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1.3 POL I TRANSCRIPTIONAL MACHINERY

In vitro pol I transcription systems have been developed in many species including *S. cerevisiae*, *Schizosaccharomyces pombe*, *A. castellanii*, *X. laevis*, *Brassica oleracea*, *Arabidopsis thaliana*, mouse, rat, and human. Each of these systems generally contain the following factors required for rRNA transcription: an activating transcription factor, a fundamental transcription factor, pol I, and one or more essential pol I associated transcription factors. A schematic representation of the factors assembled in a preinitiation complex (PIC) from *S. cerevisiae*, *A. castellanii*, human, and mouse is presented in Figure 1.2. These four species represent the best defined pol I transcription systems.

1.3.1 Pol I

DNA-dependent RNA polymerase I is a multi-subunit enzyme that is responsible for the transcription of the genes encoded by the ribosomal DNA. Pol I is best defined in *S. cerevisiae*, where it contains at least 13 subunits, some of which are unique to the enzyme, and some of which are common between the three polymerases. The subunits common to all three polymerases are, ABC27, ABC23, ABC14.5, ABC10α, and ABC10β. AC40 and AC19 are common to pol I and pol III (reviewed in Carles and Riva, 1998). The two largest subunits of eukaryotic RNA pol I share sequence homology with the largest subunits of pol II and pol III, as well as with the *E. coli* polymerase subunits β' and β. Additionally, in yeast, the AC19 and AC40 subunits exhibit sequence similarities to the bacterial α subunit (reviewed in Carles and Riva, 1998). Furthermore, the B44 (rpb3) and B12.5 (rpb11) subunits of pol II, homologous to the AC40 and AC19 pol I subunits, respectively, are positioned in the pol II crystal structure in similar

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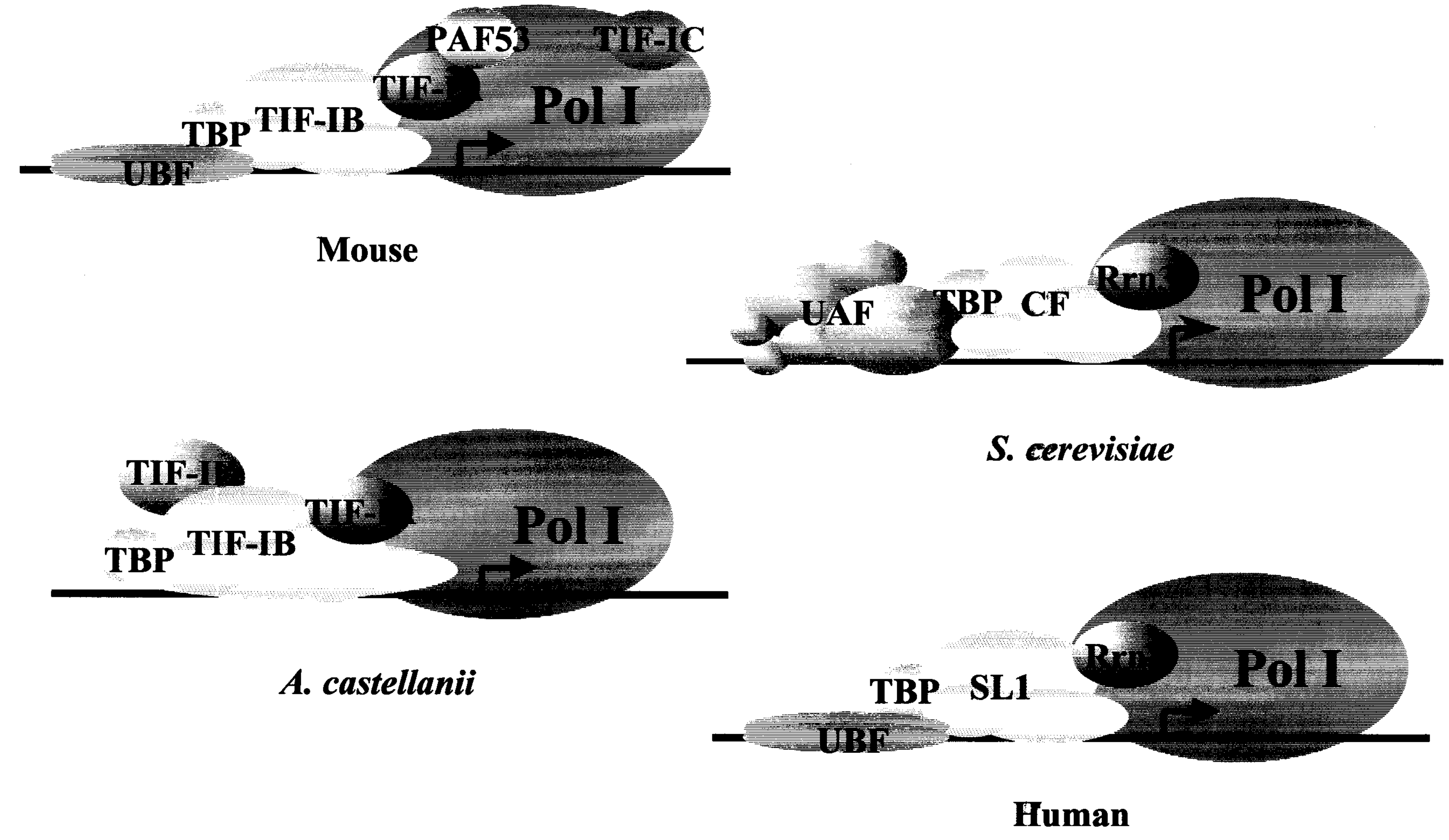

Figure 1.2. Schematic representation of preinitiation complexes (PICs). Shown are the factors required for the assembly of PICs from mouse, *S. cerevisiae*, *A. castellanii*, and human.

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locations as the α subunits in the bacterial crystal structure (Cramer et al., 2001). The subunits unique to yeast pol I are, A49, A43, A34.5, A14, and A12.2. Deletion of these subunits exhibit only conditional lethality with the exception of A43, which is required for cell growth (Thuriaux et al., 1995). The A43 subunit associates with a factor essential for transcription, TIF-IA/Rrn3p (Cavanaugh et al., 2002; Imazawa et al., 2002; Peyroche et al., 2000; Yuan et al., 2002).

Murine pol I contains 11 bona fide subunits and possibly up to three polymerase associated factors (PAFs): PAF53, PAF51, and PAF49 (Hanada et al., 1996). However, it is not clear if PAF53 is an associated factor or an actual pol I subunit. PAF53 possesses sequence similarities to the yeast A49 subunit, associates with an active form of pol I, and is capable of binding the upstream binding factor (UBF) (Hanada et al., 1996; Seither et al., 1997). An additional factor, PAF67, has been identified in mammalian systems. PAF67 possess properties similar to PAF53, in that it is tightly associated with transcription competent pol I and interacts with UBF (Seither et al., 2001).

1.3.2 Fundamental transcription factors

Factors have been identified in each of the pol I transcription systems that are responsible for recruiting the polymerase to the promoter and positioning it to start transcription at the *tis*. These fundamental transcription factors generally bind to the promoter in the core promoter region. Together the factor and the core confer species specificity to the pol I transcription machinery (Clos et al., 1986; Grummt et al., 1982; Learned et al., 1985; Miesfeld and Arnheim, 1984; Mishima et al., 1982). These factors are multi-subunit protein complexes consisting of the TATA-binding protein (TBP) and three to four TBP-associated factors (TAFs) (Paule and White, 2000). The species

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specificity characteristic of the fundamental transcription factors is conferred by the TAFs, not TBP, whereby substitution of the endogenous TBP with TBP from alternate species does not affect transcription (Rudloff et al., 1994). Direct physical interactions exist between TBP and each of the TAFs in mammalian systems, as well as between each of the TAFs themselves (Heix et al., 1997). Additionally, the TAFs of mouse and human are conserved in a manner that allows for the formation of chimeric factor complexes in all combinations (Heix et al., 1997). However, these chimeric complexes, are not functional for specific transcription initiation, highlighting their species specific characteristics. In addition to being part of the pol I transcriptional machinery, TBP is an essential component of fundamental transcription factors from the other two RNA polymerase systems, TFIID and TFIIIB (Kassavetis et al., 1992; Pugh and Tjian, 1992). TBP in the pol I system does not utilize its TATA-binding site, and therefore functions differently than in pol II or pol III systems (Beckmann et al., 1995; Radebaugh et al., 1994). Table 1.1 provides a list of the species in which fundamental pol I transcription factors have been identified, and the nomenclature used in each species.

Core factor (CF) is the fundamental pol I transcription factor for *S. cerevisiae*. CF consists of three subunits, Rrn6p, Rrn7p, and Rrn11p (Keys et al., 1994; Lalo et al., 1996; Lin et al., 1996). CF is absolutely required for rRNA transcription and is believed to recruit pol I to the promoter through protein-protein interactions. Recent results are consistent with an interaction between Rrn7p and the A190 subunit of pol I (Radebaugh et al., unpublished). CF is functionally related to the pol I fundamental transcription factors from other species, however, CF does not contain TBP as one of its essential subunits (Keener et al., 1998; Lalo et al., 1996). In fact, in contrast to the TAFs in

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FUNDAMENTAL TRANSCRIPTION FACTORS

Species	Nomenclature
A. castellanii	TIF-IB
Human	SL1, TFID
Mouse	TIF-IB, Factor D
Rat	rSL1
S. cerevisiae	CF
S. pombe	unnamed
X. laevis	Rib1

ACTIVATING/ACCESSORY FACTORS

Species	Nomenclature
A. castellanii	TIF-IE
Human	UBF
Mouse	UBF
Rat	UBF
S. cerevisiae	UAF
S. pombe	unnamed
X. laevis	UBF

ESSENTIAL POL I ASSOCIATED FACTOR TIF-IA/Rrn3p

Species	Nomenclature
A. castellanii	TIF-IA
Human	TIF-IA, hRRN3, Rrn3
Mouse	TIF-IA
S. cerevisiae	Rrn3p
S. pombe	unnamed

Table 1.1. Nomenclature of pol I transcription factors in different species.

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mammalian systems, only one of the yeast TAFs, Rrn6p, exhibits strong binding to TBP, with only weak binding being detected with Rrn7p and Rrn11p (Steffan et al., 1996). The *X. laevis* fundamental transcription factor, Rib1, also has a somewhat dynamic association with TBP (see below) (Bodeker et al., 1996). *In vitro* studies in yeast show CF alone, not CF with TBP, is required for basal levels of pol I transcription at the rRNA promoter (Keener et al., 1998). In *S. cerevisiae*, CF is able to bind the promoter specifically in the core region, but it does not commit the template to transcription (Keys et al., 1994). This loose binding is further emphasized by the fact that CF can cycle on and off the promoter upon promoter clearance and transcriptional initiation, along with pol I, Rrn3p and TBP (Aprikian et al., 2001). 5' deletions of the promoter region have shown that CF only requires sequence to –38 bp upstream of the *tis* to support basal transcription (Keener et al., 1998). The subunits of CF contain sequence similarities to subunits of other fundamental transcription factors. There is limited similarity between Rrn7p and *A. castellanii* TAF_I96 (Radebaugh et al., unpublished data), and both Rrn7p and Rrn11p have homology to *S. pombe* fundamental transcription factor subunits SpRrn7h and SpRrn11h (Boukhgalter et al., 2002). Rrn7p and SpRrn7h also possess sequence homology to the mammalian TAF_I68 subunit.

TIF-IB, the fundamental transcription factor for *A. castellanii,* is required for rRNA promoter driven transcription. *A. castellanii* TIF-IB consists of TBP and four associated TAFs, TAF_I145, TAF_I99, TAF_I95, and TAF_I91 (Radebaugh et al., 1994). DNase I footprinting has shown that TIF-IB binds to the rRNA core promoter from –67 to –17 relative to the *tis* (Bateman et al., 1985). Site-specific photo-cross-linking has been used to map the TAFs in this region (Gong et al., 1995). The binding of TIF-IB to this

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promoter region is brought about by interactions with the minor groove in a structurally conserved and sequence tolerant manner (Geiss et al., 1997; Marilley et al., 2002). Partially purified TIF-IB is able to form a committed complex on the promoter that is stable through multiple rounds of transcription, whereas TIF-IB purified to near homogeneity cannot (Iida et al., 1985; Radebaugh et al., 1998; Radebaugh et al., 1994). Similar to CF, TIF-IB alone can direct pol I initiation at the *tis*, but it requires an additional factor to commit the template for transcription. In the *A. castellanii* system a novel 141 kD factor, TIF-IE, stabilizes TIF-IB in the committed complex (Al-Khouri and Paule, 2002; Radebaugh et al., 1998).

TIF-IB, also referred to as Factor D, is the fundamental transcription factor in mouse (Clos et al., 1986; Tower et al., 1986). TIF-IB consists of TBP and three TAFs, TAF_I95, TAF_I68, and TAF_I48 (Eberhard et al., 1993). Mouse TIF-IB provides both template commitment and promoter selectivity, but its binding can be stimulated or stabilized on the promoter by upstream binding factor (UBF) (Grummt, 1998). TIF-IB binds to the core promoter region with a 3' boundary at –21 on the coding strand and –7 on the noncoding strand (Clos et al., 1986). The rat fundamental transcription factor, selectivity factor 1 (SL1), functions in a similar manner to mouse, whereby SL1 is able to direct rRNA transcription *in vitro* without the addition of UBF (Smith et al., 1993).

SL1, also called TFID, is the human fundamental transcription factor (Kato et al., 1986; Learned et al., 1985). SL1 confers species specificity to human rRNA transcription, but is not capable of binding to the promoter without the addition of UBF (Learned et al., 1986). Human SL1 contains TBP, along with three TAFs, TAF_I110, TAF_I63, and TAF_I48 (Zomerdijk et al., 1994). The SL1 subunits TAF_I110 and TAF_I63 bind directly to

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the promoter in *in vivo* UV cross-linking assays (Beckmann et al., 1995). The third TAF, TAF_I48, is responsible for directly binding UBF (reviewed in Grummt, 1999). Similar to human SL1, the *X. laevis* fundamental factor, Rib1, requires UBF for recruitment and stabilization on the rDNA promoter (Bodeker et al., 1996; McStay et al., 1991). As mentioned above, the TAFs of Rib1 are not always found in a stable association with TBP; UBF is required to provide this stabilization (Bodeker et al., 1996).

1.3.3 Activating/Accessory factors

Transcription of the ribosomal RNA genes by pol I and the fundamental factor is facilitated by an assortment of activating and/or accessory factors. Some of these factors are absolutely required for specific transcription while others have varying requirements based on whether transcription is assayed *in vitro* or *in vivo*. However, each of these factors play important roles in normal cellular growth and the regulation of that growth. Table 1 provides a list of the known activating/accessory factors along with the organism in which they were characterized.

In the yeast, *S. cerevisiae*, the upstream activating factor (UAF) is required for the pol I mediated transcription of the rRNA genes *in vivo. In vitro* UAF provides a stimulatory role to transcription. UAF consists of six separate subunits, Rrn5p, Rrn9p, Rrn10p, Uaf30p and the histones H3 and H4 (Keener et al., 1997; Siddiqi et al., 2001). UAF binds to the upstream promoter element where, along with TBP, it stimulates transcription above the levels seen with CF and pol I alone (Keener et al., 1998). Additionally, UAF provides commitment for the yeast pol I transcriptional machinery (Keys et al., 1996). The results of 5' deletions, as well as linker scanner mutants, show that UAF requires the region between –155 and –51, relative to the *tis,* for binding

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(Kulkens et al., 1991). However, Keener et al., (1998) were able to reconstitute activated transcription with UAF on a template deleted 5' to –76. Therefore, there appears to be some uncertainty about the exact sequences within the UPE that UAF requires for binding. UAF is the only yeast transcription factor that remains stably bound to the promoter following transcriptional initiation and promoter clearance (Aprikian et al., 2001).

UAF also plays a unique role in rRNA transcription in *S. cerevisiae*. Mutations of UAF result in a "polymerase switch," whereby, pol II transcribes the rRNA genes instead of pol I (Oakes et al., 1999). This switch is accompanied by an expansion in the number of ribosomal genes, and an alteration in nucleolar structure. Additionally, a mutation of the UAF subunit, Uaf30p, results in slow cellular growth, however there is a retention of rRNA transcription by pol I (Siddiqi et al., 2001). Therefore, UAF functions not only as an activator of pol I rRNA transcription, but also as a suppressor of pol II rRNA transcription.

As mentioned above, TBP is not required for basal levels of transcription from the yeast promoter. It does however, play a stimulatory role in transcription and has been shown to bind specifically to Rrn9p of UAF and Rrn6p of CF through yeast two-hybrid interactions (Steffan et al., 1996). Additionally, Rrn9p of UAF and Rrn7p of CF have been shown to interact by yeast two-hybrid (Steffan et al., 1996). UAF alone is not capable of recruiting the levels of CF required to obtain activated transcription (Keener et al., 1998), but together with TBP, the pair efficiently recruit CF.

UBF functions in a similar manner to UAF in certain systems although there is no sequence or structural conservation between the two factors. UBF generally contains four

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to five DNA-binding motifs called high mobility group (HMG) boxes, and binds at various positions within the promoter. UBF has been cloned from many species including human, rat, mouse, and *X. laevis* (Bachvarov and Moss, 1991; Hisatake et al., 1991; Jantzen et al., 1990; McStay et al., 1991; O'Mahony and Rothblum, 1991). *In vivo*, UBF exists in two separate forms, an active form, UBF1, and an inactive form, UBF2, with molecular weights of 97 and 95 kD, respectively. The two different forms are due to alternative splicing of the gene encoding UBF (Hisatake et al., 1991). In humans, UBF is required for specific transcription of rRNA. Human UBF binds to both the upstream promoter region, and the core promoter, where it is involved in recruitment of SL1. Together SL1 and UBF form the committed complex (Zomerdijk et al., 1998). Human UBF also remains stably bound to the promoter through multiple rounds of transcription (Panov et al., 2001). In mouse, UBF is not required for transcription but can be greatly stimulatory. In transcription reactions using pure proteins, UBF stimulates transcription 3 to 5 fold, however, when used in crude transcription reactions, UBF can stimulate up to 100 fold (Kuhn et al., 1993). It is believed that in the crude system, UBF stimulates transcription to a greater extent due to its ability to relieve repression from the Ku antigen (Kuhn et al., 1993). UBF contains multiple phosphorylation sites in its acidic C-terminal tail (Voit et al., 1995). Differential phosphorylation of these sites regulate the activities of UBF (reviewed by Grummt (1998)). In *X. laevis* the ability of UBF to stimulate transcription depends on its ability to stabilize the TAF-TBP interactions of Rib1 (Bodeker et al., 1996). UBF from several species has also been shown to wrap or bend DNA upon binding, defining it as an “architectural” protein (Bazett-Jones et al., 1994; Putnam et al., 1994; Stefanovsky et al., 2001; Zomerdijk et al., 1998). Additionally, UBF

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has been implicated in pol I recruitment due to its interaction with PAF53 (Hanada et al., 1996).

Analysis of the *A. castellanii* pol I transcription system has not led to the identification of either a UAF or UBF homologue. However, as discussed above, TIF-IE helps stabilize TIF-IB on the promoter and functions in template commitment (Al-Khouri and Paule, 2002; Radebaugh et al., 1998). TIF-IE does not bind to the promoter itself, and is found to tightly associate with both TIF-IB and pol I through multiple chromatographic separations.

1.3.4 Polymerase I associated factors

TIF-IC is a 65 kDa polypeptide that is required for transcription initiation from the mouse rRNA promoter. TIF-IC associates tightly with pol I in the absence of any other factors, and is necessary for the formation of a Sarkosyl-resistant PIC on the mouse rRNA promoter (Schnapp et al., 1994). Additionally, TIF-IC is required for the formation of the first phosphodiester bond, is necessary for the formation of short abortive transcripts, and stimulates transcriptional elongation by preventing or overcoming stalls (Schnapp et al., 1994). TIF-IC may be a functional homologue of the pol II transcription factor TFIIF, because both suppress non-specific initiation, aid in PIC formation, and stimulate initiation and elongation (Schnapp et al., 1994).

TIF-IA/Rrn3p is required for pol I transcription of the rRNA gene. The TIF-IA/Rrn3p factor has been identified in many systems. (A list of these organisms, along with their nomenclature for the factor, is given in Table 1.1). TIF-IA was first characterized in the mouse system as being the growth regulated factor, and was later identified in a *S. cerevisiae* genetic screen for essential pol I transcriptional components

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(Buttgereit et al., 1985; Schnapp et al., 1993; Yamamoto et al., 1996). Termed Rrn3p in yeast, this factor associates with pol I specifically, in the presence or absence of DNA, and is not believed to specifically bind to DNA (Milkereit and Tschochner, 1998; Schnapp et al., 1993; Yamamoto et al., 1996). Similar properties for TIF-IA have been described in mammalian systems (Schnapp et al., 1993). TIF-IA/Rrn3p is required to be associated with pol I for specific transcriptional initiation to occur at the rRNA promoter (Milkereit and Tschochner, 1998; Miller et al., 2001; Peyroche et al., 2000; Yamamoto et al., 1996). Less than 2% of the pol I found in a yeast cell is present in this initiation competent pol I-Rrn3p complex, with the rest of the pol I being needed for events such as elongation or possibly reinitiation (Milkereit and Tschochner, 1998). TIF-IA/Rrn3p has been cloned from both yeast and human (Bodem et al., 2000; Moorefield et al., 2000; Yamamoto et al., 1996). TIF-IA related genes have also been identified in *S. pombe*, *C. elegans, and A. thaliana.*

It has been suggested that TIF-IA/Rrn3p plays a role in the recruitment of pol I to the promoter during transcriptional initiation. TIF-IA/Rrn3p directly binds to both pol I and the fundamental transcription factor. In yeast, Rrn3p directly associates with the A43 subunit of pol I, and to both Rrn6p and Rrn7p of CF (Peyroche et al., 2000; C. Radebaugh et al., unpublished). Furthermore, mammalian TIF-IA binds to A43, and PAF53, as well as TAF_I110 and TAF_I63 of SL1 and TAF_I68 of TIF-IB (Cavanaugh et al., 2002; Miller et al., 2001; Yuan et al., 2002). The importance of these interactions, in terms of rRNA growth regulation, will be discussed below.

The Sollner-Webb group has identified a transcription factor in mouse called C*, which resembles both TIF-IA/Rrn3p and TIF-IC. As is the case with yeast and the pol I-

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TIF-IA/Rrn3p complex, C* has been found to tightly associate with pol I, combining to form a complex that is stable through several types of chromatography (Brun et al., 1994). C* must be in a complex with pol I for specific transcription to occur, and it has been shown to be consumed by transcription after an RNA product of approximately 37-40 nucleotides has been produced (Brun et al., 1994). This indicates that the pol I-C* complex remains in an "initiation competent" form until a certain number of nucleotides are incorporated into the growing RNA chain, and then is not needed for elongation, as is the case with TIF-IA.

1.4 PREINITIATION COMPLEX FORMATION AND TRANSCRIPTIONAL INITIATION

In *S. cerevisiae*, UAF is responsible for binding the promoter and providing template commitment to the PIC (Riggs et al., 1995). Together with TBP, UAF presumably recruits CF to the core promoter region by protein-protein interactions. Interactions between subunits of UAF and CF have been shown, in addition to interactions between TBP and each of the factors (Steffan et al., 1996; Radebaugh et al., unpublished). *In vitro*, CF is capable of binding to the promoter by itself, recruiting transcriptionally competent pol I and directing transcription, although this complex only produces basal levels of transcription (Keener et al., 1998). Pol I is required to be associated with Rrn3p in order to obtain transcriptional initiation (Milkereit and Tschochner, 1998). However, yeast pol I is capable of binding to the promoter without Rrn3p, although these complexes are transcriptionally inactive and cannot be rescued by the addition of Rrn3p (Aprikian et al., 2001). Pol I bound to a PIC containing UAF, TBP,

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and CF can, however, be replaced by a transcriptionally competent pol I-Rrn3p complex (Aprikian et al., 2001). Additionally, Aprikian et al., (2001) suggest a model in which CF is only recruited to the promoter in the presence of pol I. Furthermore, upon transcriptional initiation, pol I, Rrn3p, TBP, and CF are released from the PIC, leaving UAF bound and poised for multiple rounds of transcription. In transcription reactions containing partially purified fractions, Rrn3p dissociates from pol I upon transcription and is not able to bind a subsequent pol I molecule (Milkereit and Tschochner, 1998). This suggests that there is a modifying activity present in the partially purified fractions that disrupts the pol I-Rrn3p complex at a step following initiation

In *A. castellanii,* TIF-IB is capable of forming a committed complex on the rRNA promoter, that remains stably bound through multiple rounds of transcription (see above). Template commitment is provided by TIF-IE (Al-Khouri and Paule, 2002; Radebaugh et al., 1998). DNase I footprinting assays have shown that TIF-IB binds to the promoter between –67 and –17 relative to the *tis* (Bateman et al., 1985). Pol I extends the protection of the promoter downstream to approximately +19 (Bateman et al., 1985). The addition of nucleotide triphosphates results in the clearance of pol I from the promoter while TIF-IB remains (Bateman and Paule, 1988; Bateman and Paule, 1986; Kahl et al., 2000). This suggests that all of the pol I bound to the *A. castellanii* promoter is competent for transcription. In other words, all of the pol I bound is associated with TIF-IA.

In mouse, the *in vitro* assembly of the PIC proceeds in a stepwise manner. First, TIF-IB binds to the core promoter and is stably bound through multiple rounds of transcription (Schnapp and Grummt, 1991). UBF does not bind to the promoter alone but does stabilize the binding of TIF-IB (Schnapp and Grummt, 1991). The binding of UBF

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is followed by the recruitment of pol I and TIF-IC (Schnapp and Grummt, 1991). Finally, TIF-IA is bound, forming a PIC that is competent for transcriptional initiation (Schnapp and Grummt, 1991). When nucleotide triphosphates are added to PICs formed with purified transcription factors at the mouse rRNA promoter, transcription occurs. This transcription results in the release of TIF-IA in a form that is capable of associating with a subsequent PIC and conferring transcriptional activity to the PIC (Schnapp et al., 1993). However, it is not clear if TIF-IA is released on its own and associates with pol I containing PIC, or if in the pure system, the pol I-TIF-IA complex is being released and associating with a new PIC.

In human pol I transcription, UBF is required for SL1 binding to the promoter, and together they form a committed complex (Zomerdijk et al.,1998). Human Rrn3 directly interacts with SL1, this interaction is essential for pol I recruitment (Miller et al., 2001). Upon transcription, both UBF and SL1 stay bound to the promoter poised for multiple rounds of transcription (Panov et al., 2001). Additionally, in crude S100 extracts, Rrn3 is inactivated during transcription, resulting in a dissociation from pol I (Hirschler-Laszkiewicz et al., 2003). This inactivated Rrn3 is not capable of binding to a new pol I molecule or reactivating a subsequent transcription.

1.5 MECHANISMS REGULATING POL I TRANSCRIPTION

Ribosomal RNA transcription is regulated on many levels according to specific environmental conditions, as well as during the cell cycle. Two general levels of regulation have emerged. One in which transcription is regulated by pol I or a closely associated factor, and one in which the formation of the active committed complex is

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regulated. The former mechanism provides for the rapid increase or decrease of transcriptional activity as cells are deprived of essential nutrients or are entering a stationary growth phase. The latter mechanism provides for the regulation of transcription during events such as the cell cycle or the response to tumor formation.

As mentioned previously, rRNA genes are present in multiple copies within the cell, although cells in log phase growth generally only use half of their rRNA genes for synthesis (Conconi et al., 1989; Dammann et al., 1993; Lucchini and Sogo, 1992). Additionally, some reports have shown that both mammals and *S. cerevisiae* can regulate the number of active gene copies as cells encounter differential growth phases (Dammann et al., 1993; Haaf et al., 1991). Therefore, it had been proposed that rRNA transcription could be regulated by the number of active gene copies. However, a recent report has revealed that the initiation rate of rRNA transcription is the mechanism responsible for regulation, not the percentage of active gene copies. Two strains of yeast with different numbers of rRNA gene copies, 143 or 42, grew equally well in exponential growth phase (French et al., 2003). Counts of transcribing polymerases in Miller spreads showed the cells with fewer copies had approximately the same total numbers of transcribing polymerases. This demonstrated that transcription was not regulated by the number of active gene copies, but rather by the transcription initiation rate on the active genes.

1.5.1 Growth-dependent transcription regulation

The rate of rRNA transcription closely parallels the rate of cell growth. As cells encounter non-optimal growth conditions, the rRNA transcription rate decreases dramatically, possibly to conserve energy needed for other cellular events (Clarke et al., 1996; Riggs et al., 1995). The target for this rapid reduction in transcription is believed to

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be the assembly of the transcriptionally competent pol I-TIF-IA/Rrn3p complex. Direct physical interactions between TIF-IA/Rrn3p and specific subunits of pol I are affected by phosphorylation/dephosphorylation (Cavanaugh et al., 2002; Imazawa et al., 2002; Peyroche et al., 2000; Yuan et al., 2002). This suggests that formation of the pol I-TIF-IA/Rrn3p complex is a target of regulation through reversible phosphorylation. Depending on the particular system, the target for this regulatory mechanism has been shown to be either pol I or TIF-IA/Rrn3p (Bateman and Paule, 1986; Cavanaugh et al., 2002; Tower and Sollner-Webb, 1987; Yuan et al., 2002).

In *S. cerevisiae*, Rrn3p must be associated with pol I for specific transcriptional initiation to occur at the rRNA promoter (Milkereit and Tschochner, 1998; Peyroche et al., 2000; Yamamoto et al., 1996). This association is mediated through the direct interaction of Rrn3p and the A43 pol I subunit (Peyroche et al., 2000). Milkereit et al., (1998) have demonstrated that the cellular concentrations of Rrn3p and pol I are similar in both actively growing and stationary phase cells. However, the specific transcription initiation competent pol I-Rrn3p complex is not present in stationary phase. The phosphorylation state of the pol I A43 subunit is believed to be important for the critical pol I-Rrn3p interaction. Fath et al., (2001) examined the phosphorylation states of the pol I A43 and A190 subunits from free pol I and pol I-Rrn3p complex. They report the phosphorylation ratio A43/A190 is much higher in the pol I-Rrn3p complex than in free pol I (Fath et al., 2001). As a result, the authors suggest, that specific phosphorylations, such as that of A43, are required for the association of pol I with Rrn3p. Furthermore, phosphatase treated pol I does not associate with Rrn3p, while mock-treated pol I does. Interestingly, their work in the yeast system also demonstrated that Rrn3p is capable of

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forming an initiation competent complex with pol I, independent of its specific phosphorylation state. This finding strengthens the argument that, in yeast, the modification of a specific pol I subunit(s) is the target for growth-dependent rRNA transcriptional regulation.

Similar to the yeast system, mammalian TIF-IA/Rrn3 interacts directly with the A43 subunit of pol I (Cavanaugh et al., 2002; Miller et al., 2001; Yuan et al., 2002). Additionally, the levels of pol I and TIF-IA/Rrn3 are similar in actively growing and stationary phase cells (Cavanaugh et al., 2002; Yuan et al., 2002). However, in contrast to the yeast system, it appears phosphorylation of TIF-IA is necessary for the formation of the pol I-TIF-IA/Rrn3 complex. TIF-IA/Rrn3 produced in Sf9 cells, and therefore believed to be correctly phosphorylated, physically interacts with A43 (Cavanaugh et al., 2002). Bacterially expressed TIF-IA is not capable of this interaction (Cavanaugh et al., 2002). Additionally, phosphatase treatment of FLAG-TIF-IA inhibits both transcriptional activity and the ability to associate with A43 (Cavanaugh et al., 2002). Furthermore, cycloheximide treatment, suppresses rRNA transcription, inhibits the phosphorylation of murine TIF-IA, and is associated with the loss of pol I-TIF-IA interaction (Cavanaugh et al., 2002). Interestingly, both Sf9 and bacterially expressed TIF-IA preparations are able to bind to the TAF_I68 subunit of SL1, indicating a specific phosphorylation event is not necessary for interaction with the fundamental transcription factor.

In the murine pol I transcription system, five purified components are required to reconstitute specific transcription from the rRNA promoter: pol I, TIF-IB, UBF, TIF-IC, and TIF-IA (Schnapp and Grummt, 1991; Schnapp et al., 1993). In inactive extracts prepared from cells in stationary phase growth, the addition of TIF-IA alone restores

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rRNA transcriptional activity (Schnapp et al., 1993). Transcription from the stationary phase extract was not restored by the addition of pol I, UBF, TIF-IB, or TIF-IC (Schnapp et al., 1993). This data is consistent with the notion that TIF-IA in mammalian cells is the target of regulation as cells undergo the transformation from actively dividing to stationary phase growth.

TIF-IA is a phosphoprotein that contains multiple phosphorylation sites, and its phosphorylation pattern is affected by growth conditions (Zhao et al., 2003). Recent results from the Grummt laboratory have indicated that two specific serine residues of mammalian TIF-IA (S633 and S649) require phosphorylation for transcriptional activity (Zhao et al., 2003). The S633 and S649 residues are phosphorylated by ERK and RSK kinases, providing activation of transcription upon growth factor stimulation. Substitutions of these serines with aspartic acid residues resulted in the ability of the mutant added exogenously to rescue extracts from density-arrested cells, whereas, substitution with alanines did not lead to transcriptional rescue. These results indicate a requirement for a net negative charge, provided by an amino acid or a phosphate group, at these important positions for both transcriptional activity and pol I binding.

Unfortunately, the contradictory evidence presented from the different organisms makes it unclear which phosphorylation is required for the formation of pol I-TIF-IA/Rrn3p complex. It seems that in lower eukaryotes, such as *S. cerevisiae*, a specific phosphorylation of a pol I subunit (A43) is required for the pol I-TIF-IA complex to form. In contrast, specific phosphorylation of TIF-IA itself is required in mammals. However, the data is consistent in that a regulatory phosphorylation/dephosphorylation is required for the formation of the active pol I-TIF-IA/Rrn3p complex. As cells undergo

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the switch from actively dividing to stationary phase growth, a dephosphorylation event disrupts the complex resulting in down regulation of rRNA transcription.

1.5.2 Cell cycle regulation of rRNA transcription

In mammalian cells, the rate of rRNA transcription is influenced by the cell cycle. Ribosomal RNA transcription rates are highest in S and G2, shut down in mitosis and recover during G1. Data has shown that the activity of TIF-IA is not repressed during mitosis (Schnapp et al., 1993). The down regulation of transcription during the cell cycle is, however, modulated by TIF-IB/SL1 and UBF, the factors required for PIC formation. Two subunits of human SL1, TBP and TAF_I110, are phosphorylated by cdk1/cyclinB as cells enter mitosis (Heix et al., 1998; Kuhn et al., 1998). The phosphorylation of TAF_I110 is responsible for the cdk1/cyclin B specific down regulation of rRNA transcription through the loss of interaction with UBF, thus disrupting the preinitiation complex. As mentioned above, human SL1 exhibits no sequence specific binding at the rRNA promoter (Learned et al., 1986). Therefore, loss of interaction with UBF results in the lack of stable SL1 binding. The activity of murine TIF-IB was shown to quickly recover at the exit of mitosis, suggesting the slow recovery of rRNA transcription during G1 was not due to SL1/TIF-IB activity (Klein and Grummt, 1999).

UBF is also inactivated during mitosis. This inactivation is due to a phosphorylation event that is reversed by an okadaic acid-sensitive phosphatase (Klein and Grummt, 1999). Additionally, as mammalian cells progress through the G1 phase, UBF is activated by the specific phosphorylations of serine 484 by cdk4/cyclin D1, and serine 388 by cdk2/cyclin A and cdk2/cyclin E (Voit and Grummt, 2001; Voit et al., 1999). The

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phosphorylation of serine 388 enables UBF to associate directly with pol I, presumably aiding in the recruitment of pol I to the promoter (Voit and Grummt, 2001).

1.5.3 Tumor suppressor proteins pRb and p53

Tumor formation corresponds with rapid and unregulated cellular growth. Under these conditions, the need for ribosome production is great. Therefore, it is logical to suggest that tumor suppressors could be involved in the regulation of rRNA transcription and/or 5S RNA transcription. Consistent with this theory, the tumor suppressor proteins, pRb and p53, have been shown to localize to the nucleolus (Bukovsky et al., 1995; Rubbi and Milner, 2000). Following this observation, direct evidence has been presented indicating both proteins play a role in rRNA regulation.

The retinoblastoma susceptibility gene encodes the 110 kD nuclear phosphoprotein (Rb). pRb has been shown to function in transcriptional repression for all three RNA polymerases. pRb represses transcription from pol III by direct interaction with pol III transcription factors (Chu et al., 1997; Sutcliffe et al., 2000). Similarly, pRb interacts with UBF in a manner that specifically represses rRNA transcription (Cavanaugh et al., 1995). The C-terminal part of pRb binds to HMG boxes 1 and 2 of UBF, disrupting promoter binding, but not the binding of UBF to either TIF-IB or pol I (Voit et al., 1997). Additionally, as the density of cultured cells increases and the cell cycle is arrested, hypophosphorylated pRb accumulates in the nucleoli (Hannan et al., 2000). This hypophosphorylated form of pRb associates with UBF in a manner corresponding to a decrease in rRNA synthesis. Overexpression of pRb was shown to cause a reduction in UBF-dependent activation of rRNA transcription (Hannan et al., 2000). The acetyltransferase, CBP, shown to activate rRNA transcription through the acetylation of

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UBF, competes with pRb for UBF binding (Hirschler-Laszkiewicz et al., 2001; Pelletier et al., 2000). Furthermore, p130, the pRb related pocket protein, was shown to interact with UBF and repress pol I transcription (Ciarmatori et al., 2001). The competition for binding to UBF demonstrates a mechanism by which the activity of UBF regulates transcriptional initiation in the context of tumor suppression.

Many tumors are characterized by mutation of the tumor suppressor p53. Mice that are homozygous null for p53 have an increased susceptibility for tumor formation (Donehower et al., 1992). Similar to pRb, p53 also has been shown to affect transcription of 5S RNA by pol III (Cairns and White, 1998), and function in down regulating rRNA transcription. Overexpression of p53 inhibits transcription from a murine reporter plasmid containing a rRNA promoter (Budde and Grummt, 1999). Additionally, p53 represses transcription in the human system *in vivo* and *in vitro*, presumably by direct binding to two subunits of SL1 (TBP and TAF_I110) (Zhai and Comai, 2000). This binding disrupts the interaction of UBF and SL1, inhibiting PIC formation and reducing the levels of rRNA transcription. Together, the tumor suppressor proteins, pRb and p53, are able to regulate rRNA transcription to prevent the uncontrolled growth phenotype characteristic of tumors.

1.5.4 Additional factors that function in rRNA regulation

Many factors and modifying enzymes are reported to play roles in rRNA transcriptional regulation. Some of these factors are responsible for coordinating events during the cell cycle, while others are reported to be part of a controversial pol I "holoenzyme" complex. In each case, the following factors/enzymes have been shown to affect pol I transcription in some manner.

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TFIIH is found associated with pol I in a holoenzyme complex. This holoenzyme contains many activities in addition to pol I and its general transcription factors (for review see (Grummt, 2003)). In mouse, TFIIH is required *in vivo* and *in vitro* for rRNA transcription (Iben et al., 2002). Both TIF-IB and pol I associate with TFIIH through multiple rounds of purification, and exogenously added TFIIH is able to reactivate transcription reactions lacking TFIIH (Iben et al., 2002). The requirement for TFIIH in rRNA transcription is at a step subsequent to transcription initiation. Transcriptional activation does not rely on the ATPase, helicase, nor protein kinase activity of TFIIH (Iben et al., 2002).

CSB is a protein found mutated in patients with Cockayne's syndrome. CSB is part of a multifunctional enzyme complex, containing pol I, TFIIH, and TIF-IB, that is competent for rRNA transcription (Bradsher et al., 2002). Extracts deficient in CSB exhibit reduced amounts of rRNA transcription, and the addition of exogenous recombinant CSB stimulates transcription by restoring the association between pol I and TFIIH (Bradsher et al., 2002). The ability of CSB and TFIIH to regulate rRNA synthesis links DNA repair mechanisms to rRNA transcription.

As mentioned above, TTF-1 functions in termination of rRNA transcription. TTF-1 is also responsible for the recruitment of the p300/CBP-associated factor, PCAF, to the promoter (Muth et al., 2001). PCAF subsequently acetylates the TAF_I68 subunit of TIF-IB, enhancing its binding to the promoter, and stimulating rRNA transcription (Muth et al., 2001). Reversal of the PCAF mediated acetylation of TIF-IB is accomplished by the deacetylase Sir2a, resulting in repression of pol I transcription (Muth et al., 2001). These

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reactions provide a mechanism in which rRNA transcription is regulated by reversible acetylation of pol I transcription factors.

DNA-dependent protein kinase (DNA-PK) consists of a 350 kD catalytic subunit (p350) and the human autoantigen Ku (Gottlieb and Jackson, 1993). As discussed above, UBF activates rRNA transcription by repressing the promoter binding effects of Ku (Kuhn et al., 1993). DNA-PK represses pol I transcription in an ATP hydrolysis-dependent manner (Kuhn et al., 1995; Labhart, 1995), suggesting a phosphorylation event is responsible for transcriptional down regulation. This repression is overcome by competition between the fundamental transcription factor, SL1/Rib1, and Ku for rRNA promoter binding (Labhart, 1995; Michaelidis and Grummt, 2002).

Hmo1 is a HMG-box protein found in *S. cerevisiae*, that may function in a role similar to the vertebrate factor UBF. Overexpression of Hmo1 suppresses mutations of the pol I subunit Rpa49, stimulating rRNA transcription (Gadal et al., 2002). Rpa49 is the yeast homologue to mammalian PAF53, a pol I associated factor known to associate with UBF (Hanada et al., 1996). The similarities in function and interactions between Hmo1 and UBF, with their respective pol I machinery, suggests that Hmo1 may also play an architectural role in organizing the yeast promoter for PIC formation and transcriptional initiation.

TAF1, formerly $TAF_{II}250$, is a subunit of the pol II transcription factor TFIID. TAF1 may also function as an activator for the transcription of rRNA genes. TAF1 interacts with UBF in yeast two-hybrid and coimmunoprecipitation assays, and localizes with UBF in the nucleolus (Lin et al., 2002). TAF1 stimulates rRNA transcription in cell-free transcription and cotransfection assays (Lin et al., 2002). This finding has prompted the

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authors to suggest TAF1 plays an important role in the regulation of genes required for cell growth, from both pol I and pol II transcription systems.

Casein kinase II (CKII) phosphorylates the C-terminal tail of UBF (Voit et al., 1995). Voit et al., (1995) report that the cellular localization of UBF is not altered between growing and quiescent cells, although serum starvation changes UBF's phosphopeptide pattern. CKII phosphorylation of UBF aids in the activation of transcription, but the CKII mediated phosphorylation of the C-terminal tail of UBF is not fully responsible for transcriptional activation mediated by UBF (Voit et al., 1995).

UBF is also regulated by ERK1/2 kinase (Stefanovsky et al., 2001). UBF is phosphorylated, both *in vivo* and *in vitro*, by ERK1/2 at amino acids residues 117 and 201 within HMG boxes 1 and 2 (Stefanovsky et al., 2001). These phosphorylations diminish the interactions of UBF with the promoter, resulting in repressed rRNA transcription (Stefanovsky et al., 2001). The direct phosphorylation of UBF by the MAP kinase (ERK) pathway provides a link between growth factor signaling and rRNA transcriptional regulation.

1.6 OBJECTIVES OF THE DISSERTATION

The original objective of the dissertation was to study the biochemical properties of the yeast pol I transcription factors. We planned to develop assays that would have enabled us to map the locations of the individual subunits of the yeast rRNA promoter. We then planned to use these assays to study the role of Rrn3p in pol I recruitment. Additionally, we hoped to gain insight into the mechanisms that regulate rRNA transcription in yeast. The purification of the yeast transcription factors, institution of a

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reconstituted transcription assay, and initial characterization of factor DNA binding properties are summarized in Chapter 2. The availability of factors from the yeast system allowed us to assay for TIF-IA activity in the *A. castellanii* system. In Chapter 3, I report the identification of an *A. castellanii* TIF-IA homologue and characterize its activity in transcription initiation. Chapter 4 contains a manuscript examining protein-protein interaction in the yeast pol I transcription system. Finally, Chapter 5 contains additional observations and future directions.

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CHAPTER 2

PURIFICATION AND CHARACTERIZATION OF *SACCHAROMYCES CEREVISIAE* POL I TRANSCRIPTION FACTORS

This chapter describes the purification of the transcription factors required for the reconstitution of *in vitro* transcription in yeast. Additionally, EMSA, footprinting, and photo-cross-linking studies were initiated in order to determine the activities of the yeast pol I transcription factors. I purified most of the factors used in these experiments. Control proteins were provided by the Nomura laboratory (UC Irvine), Tara Towers purified TBP, and Dr. Cathy Radebaugh was instrumental in the initial development of the transcription assays.

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PURIFICATION AND CHARACTERIZATION OF *SACCHAROMYCES CEREVISIAE* POL I TRANSCRIPTION FACTORS

2.1 ABSTRACT

Transcription of rRNA can account for up to 60% of the total cellular RNA that is being transcribed in an actively dividing cell. Due to the large amounts of energy this transcription requires, it is of utmost importance for the cell to be able to regulate rRNA transcription. Pol I from *S. cerevisiae* utilizes a variety of transcription factors to support the levels of rRNA required by the cell. These transcription factors include, upstream activation factor (UAF), TATA-binding protein (TBP), core factor (CF), and Rrn3p. In order to study the mechanisms that regulate rRNA, a fundamental understanding of how the pol I transcription factors interact with each other and with the promoter is critical. The goal of this work was to characterize these interactions in the yeast system, with the hope of mapping the factors to the promoter and identifying the interactions necessary for transcriptional initiation. We instituted a reconstituted *in vitro* transcription assay with the help of the Nomura laboratory. We then analyzed the DNA-binding activities of the pol I transcription factors using EMSAs, footprinting, and photo-cross-linking assays. UAF was found bind to the rRNA promoter in an EMSA, although no footprint was observed. We have also obtained preliminary photo-cross-linking data for CF, UAF, and TBP.

2.2 INTRODUCTION

DNA-dependent RNA Polymerase I (pol I) is one of three nuclear RNA polymerases found in eukaryotic cells. The rRNA genes in the yeast, *Saccharomyces*

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cerevisiae, are found in a repeated head to tail array of approximately 150 copies on chromosome XII. Within the spacer regions between each 35S rRNA transcription unit lies the gene encoding the 5S RNA. This gene is transcribed by pol III, and is oriented in the opposite direction of the 35S rRNA gene. Following transcription, the 35S precursor rRNA is processed into the 28S, 18S, and 5.8S rRNAs. In an actively dividing yeast cell, transcription from pol I can account for up to 60% of the total cellular RNA being produced (Warner, 1999). As the cell leaves optimal growth conditions the rRNA transcription rate dramatically decreases, possibly to conserve energy needed for other cellular events (Clarke et al., 1996; Riggs et al., 1995). Regulation of this process is not completely understood. To uncover the precise mechanism by which the cell regulates rRNA transcription, we must first elucidate the steps involved in preinitiation complex formation and the subsequent clearance of the promoter during transcriptional initiation.

The ability of the pol I transcriptional machinery to specifically transcribe the repeating ribosomal gene units is facilitated through DNA-protein interactions with the rRNA promoter. The yeast promoter contains two functional domains, the core promoter, closest to the transcription initiation site (*tis*), and the more distal upstream promoter element (UPE) (Paule, 1998b). Pol I is recruited to the promoter via protein-protein interactions with specific *trans*-acting protein factors. In the yeast, *S. cerevisiae*, these transcription factors are upstream activation factor (UAF), core factor (CF), TATA-binding protein (TBP), and Rrn3p (Keys et al., 1996; Lalo et al., 1996; Steffan et al., 1996; Yamamoto et al., 1996).

The yeast pol I transcription factors contain subunits encoded by the *RRN* genes, isolated and characterized by the Nomura laboratory (Nomura, 1998). Using a high copy

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plasmid containing the 35S rRNA coding region downstream of a strong pol II promoter (*GAL7*), they were able to isolate mutants in the genes required for rRNA transcription by pol I (Nogi et al., 1991). When grown on galactose, cells containing this plasmid, pNOY103, can produce functional rRNA utilizing the pol II transcriptional machinery. When a cell carrying a mutation in a gene essential for rRNA transcription is switched to glucose, repressing the *GAL7* promoter, growth is prohibited, or significantly reduced. From this screen, twelve *RRN* genes have been characterized; seven encode subunits of pol I transcription factors and five encode unique pol I subunits (Nomura, 1998).

CF is the fundamental pol I transcription factor for *S. cerevisiae*. It is absolutely required for transcription and is believed to recruit pol I to the promoter through protein-protein interactions. CF consists of three subunits, Rrn6p, Rrn7p, and Rrn11p, encoded by the *RRN6*, *RRN7*, and *RRN11* genes, respectively (Keys et al., 1994; Lalo et al., 1996; Lin et al., 1996). CF is functionally related to the pol I fundamental transcription factors from other species: TIF-IB (mouse and *Acanthamoeba castellanii*), SL1 (human and rat), factor D or TFID (human, mouse, and rat), and Rib1 (*X. laevis*) (Paule, 1998b; Bell et al., 1988; Bodeker et al., 1996; Kato et al., 1986; Schnapp et al., 1990; Tower et al., 1986). However, CF differs from these other factors in one important feature, CF does not contain TBP as one of it's essential subunits (Keener et al., 1998; Lalo et al., 1996). The fundamental pol I transcription factors from the other species mentioned consist of TBP along with TBP-associated factors (TAFs), although the TAFs of Rib1 have been shown to have a somewhat dynamic association with TBP (Bodeker et al., 1996). TBP has also been shown to be an essential component of the fundamental transcription factors from the other RNA polymerase systems, such as TFIID and TFIIIB (Kassavetis et al., 1992;

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Pugh and Tjian, 1992). *In vitro* studies in yeast have shown that CF alone, not CF with TBP, is required for basal levels of *in vitro* transcription by pol I at the rRNA promoter (Keener et al., 1998). In *S. cerevisiae*, CF is able to bind the promoter specifically in the core region, but does not commit the template to transcription (Keys et al., 1994). This loose binding is further emphasized by the fact that CF can cycle on and off the promoter upon promoter clearance and transcriptional initiation, along with pol I, Rrn3p, and TBP (Aprikian et al., 2001). 5' deletions of the promoter region have shown that CF only requires sequence to –38 bp upstream of the *tis* to support basal transcription (Keener et al., 1998).

UAF consists of six separate subunits, Rrn5p, Rrn9p, Rrn10p, Uaf30p and the histones H3 and H4 (Keener et al., 1997; Siddiqi et al., 2001). UAF binds to the upstream promoter element where, along with TBP, it stimulates transcription above that seen in basal transcription (Keener et al., 1998). Additionally, UAF is the commitment factor for rRNA transcription (Keys et al., 1996). The results of 5' deletions, as well as linker scanner mutants, show that UAF requires the region between –155 and –51, relative to the *tis,* for binding (Kulkens et al., 1991). However, Keener et al., (1998) were able to reconstitute activated transcription with UAF on a template deleted 5' to –76. Therefore, there appear to be some uncertainties about the exact sequences within the UPE that UAF requires for binding. It was recently shown that UAF is the only yeast transcription factor that remains stably bound to the promoter following promoter clearance (Aprikian et al., 2001). Pol I, Rrn3p, TBP, and CF all cycle on and off the promoter upon transcriptional initiation.

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The mammalian transcription factor, UBF, functions in a similar manner to UAF in certain systems. UBF also remains stably bound to the promoter through multiple rounds of transcription (Panov et al., 2001). UBF generally contains four to five DNA-binding motifs called HMG boxes, and has been shown to bind at the UPE, the core promoter element, and to enhancer elements. In humans, UBF is required for specific transcription of rRNA. UBF is able to bind to both the upstream region, as well as the core promoter, where it is involved in recruitment of the human selectivity factor, SL1. Together SL1 and UBF form the committed complex (Zomerdijk et al., 1998). In mouse, UBF is not required for transcription but can be greatly stimulatory. In transcription reactions using pure proteins, UBF stimulates transcription 3 to 5 fold, however when used in crude transcription reactions, UBF can stimulate up to 100 fold (Kuhn et al., 1993).

UBF has been defined as an "architectural" protein, in that UBF is able to wrap or bend DNA upon promoter binding (Bazett-Jones et al., 1994; Putnam et al., 1994; Zomerdijk et al., 1998; Stefanovsky et al., 2001). It is not known if UAF employs similar types of interactions to bind to the yeast rDNA promoter. No sequence similarity exists between the subunits of UAF and UBF, however, it is not clear if the two factors exhibit functional homology.

TBP is not required for basal levels of transcription from the yeast promoter. It does however, play a stimulatory role in transcription and has been shown by yeast two-hybrid interactions to bind specifically with Rrn9p of UAF and Rrn6p of CF (Steffan et al., 1996). Additionally, Rrn9p of UAF and Rrn7p of CF interact in a yeast two-hybrid assay (Steffan et al., 1996). UAF alone is not capable of recruiting the levels of CF

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required to obtain activated transcription (Keener et al., 1998), but together with TBP, the pair have the ability to efficiently recruit CF.

Rrn3p is an essential pol I transcription factor, whose role in transcription is not completely understood. Rrn3p associates with pol I specifically, in the presence or absence of DNA, and is not believed to specifically bind to DNA (Yamamoto et al., 1996). Rrn3p needs to be associated with pol I for specific initiation to occur at the rRNA promoter (Milkereit and Tschochner, 1998). Less than 2% of the pol I found in the yeast cell is present in this initiation competent pol I-Rrn3p complex, with the rest of the pol I possibly being used in events such as elongation (Milkereit and Tschochner, 1998). Depending on conditions, *S. cerevisiae* can be isolated in an actively growing state or a non-growing (stationary) phase. Milkereit et al., (1998) have demonstrated that the cellular concentrations of both Rrn3p and pol I from actively growing and stationary phase cells are similar, but the specific transcription initiation competent pol I-Rrn3p complex is not present in the stationary phase cells. It has been suggested that the formation of this complex may be the target for growth-dependent regulation of rRNA synthesis. Direct physical interactions between Rrn3p and specific subunits of pol I have been shown to be regulated by phosphorylation/dephosphorylation (Cavanaugh et al., 2002; Imazawa et al., 2002; Peyroche et al., 2000; Yuan et al., 2002). Additionally, it has been shown that Rrn3p interacts with subunits of the fundamental transcription factors such as CF (Miller et al., 2001; Peyroche et al., 2000; Yuan et al., 2002). These observations suggest that Rrn3p may provide a role in bridging pol I and the fundamental transcription factor, aiding in the recruitment of pol I to the promoter. However, the

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precise mechanism by which Rrn3p functions in rRNA transcription is not completely understood.

The goal of this work was to purify the factors required for *in vitro* transcription in the yeast system. Once purified, factor activity was assayed in reconstituted *in vitro* transcription assays. DNA binding, protein-protein interactions, and enzymatic activities of the factors were assayed using gel shift, DNA protection, transcription, and UV cross-linking assays. We hoped to develop these assays in order to determine the physical location of the factors along the promoter and exactly how, in concert with the rRNA promoter, these factors interacted. We hoped to elucidate the roles each factor plays in transcriptional initiation and gain insight into how regulatory mechanisms target the transcription of rRNA in a growth-dependent manner.

2.3 MATERIALS AND METHODS

2.3.1 Purification of pol I transcription factors from *Saccharomyces cerevisiae*

Pol I, CF, Rrn3p, and TBP were purified as previously described (Keener et al., 1998), with the following exceptions. Polymerase I was purified according to protocol using a protease deficient cell line, BJ926 (Table 2.1), with the exception of a 1 ml heparin-Sepharose CL-6B column (Amersham Pharmacia Biotech) that was substituted for the 1 ml HiTrap column, Figure 2.1. Pol I activity was measured after each column in a non-specific transcription assay (see below). CF was purified using the NOY797 (Table 2.1) cell line (a kind gift of the M. Nomura laboratory), which contains a triple HA1 tag at the N-terminus of the CF subunit Rrn7p, as well as a hexahistidine tag at the C-terminus. The purification was carried out as described according to protocol with the

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Strain	Description
BJ926	*Matα/Matα, prb1-1122/prb1-1122, prc1-126/prc1-126, pep4-3/pep4-3 can1/can1, gal2/gal2, his1/+, +/trp1*
NOY797	*Matα, ade2-1, ura3-1, lue2-3, 112, trp1-1, his3-11, can1-100 rrn7::LEU2*, pNOY403 [*TRP1*, *RRN7*-$(HA1)_3$-$(His)_6$]
NOY798	*Matα*, *ade2*, *ade3*, *lue2*, *ura3*, *trp1*, *his*, *can1*, *rrn5*::*TRP1*, pNOY402 [*LEU2*, *RRN5*-$(HA1)_3$-$(His)_6$]

Table 2.1. Yeast strains used in this study.

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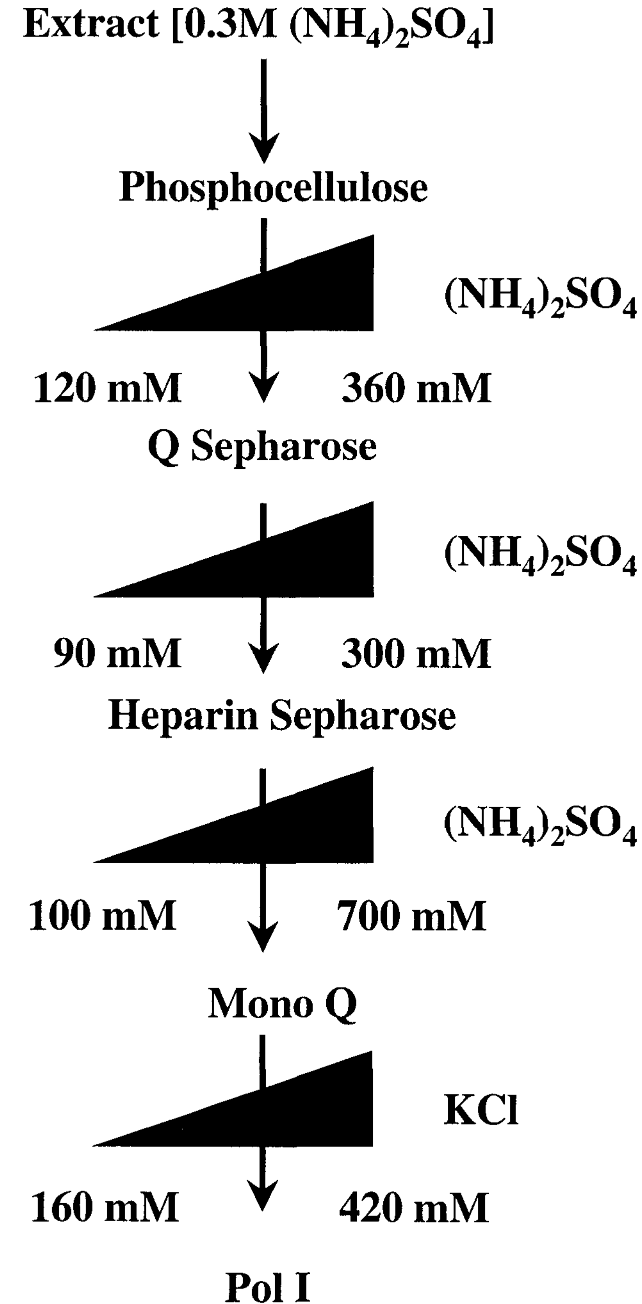

Figure 2.1. Flow diagram for the purification of *S. cerevisiae* pol I. Solid triangles represent linear salt gradients, with the starting salt concentrations listed on the left and the final salt concentrations on the right.

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following exceptions. First, the supernatant from 155 grams of lysed cells was added to 45 ml of His-Bind resin (Novagen), incubated for 2 hours at 4°C and then poured into a column containing an additional 5 ml of resin. Second, peak-containing fractions from the His-Bind column were loaded onto a 4 ml heparin-Sepharose CL-6B column, then washed and eluted as per protocol. Third, the anti-HA monoclonal antibody affinity column was eliminated. Following the heparin-Sepharose column, peak-containing fractions were pooled and diluted to 240 mM KCl in gradient buffer and then loaded directly onto a 2 ml Q-Sepharose fast-flow column (Amersham Pharmacia Biotech) as shown in Figure 2.2. CF was eluted with a gradient from 240-600 mM KCl in gradient buffer, with peak fractions eluting at approximately 340 mM KCl. Rrn3p was purified according to protocol as outlined in Figure 2.3. TBP was purified by Tara Towers according to protocol as outlined in Figure 2.3.

UAF was purified as described by (Keener et al., 1997), with the following exceptions, as shown in Figure 2.2. UAF was purified using the NOY798 (Table 2.1) cell line (a kind gift of the M. Nomura laboratory) containing a triple HA1 tag and a hexahistidine tag at the C-terminus of the UAF subunit Rrn5p. The supernatant from 155 grams of lysed cells was added to 55 ml of His-Bind resin, incubated for 3.5 hours at 4°C then poured into a column containing an additional 5 ml of resin. Elution from the His-Bind resin went directly onto a heparin-Sepharose CL-6B column. The anti-HA monoclonal antibody affinity column was substituted with a 1 ml anti-His_6 monoclonal antibody affinity column (Sigma). UAF was eluted from the anti-His_6 monoclonal antibody affinity column directly onto the second heparin-Sepharose column with 1mg/ml His_6 peptide in gradient buffer.

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Extract [0.4M $(NH_4)_2SO_4$]

Ni++ Column

Imidazole (250 mM)

Heparin Sepharose

KCl

0.4 M 1 M

anti-His_6 mAb affinity column

His_6 peptide

Heparin Sepharose

KCl

0.4 M 1 M

UAF

Extract [0.3M $(NH_4)_2SO_4$]

Ni++ Column

Imidazole (250 mM)

Heparin Sepharose

KCl (550 mM)

Q Sepharose

KCl

0.24 M 0.6 M

CF

Figure 2.2. Flow diagrams for the purification of UAF and CF. Solid triangles represent linear salt gradients, with the starting salt concentrations listed on the left and the final salt concentrations on the right. Salts concentrations are listed for columns being eluted with a bump.

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Extract
↓
Ni++ Column
↓ Imidazole (250 mM)
Q Sepharose
KCl
0.19 M ↓ 0.55 M
Mono Q
KCl
0.19 M ↓ 0.55 M
Rrn3p

Extract
↓
Ni++ Column
↓ Imidazole (250 mM)
Heparin Sepharose
KCl
0.24 M ↓ 0.6 M
Mono S
KCl
0.1 M ↓ 0.36 M
TBP

Figure 2.3. Purification of HIS-Tagged Rrn3p and TBP. Solid triangles represent linear salt gradients, with the starting salt concentrations listed on the left and the final salt concentrations on the right. Salts concentrations are listed for columns being eluted with a bump.

2.3.2 Non-Specific transcription assays

Pol I fractions were monitored through the purification process by their non-specific RNA chain elongation activity. Non-specific transcriptions followed protocol as previously described (Spindler et al., 1978), with the following exceptions. The standard 60 μl reaction contained 20 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 300 μg/μl calf thymus DNA, 100 mM KCl, 200 μM each of GTP and CTP, 450 μM ATP and 1.5 μCi [α-^{32}P]UTP (Perkin-Elmer; 3000 Ci/mmol).

2.3.3 Reconstituted transcription assays

The conditions required for the *in vitro* reconstituted transcription assays were optimized based on previous protocol (Keener et al., 1998), using a basal transcription assay, containing CF, pol I, and Rrn3p. The reactions were carried out at 25°C in a final volume of 20 μl. The reactions contained: 20 mM Tris acetate, pH 7.9, 4 mM magnesium acetate, 8% glycerol, 2 mM DTT, 100 mM K^+ glutamate, 0.25 mg/ml acetylated bovine serum albumin, 0.025 units/μl RNasin (Promega), 10 ng template DNA, and nucleotides as follows: 200 μM ATP, GTP, and CTP, 5 μM UTP, and 2.5 μCi of [α-^{32}P]UTP (Perkin-Elmer; 3000 Ci/mmol). The reactions also contained small amounts of KCl from the protein fractions, the amount of KCl was equalized in each reaction and the total amount of K^+ ions were brought to 100 mM with K^+ glutamate. Depending on the particular assay, one of two templates were used, either pYR11-316 or pBS/35Sp. The pYR11-316 plasmid was kindly provided by the R. Reeder laboratory. It contains the *Sma*I-*Hind*III sequence surrounding the rDNA start site (Schultz et al., 1991). This template was found to have mutations at -130 and -110, in addition to an extra 10 bp as described by (Choe et al., 1992). Therefore, the plasmid pBS/35Sp was created

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containing the rDNA *Sma*I-*Hind*III fragment cloned from yeast genomic DNA and inserted into the pBS(-) plasmid at the *Sma*I and *Hind*III sites. The plasmid contains the rDNA sequence from –210 to +535. Both pYR11-316 and pBS/35Sp were linearized with *Eco*RV giving runoff transcripts of 420 nucleotides.

In a typical transcription reaction, all components were added to the mixture including proteins, with the NTPs being added last to start transcription. Each reaction contained the protein amounts as outlined by Keener et al., (1998), unless otherwise noted. Pol I and Rrn3p were allowed to preincubate as previously described (Keener et al., 1998), unless otherwise noted. Transcription was allowed to proceed for 30 minutes. The reactions were terminated by the addition of 80 μl stop buffer containing 0.625 mg/ml proteinase K and 0.625% SDS, followed by incubation at 50°C for 60 minutes. Nucleic acids were precipitated by addition of 75 μl of 4.68 M ammonium acetate, 0.33 mg/ml linear polyacrylamide, and 450 μl of 95 % ethanol. Nucleic acids were then pelleted by centrifugation for 30 minutes at 14,000 RPM in an Eppendorf centrifuge. Pellets were washed with 500 μl of 70% ethanol and dried under vacuum. The transcripts were then processed and detected as previously described (Radebaugh et al., 1998).

2.3.4 Immunoblotting

The presence of protein containing fractions from the column chromatography of UAF, CF, and Rrn3p was detected by immunoblotting. Protein fractions were either chloroform-methanol precipitated as previously described (Wessel and Flugge, 1984), and resuspended in sodium dodecyl sulfate (SDS) loading buffer, or diluted directly with 2X SDS loading buffer. Samples were then resolved by electrophoresis on either 10% or 11% SDS-PAGE gels, transferred to PVDF membrane (Pall Life Science) using a Genie

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electrophoretic blotter as specified by the manufacturer (Idea Scientific). Membranes were blocked in TBS with 0.05% Tween 20 and 3% milk (TBST-milk) overnight at 4°C, then incubated for 5 hours at room temperature with either anti-His_6 or anti-HA monoclonal antibodies at a dilution of 1:1,000 in TBST-milk. The membranes were then washed twice briefly, once for 20 minutes and twice for 5 minutes with TBST. The membranes were incubated with anti-mouse IgG conjugated to alkaline phosphatase (Sigma) at a dilution of 1:30,000 in TBST-milk for 1.5 hours at room temperature. The membranes were washed as described above and antibody cross-reactivity was detected using ECF reagent (Amersham) on a STORM860 scanner (Molecular Dynamics).

2.3.5 Electrophoretic Mobility Shift Assays (EMSAs)

pBS/35Sp was digested with *Sma*I and *Bgl*II to produce a 350 bp promoter containing fragment that was 5'-end-labeled for EMSAs. Labeling was carried out as described previously (Al-Khouri and Paule, 2002). Conditions for EMSAs were determined experimentally and optimized to contain: 20 mM Tris-Cl pH 8.0, 8 mM $MgCl_2$, 10% glycerol, 0.1% Tween-20, 0.2 mM EDTA, 2 mM DTT, 300 μg/ml BSA, 85 mM KCl, and 20,000 CPM/reaction of the ^{32}P labeled promoter fragment in a 30 μl reaction. Proteins were incubated with labeled DNA for 30 minutes at 25°C and immediately loaded onto a 5% non-denaturing polyacrylamide gel. Gels were run for 2 hours in TGE, dried, and subsequently exposed to phosphor storage screens (Kodak) overnight and visualized on a Storm860 scanner (Molecular Dynamics).

2.3.6 Photo-cross-linking

In order to photo-cross-link transcription factors to the yeast rRNA promoter, fragments from the promoter containing plasmid pBS/35Sp were made that contain a

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radioactive label and a reactive species that allow for the covalent bonding of the factor(s) to the DNA as previously described in detail (A. Bric, C. Radebaugh, and M. Paule, submitted manuscript), with the following exceptions. The labeled primers, along with the T3 primer, were bound to single stranded plasmid DNA, pBS/35Sp, containing the yeast promoter. The region of interest was then cut out of the plasmid, by cleavage with *Eco*RI, and purified. The 282 bp derivatized templates were then used to photo-cross-link the pol I factors to the derivatized sites. The transcription factors were incubated with the derivatized template in a typical binding reaction containing: 20 mM Tris acetate, pH 7.9, 4 mM magnesium acetate, 8% glycerol, 2 mM DTT, 100 mM K^+ glutamate, and 0.25 mg/ml acetylated bovine serum albumin. Binding reactions were incubated for 30 minutes at 25°C. Bound proteins were photo-cross-linked as per protocol (A. Bric, C. Radebaugh, and M. Paule, submitted manuscript). Primers were constructed that allowed for the derivatization of sites at -5/-4, -1/+1, +5/+6, -40/-41, -45/-46, -50/-51, -55/-56, and -60/-61 of the rDNA promoter.

2.4 RESULTS

2.4.1 Purification of yeast transcription factors needed for reconstituted transcription.

Purified heparin-Sepharose pool pol I, labeled J-Pol I, was preincubated with Rrn3p provided by the Nomura laboratory before being tested for activity in a reconstituted specific transcription assay. Pol I was shown to be active for specific transcription, using transcription components obtained from the Nomura laboratory, labeled N-factors, as positive controls (Figure 2.4, lane 6). CF was tracked through purification by western

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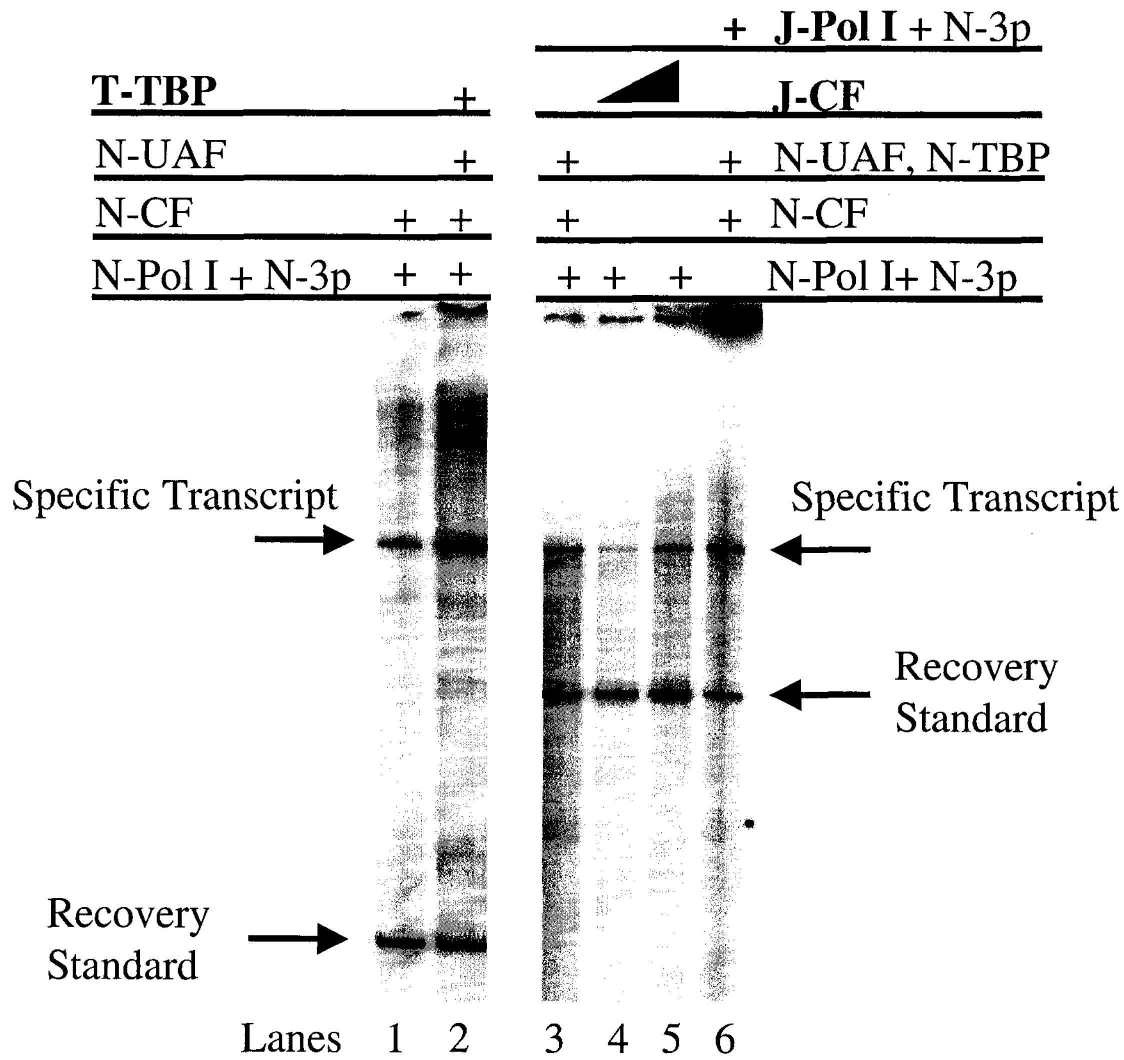

Figure 2.4. Factor activity in a reconstituted transcription assay. T-TBP was added into an activated transcription reaction (lane 2). Q-Sepharose CF, J-CF, (1 and 2 μl) was titrated into a transcription assay with Nomura factors (lanes 4 and 5). Heparin-Sepharose pol I, J-Pol I, was preincubated with Nomura Rrn3p before addition into a transcription reaction (lane 6).

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blotting using an anti-HA monoclonal antibody. CF was also shown to be active in the reconstituted transcription assay, whereby increasing amounts of CF gave increasing amounts of transcription (Figure 2.4, lane 4 and 5). Additionally the transcription activity of CF fractions from the Q Sepharose column corresponds with the protein levels as assayed by western blotting (Figure 2.5). TBP purified by Tara Towers, T-TBP, was shown to have DNA binding activity by electrophoretic mobility shift assay (Figure 2.6), and was shown to be active in an "activated" transcription assay, in which adding UAF and TBP together, transcription is stimulated over basal levels (Figure 2.4, lane 2). Rrn3p purified according to protocol was not found to be active in the yeast transcription assay (data not shown). Therefore, the Rrn3p used in these and the following experiments has been provided by the Nomura laboratory. UAF purified in this lab was shown to be active in reconstituted transcription assays by John Keener from the Nomura laboratory (Figure 2.7). Unfortunately we have not seen consistent UAF stimulatory activity in reconstituted transcription assays performed in this laboratory. Even when activated transcription was observed, it was only moderately stimulated over basal levels (Figure 2.8). We believe our inability to consistently obtain activated transcription may be due to unidentified differences in the mechanics of the transcription reactions performed in our laboratory and that of Nomura's laboratory. In any case, it is clear that the UAF we have purified is active.

2.4.2 UAF specifically binds the rDNA promoter in a weak fashion, whereas CF binds the promoter construct non-specifically.

The general regions that the yeast pol I transcription factors bind within the rRNA promoter have roughly been mapped out by deletion and linker scanner analysis as well

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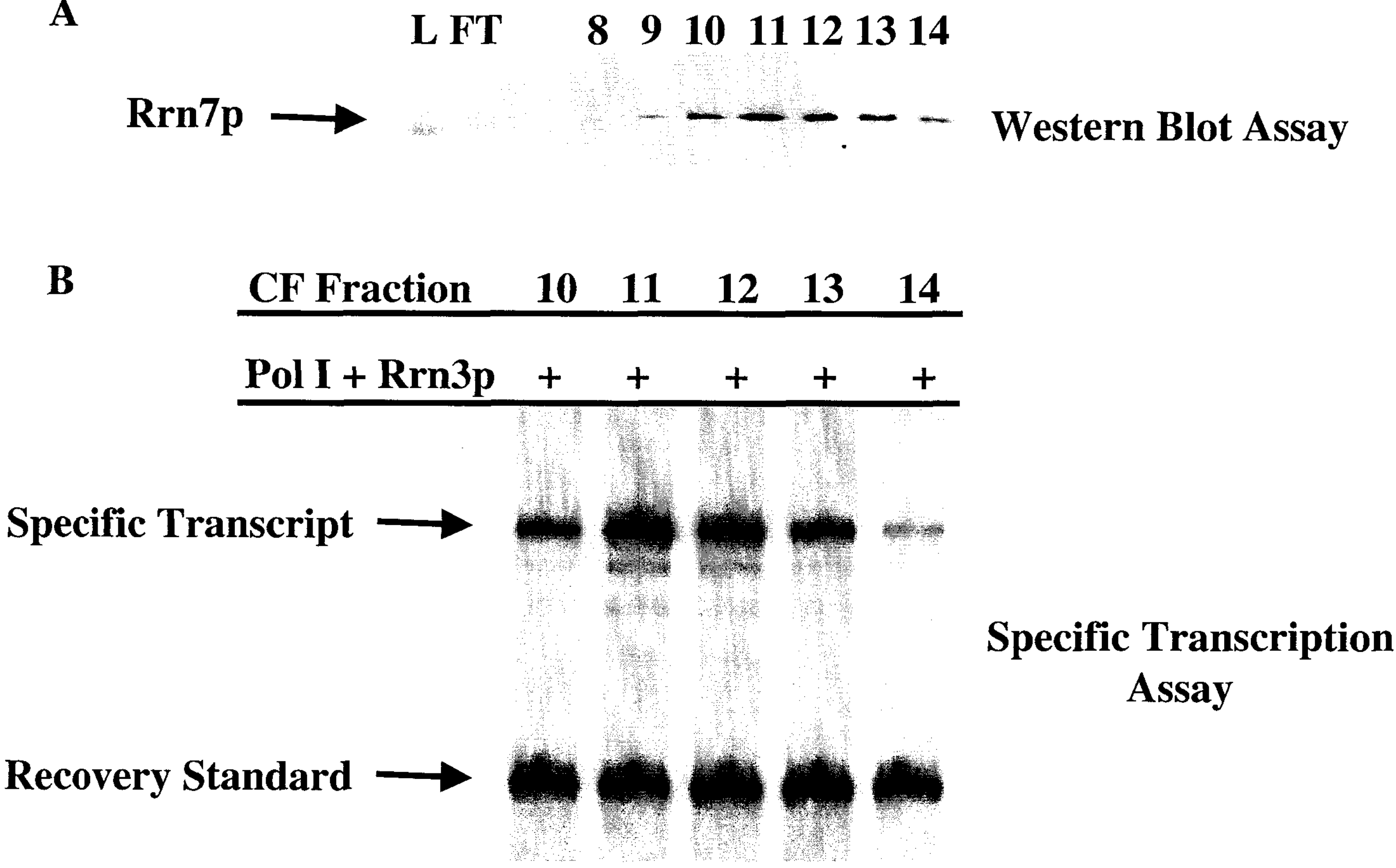
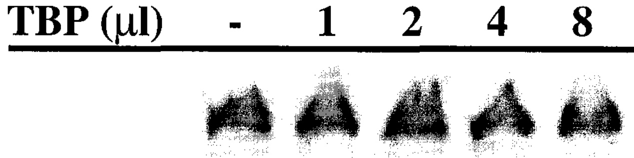


Figure 2.5. Core Factor Q-Sepharose fractions correspond to transcriptional activity. **A)** 5 μl of Q-Sepharose CF were electrophoresed and western blotted. Protein levels were assayed via cross-reactivity to His_6-HA tagged Rrn7p with an anti-HA mAb. **B)** 1 μl of the same CF fractions were used in a basal transcription assay. PhosphorImage files jg053003 and jg102200.



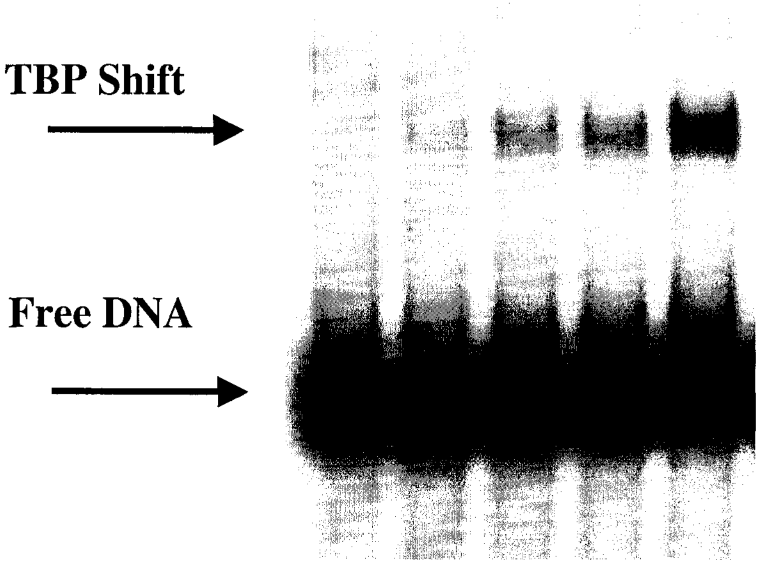


Figure 2.6. Increasing amounts of TBP provide increasing amounts of complex. EMSA with indicated amounts of MonoQ purified TBP on the pBS/35Sp promoter fragment. PhosphorImage file jg040500.

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UAF 0.5 μl (fxn#)					**14**	**15**	**16**	
UAF 0.2 μl (fxn#)		**14**	**15**	**16**				
Pol I-Rrn3p, CF, TBP	+	+	+	+	+	+	+	+

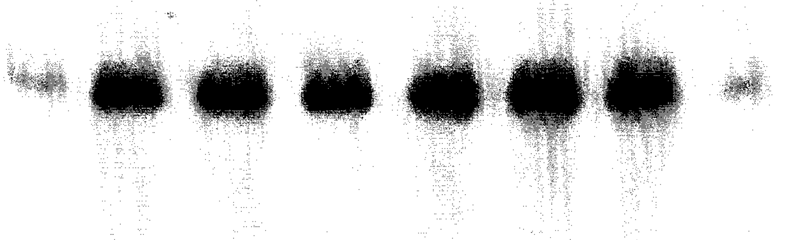

Figure 2.7. UAF activates transcription over basal levels. UAF fractions from the second heparin-Sepharose column were assayed for their ability to activate transcription over basal levels.

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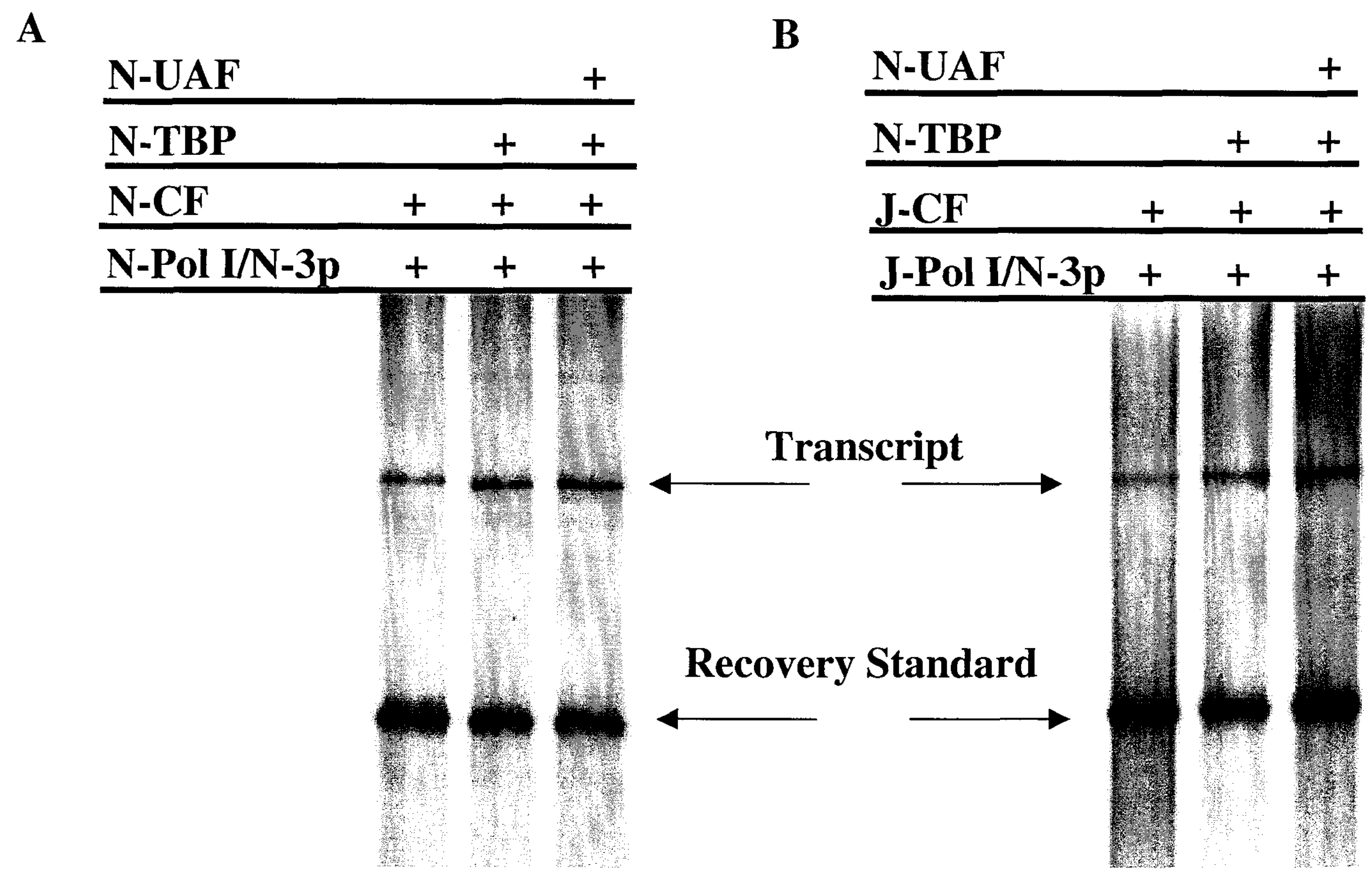

Figure 2.8. Activated transcription assays. A) Activated transcription with all Nomura's factors. **B)** Activated transcription with J-Pol I and J-CF. PhosphorImage files jg111000 and jg03090.

as *in vivo* footprinting (Bordi et al., 2001; Keener et al., 1998; Kulkens et al., 1991). UAF binds the promoter in the region of the UPE and CF binds the promoter in the core promoter region. Initially, gel shifts were obtained from the CF purified in the laboratory that seemed to correspond with protein levels. Additionally, the gel shifts seemed to be specific, partially withstanding competition by non-specific competitor DNA (data not shown). The shifts initially observed were seen using a DNA construct containing the entire promoter region. When the same experiments were performed using the construct that lacked the core promoter region, the shift remained (data not shown). This indicated there must be a DNA-binding activity in the "partially" pure CF sample that binds to the yeast promoter construct, most likely in the UPE. We believe that the elimination of the antibody affinity column is yielding impure CF samples and therefore, not yielding accurate information in this type of binding experiment. Interestingly, when the same CF prep is used in a DNase I footprinting assay containing the full promoter, no DNA protection is detected from either CF or the contaminating DNA binding activity. Furthermore, the addition of UAF and TBP along with CF also failed to produce any protection of the rRNA promoter in a DNase I footprinting assay.

Several attempts were made to purify active UAF. However, UAF activity could not be detected due to the inability to achieve "activated" transcription in a reconstituted transcription assay. Therefore, we questioned the activity of each preparation. Attempts to gel shift the various UAF preparations in electrophoretic mobility shift assays yielded varying shifts (data not shown). However, a shift was obtained by the UAF fraction that was later found to be active by the Nomura laboratory. Figure 2.9A shows the peaks of UAF eluted from the first and second heparin-Sepharose

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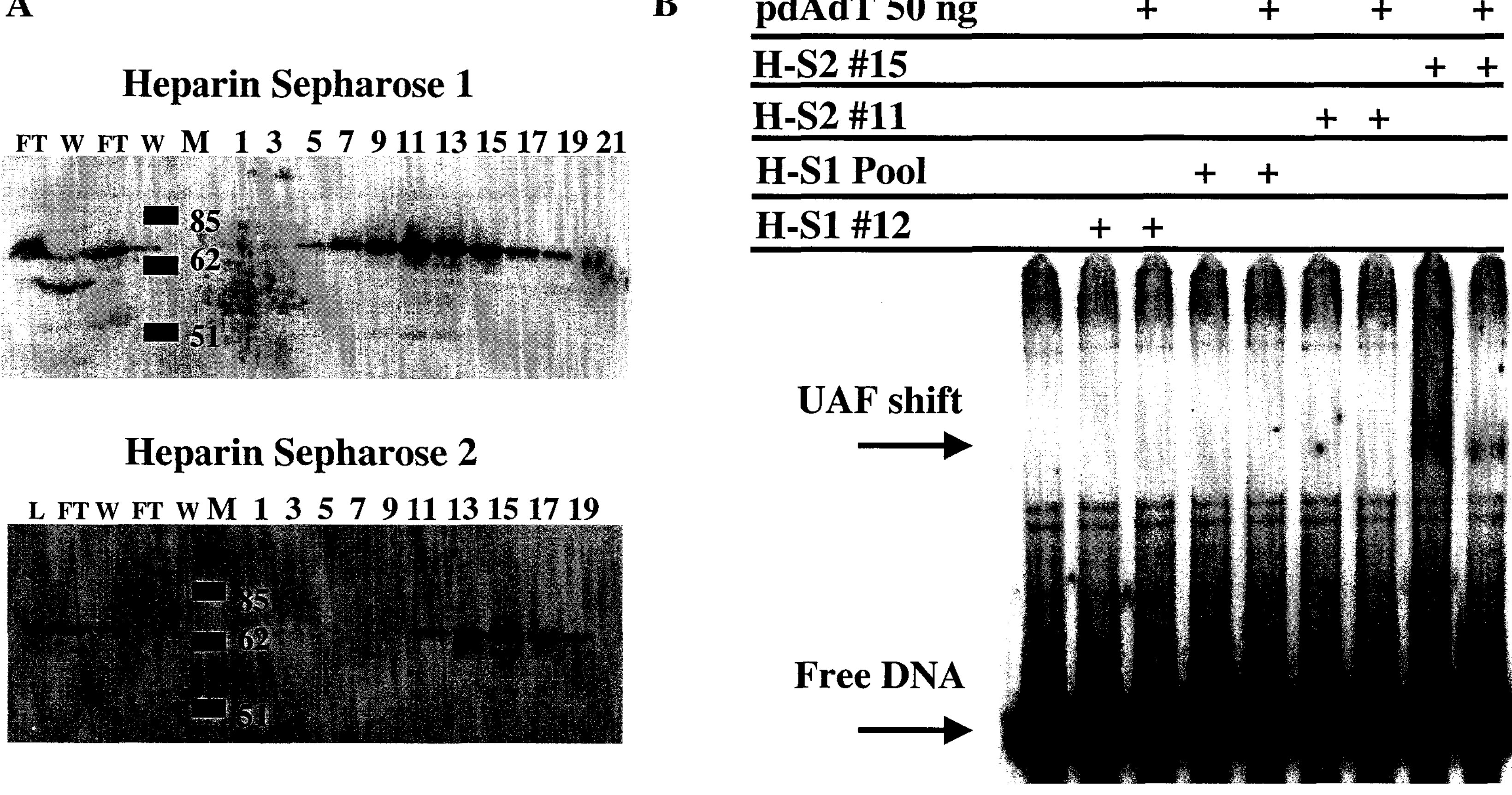

Figure 2.9. Purified UAF produces a gel shift. **A)** 10 μl of each heparin-Sepharose fraction were electrophoresed on 11% SDS-PAGE gels and subjected to western blotting. UAF was detected by cross-reactivity of Rrn5p to anti-HA mAb. **B)** 3 μl of peak fractions were analyzed in an EMSA. 50 ng poly dAdT was used as a non-specific competitor. PhosphorImage files jg042000, jg042300, and jg042500.

columns, as assayed by antibody cross-reactivity on western blots. Figure 2.9B shows a slight gel shift from fraction 15 of the second heparin-Sepharose column, no shift is seen from the less pure UAF fractions. The persistence of the shift with the addition of the non-specific competitor, poly dAdT, verifies that the binding of UAF to the promoter fragment is specific. Additionally the same shift is seen in fraction 15 and 16 from the second heparin-Sepharose column, corresponding to the two peak UAF containing fractions, as determined by western blotting (Figure 2.10). Therefore, it is likely that this shift is truly a UAF-specific shift. Attempts were also made to footprint this same UAF fraction, however no detectable protection was observed.

2.4.3 Photo-cross-linking of the yeast pol I transcription factors.

At the time of the initial photo-cross-linking experiments, it had not been determined whether or not the UAF purified in the laboratory was active or not. Therefore, the first cross-linking experiments were performed using CF only. Initially, CF was cross-linked on templates derivatized at three positions, -5/-4, -1/+1, and +5/+6. Figure 2.11 shows at least three proteins cross-link to the yeast promoter at the different derivatized sites. Cross-linked proteins are visible at 118 kD, 66 kD, and 47 kD. The strongest cross-linking occurs at position –5/-4, where the 66 kD and 47 kD bands appear to be specifically cross-linked. The cross-linked bands are competed away with specific competitor DNA, promoter fragment, but not with non-specific competitor, pBR322. The three CF subunits Rrn6p, Rrn7p, and Rrn11p have respective molecular weights of 115 kD, 56 kD, and 66 kD. It is possible the 118 kD band corresponds to Rrn6p, and the 66 kD band may correspond to either the Rrn7p or Rrn11p subunits. The lower band does not seem to correspond to any of the subunits, based on molecular weight. However, it is

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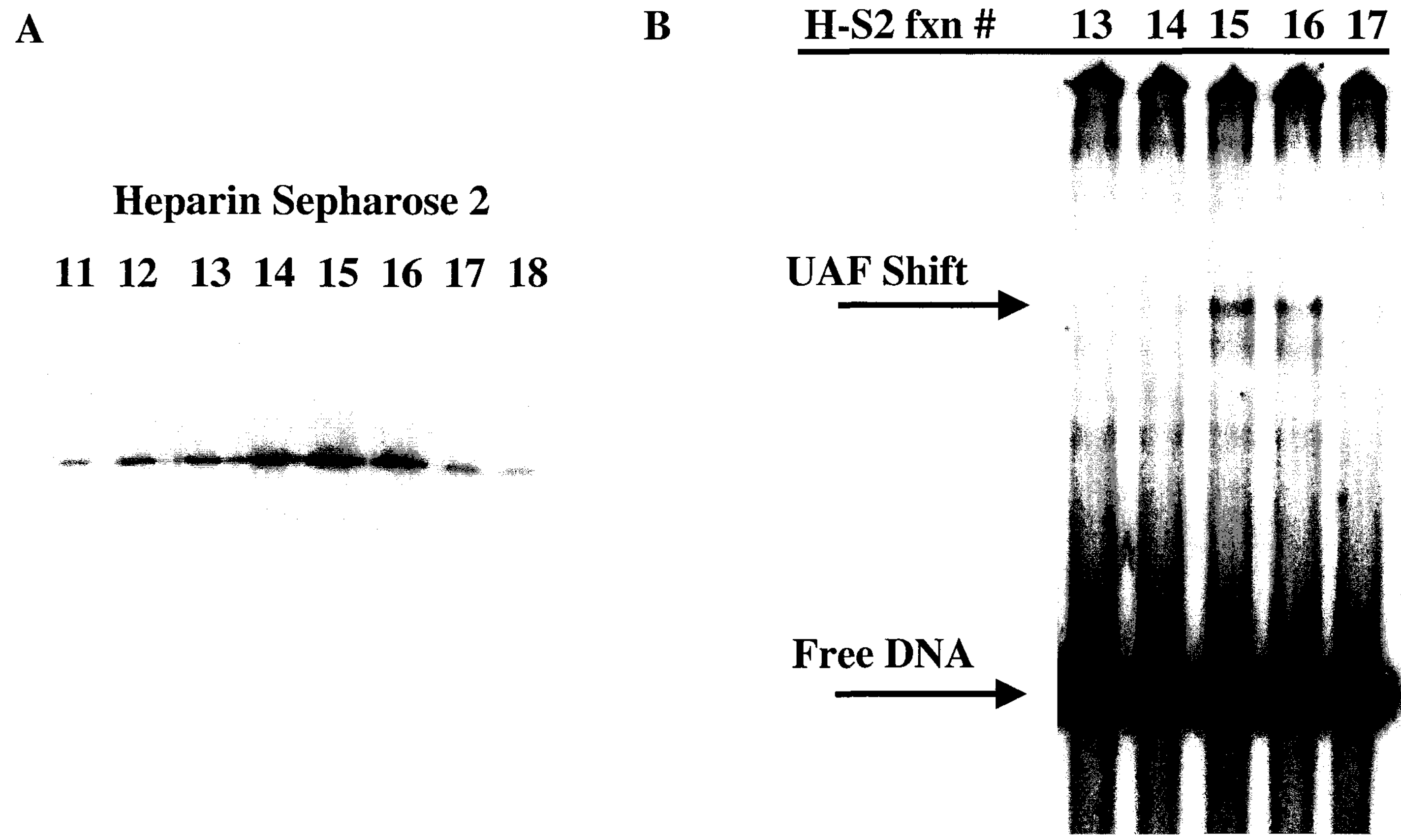

Figure 2.10. UAF heparin-Sepharose 2 fractions correspond with promoter DNA binding activity. A) 5 μl heparin-Sepharose 2 fractions were electrophoresed in an 11% acrylamide gel and subjected to western blotting. UAF was detected by cross reactivity of Rrn5p to an anti-HA mAb. **B)** 3 μl UAF fractions were assayed in an EMSA. PhosphorImage files jg060303 and jg072501.

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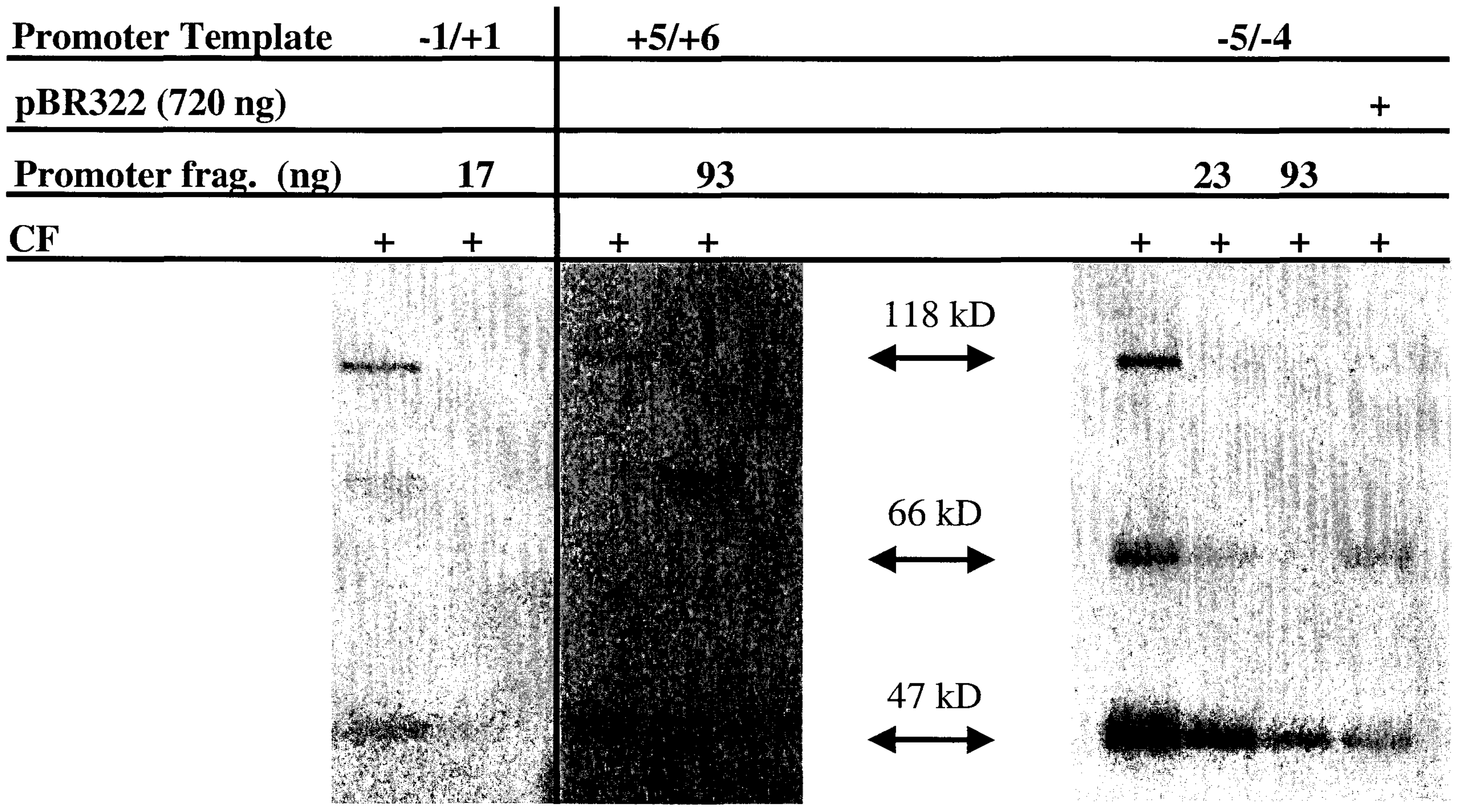

Figure 2.11: Photo-cross-linking of CF. Q-Sepharose CF (2 μl) was cross-linked to the -1/+1, +5/+6, and the -5/-4 derivatized templates. Cross-links were challenged by addition of specific competitor (pBS/35Sp SmaI-BglII fragment) or non-specific competitor (pBR322) as indicated prior to protein binding. PhosphorImage file jg063000a, jg071700(-5/+5), and jg071700(-5).

possible that a CF subunit bound to DNA could have an altered electrophoretic mobility. This CF prep is not homogenous and based on results from the electrophoretic mobility shift assays, it appears that there is at least one other protein in these preparations that binds to the yeast rRNA promoter.

When UAF and TBP are added to the cross-linking reactions, additional bands are seen on the gels. As shown in Figure 2.12, bands of approximately 52 kD and 34 kD are found cross-linked to the –5/-4 site. The molecular weights of the UAF subunits are: 42 kD, 50 kD, 17 kD, 30 kD, 18 kD, and 15 kD, for Rrn5p, Rrn9p, Rrn10p, Uaf30p, H3, and H4, respectively. The 52 kD and 34 kD bands seem to be specifically cross-linked as judged by loss of the bands with specific competitor and a very slight presence of the bands when challenged with non-specific competitor (Figure 12.2, lane 6). The 52 kD band could possibly be Rrn9p, while the 34 kD band could be either Uaf30p or TBP. Also shown is the 47 kD band associated with CF. The bands between 60 kD and 80 kD have been determined to be non-specific contaminating bands.

Following cross-linking studies of templates near the *tis*, primers were constructed to produce templates further upstream. Primers were made with derivatized sites at -40/-41, -45/-46, -50/-51, -55/-56, and -60/-61. Unfortunately, these templates label inefficiently. Consequently, the cross-linkings at each of these sites were difficult to discern. However, some cross-linking data was obtained from the -40/-41, -55/-56, and -60/-61 sites. Figure 2.13 shows cross-linking at each of these sites with CF, TBP, and UAF. Present at each site is the 47 kD band associated with CF and the 52 kD and 34 kD bands associated with either UAF alone or UAF with TBP (lanes 1, 4, and 7). The 52 kD band does not appear to produce specific cross-linking to these sites as judged by the loss

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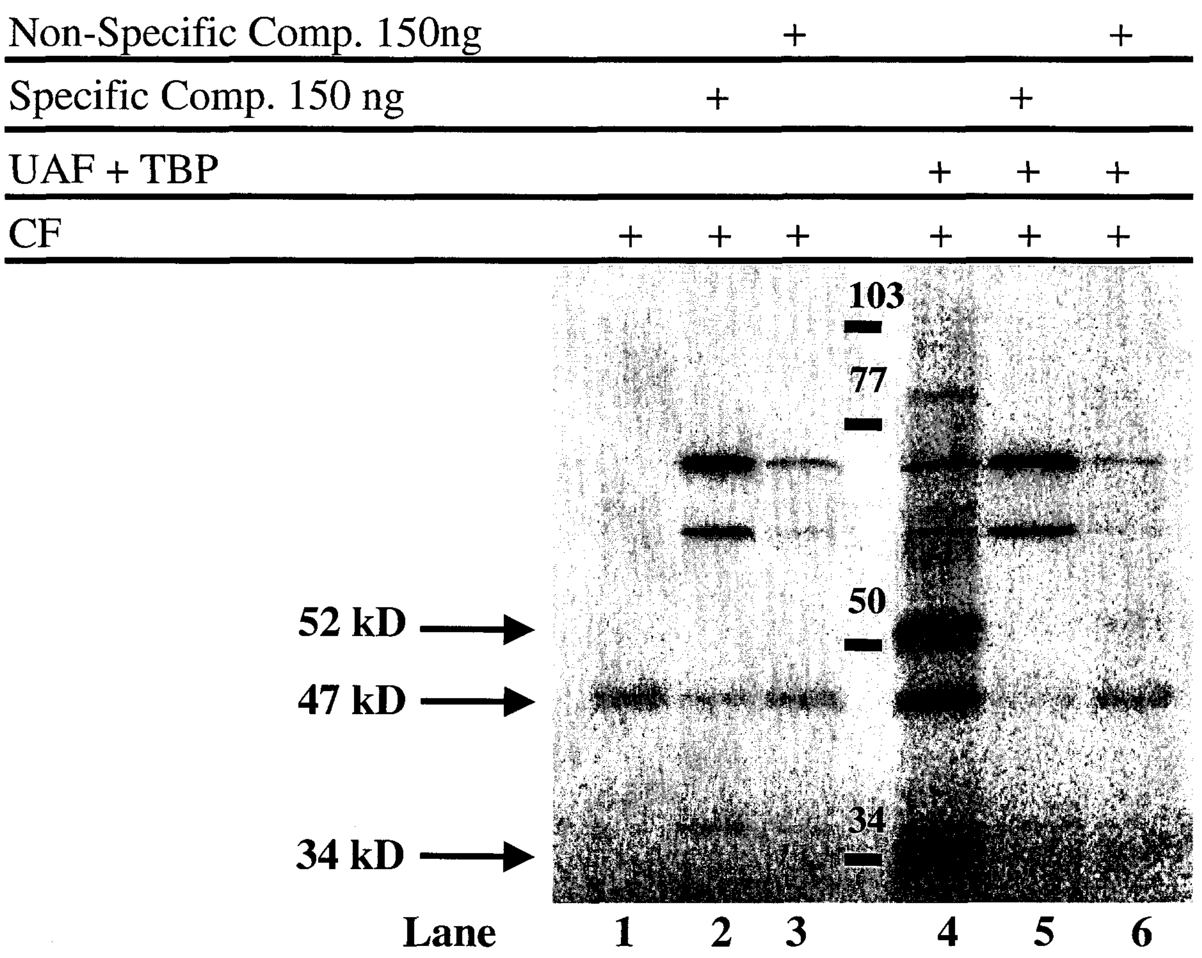

Figure 2.12. Cross-linking CF, UAF, and TBP to -5/-4 derivatized site. CF (1 μl), UAF (1 μl) and TBP (0.25 μl) were cross-linked to derivatized template -5/-4 as indicated. Non-Specific competitor pBS(-) and specific competitor pBS/35Sp were added as indicated prior to protein binding. PhosphorImage file jg122801a.

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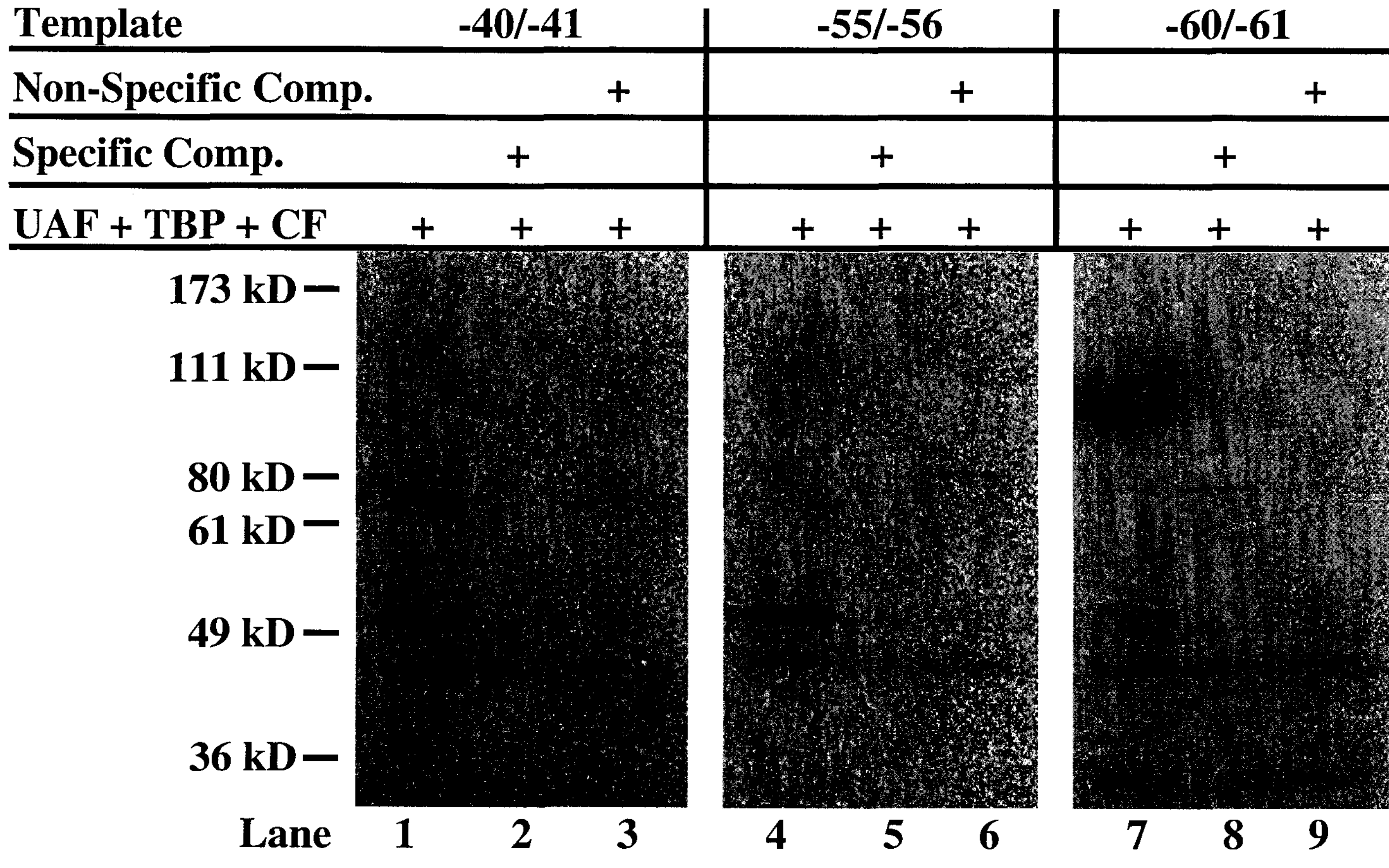

Figure 2.13. Photo-cross-linking of the -40/-41, -55/-56, and -60/-61 sites. CF (2 μl), UAF (1 μl), and TBP (0.25 μl) were cross-linked to the -40/-41, -55/-56, and -60/-61 derivatized templates. Non-specific competitor 150 ng of pBS(-) and specific competitor 150 ng of pBS/35Sp were added as indicated prior to protein binding. PhosphorImage file jg011702a, jg011702b, and jg122801b.

of the band upon incubation with non-specific competitor DNA (lanes 3, 6, and 9). The 47 kD band cross-links at each site strongly, being only slightly competed away even with specific competitor (lanes 2, 5, and 8). The 34 kD band gave fairly specific cross-linking at the –60/-61 site, being competed with specific competitor and not with non-specific competitor (lanes 7, 8, and 9).

Attempts were made to cross-link Pol I and Rrn3p along with CF-TBP-UAF at sites near the *tis*, although various bands were present, these cross-linkings were weak and provided data that was difficult to interpret.

2.5 DISCUSSION

While *S. cerevisiae* has proven to be a model organism for the study of protein-protein interactions and the identification/cloning of the factors required for rRNA transcription, it has proven to be a difficult organism in which to retrieve consistent biochemical data. It is possible that our inability to purify the components required for rDNA transcription to homogeneity may have hindered our characterization of their DNA binding properties. It has been shown by the Nomura laboratory that it is possible to get activated *in vitro* transcription from purified UAF (Keener et al., 1998). However no additional biochemical data has been published from their laboratory.

The Camilloni laboratory has published *in vivo* footprints of the yeast transcription factors, however these DNase I footprints are difficult to interpret. They reported *in vivo* footprints of the 35S rRNA promoter and found protected regions from –10 to –70 and –70 to –154 that they have attributed to CF and UAF, respectively (Vogelauer et al., 1998). They later reported the yeast pol I factors bind in a specific hierarchy, with UAF

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binding first to the UPE independently, then CF is bound in a UAF dependent manner (Bordi et al., 2001). Additionally they refer to a personal communication from Masayasu Nomura reporting an *in vitro* footprint of UAF between –45 and –110. We also have been alerted of this footprint, but we have not personally seen any data. The footprinting produced from the Camillioni laboratory are in general agreement with *in vitro* and *in vivo* linker scanner mutations and deletion analysis from the Planta and Nomura laboratories (Kulkens et al., 1991; Musters et al., 1989). They report three functionally important domains in the yeast promoter: domain I (+8 to –28) domain II (-51 to –76) and domain III (-91 to –146). With the 5' border needed for minimal core promoter function lying somewhere between –26 and –38 (Kulkens et al., 1991). This data is in agreement with recent deletion analysis from a purified *in vitro* system, showing the requirement of sequence to –38 for basal levels of transcription supported by CF (Keener et al., 1998). However, there appears to be a slight discrepancy in the sequence needed for UAF binding. The *in vivo* footprinting data indicates UAF binding requires promoter sequence well into domain III, while deletion analysis has shown activated transcription can occur with templates deleted 5' to –76 (Bordi et al., 2001; Keener et al., 1998; Kulkens et al., 1991; Vogelauer et al., 1998). It is possible that UAF binds, albeit weakly, to the downstream part of the UPE and that sequence upstream of the –76 site is needed for fully stable promoter binding. *In vitro* footprinting and UV photo-cross-linking analysis of this region are important to elucidate the exact positions that each of the UAF subunits are binding to the UPE.

As mentioned above, UAF and UBF appear to posses similar activities in rRNA transcriptional initiation. Both provide transcriptional stimulation and have been

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shown to stabilize the binding of the fundamental transcription factor. However, there is no sequence homology between any of the subunits of UAF and UBF. UBF has been shown to induce bending in the upstream promoter region resulting in activation of transcription. UAF contains the histone proteins H3 and H4, which are known to aid in the wrapping of DNA around the nucleosome. Therefore, it is of interest to study whether or not UAF induces conformational changes in the rRNA promoter regions, similar to UBF, as part of its ability to stimulate transcription. With the successful development of an *in vitro* EMSA, it may be possible to answer these questions using pBEND templates.

It has recently been reported in yeast that over-expression of TBP in a UAF deficient strain led to increased levels of basal transcription (Aprikian et al., 2000). This data seems to indicate that TBP may bind to the promoter in a manner that stabilizes CF binding, even though a later report refuted these findings (Siddiqi et al., 2001). It has also been shown that in the *A. castellanii* system, TBP is located in close proximity to the promoter through photo-cross-linking, even though TBP does not bind DNA using its TATA-box binding surface in rRNA transcription (Gong et al., 1995; Radebaugh et al., 1994). Furthermore, it was shown that recombinant yeast TBP purified in this laboratory is capable of binding to a fragment of DNA containing the yeast rRNA promoter (see Figure 6). This suggests that in addition to TBP's role as a bridging molecule for UAF and CF, it may also help stabilize the preinitiation complex by binding to the yeast promoter. This contrasts with the *A. castellanii* situation, possibly because yeast TBP is not tightly associated with TAFs, while TBP in *A. castellanii* is tightly associated with the TAFs of TIF-IB. In *A. castellanii,* free TBP binds and footprints a cryptic TATA box in the rRNA promoter, but it does not associate with this region of the promoter in the

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context of TIF-IB. Photo-cross-linking of the purified pol I transcription factors may provide insight into the exact role TBP plays in *S. cerevisiae* pol I transcriptional initiation.

Recent studies have provided conflicting results as to whether Rrn3p is needed for recruitment of pol I to the rRNA promoter or whether Rrn3p is required at a subsequent step, such as DNA melting (Aprikian et al., 2001; Miller et al., 2001). We hoped to use the photo-cross-linking experiments and potassium permanganate footprinting to elucidate the role of Rrn3p in transcriptional initiation. Attempts were made to cross-link pol I with and without Rrn3p to a promoter containing UAF, TBP, and CF. The cross-linking results were weak and difficult to interpret. It is our belief that stronger cross-linking of the factors to the yeast rRNA promoter will provide the data needed to elucidate the role of Rrn3p in transcriptional initiation. Additionally, potassium permanganate footprinting has been used in the laboratory to determine that *A. castellanii* TIF-IB/TIF-IE and pol I (probably associated with TIF-IA; see Chapter 3) are the only proteins required for promoter opening (Kahl et al., 2000). If photo-cross-linking experiments indicate Rrn3p was not needed for pol I recruitment, potassium permanganate footprinting could be utilized to determine if Rrn3p is needed for DNA melting.

It is possible that in attempting to optimize basal transcription reactions we may have selected for conditions that do not allow UAF to activate transcription. This subsequently may have hindered the development of DNA binding assays such as photo-cross-linking, DNase I footprinting, and EMSAs. However, even when attempting to follow the published protocol (Keener et al., 1997), only slight activation of transcription

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could be detected upon addition of UAF. The presence of the slight UAF gel shift on the yeast promoter indicates that the conditions for optimal binding may be achievable. It may then be possible to photo-cross-link UAF, along with the remaining pol I transcription factors. This would then allow for the elucidation of a detailed map indicating the exact regions where the different pol I factors are contacting the yeast rRNA promoter.

In order to utilize the yeast system to answer the questions posed here, I believe that the laboratory must be able to either purify the factors closer to homogeneity, or to develop binding assays using less pure extracts. Purification of the factors to homogeneity would require the ability to make the HA-antibody affinity column. Alternatively, it may be necessary to tag the factors with alternative epitopes, with more readily available antibody affinity columns. In addition, it will be absolutely necessary to be able to purify Rrn3p in an active state. Using epitope tagged Rrn3p, it should be possible to purify the factor in an active state from *S. cerevisiae*. Recently, work has also begun to purify Rrn3p from Sf9 cells. It is believed that recombinant Rrn3p from these eukaryotic cells may be more active than the recombinant protein purified from *E. coli*. If a post-translational modification is required for the full activity of Rrn3p, then the Sf9 purification may be better suited for producing active Rrn3p, as is the case in mammalian systems (Cavanaugh et al., 2002). Additionally more highly purified factors may make the photo-cross-linking studies easier to interpret. Alternatively, it is possible that pol I transcription factors purified to homogeneity may be missing other critical factors, such as TFIIH, Hmo 1, or a TIF-IC homologue. It may be possible to utilize bead bound promoters to capture the required pol I transcriptional machinery necessary for stable

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preinitiation complex formation. I believe it is possible to develop the assays required to answer the important questions posed herein, and that the answers to these questions would provide great insight into how transcriptional initiation may be targeted by regulatory mechanisms.

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CHAPTER 3

TIF-IA MEDIATES PROMOTER RECRUITMENT AND REGULATION OF RIBOSOMAL RNA TRANSCRIPITON

The work presented in this chapter involves the identification of a TIF-IA homologue in *A. castellanii*, and the study of its activity in phosphatase treated extracts as well as extracts purified from encysted cells. I performed all of the experiments presented here, and purified most of the proteins necessary for those experiments. John Anderson assisted in the preparation of the cyst pol I. This work is to be submitted to the *Journal of Biological Chemistry* for publication.

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TIF-IA MEDIATES PROMOTER RECRUITMENT AND REGULATION OF RIBOSOMAL RNA TRANSCRIPITON

3.1 ABSTRACT

The transcription of the ribosomal RNA genes by RNA polymerase I closely follows cellular growth rate. Extracts prepared from cells that have undergone nutrient deprivation or have entered a stationary phase of growth, are not capable of specifically transcribing rRNA. The interaction of pol I with an essential transcription factor, TIF-IA, is involved in this down regulation of transcription. Pol I and TIF-IA are found tightly associated in pol I fractions competent for specific transcription. Pol I purified from stationary phase cells is not associated with TIF-IA. The disruption of the pol I-TIF-IA complex is mediated by a specific dephosphorylation of either pol I or TIF-IA. We report here that phosphatase treatment of *A. castellanii* pol I fractions results in a down-regulation of transcriptional activity and promoter binding, reminiscent of pol I fractions purified from encysted cells. Additionally, this down-regulation coincides with an altered electrophoretic mobility of the *A. castellanii* TIF-IA homologue. Furthermore, we show that in *A. castellanii* the pol I-TIF-IA complex is required for pol I recruitment to the rRNA promoter.

3.2 INTRODUCTION

Polymerase I (pol I) is one of three eukaryotic DNA-dependent RNA polymerases whose sole responsibility is the transcription of the ribosomal RNA genes. Ribosomal RNA (rRNA) transcription closely follows cellular growth rate; as a cell approaches

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stationary phase or encounters conditions that negatively effect either growth rate or protein synthesis, rRNA transcription is decreased. Ribosomal RNA transcription can account for up to 60% of all RNA being transcribed in an actively dividing cell. Due to the large amounts of energy this transcription requires, it is of utmost importance for the cell to efficiently regulate rRNA transcription. Additionally, recent studies have shown a direct link between the transition from normal to neoplastic growth; indeed, it is not clear whether uncontrolled rRNA transcription might lead to abnormally high cellular growth and division. Thus, uncovering the mechanism(s) that regulate this transcription may lead to an understanding of cellular growth control in general, and to possible avenues for attacking uncontrolled cell cycling.

A particular pol I transcription factor, found to be tightly linked to growth rate has been shown in many organisms to be essential for pol I transcription. This factor, TIF-IA, was first characterized in the mouse system as being the growth regulated factor, and was later identified in a *S. cerevisiae* genetic screen for essential pol I transcriptional components (Buttgereit et al., 1985; Schnapp et al., 1993; Yamamoto et al., 1996). Termed Rrn3p in yeast (but herein called scTIF-IA), this factor associates with pol I specifically, in the presence or absence of DNA, and is not believed to bind to DNA (Milkereit and Tschochner, 1998; Schnapp et al., 1993; Yamamoto et al., 1996). Similar properties for TIF-IA have been described in mammalian systems (Schnapp et al., 1993). ScTIF-IA is required to be associated with pol I for specific transcriptional initiation to occur at the rRNA promoter (Milkereit and Tschochner, 1998; Miller et al., 2001; Peyroche et al., 2000; Yamamoto et al., 1996). Less that 2% of the pol I found in a yeast cell is present in this initiation competent pol I-TIF-IA complex, with the rest of the pol I

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being needed for such events as elongation or possibly reinitiation (Milkereit and Tschochner, 1998). TIF-IA has been cloned from both yeast and human (Bodem et al., 2000; Moorefield et al., 2000; Yamamoto et al., 1996). Additionally, TIF-IA related genes have been identified in *S. pombe, C. elegans, and A. thaliana.* When extracts are prepared from cells under non-favorable conditions, such as yeast cells in stationary phase, the levels of both pol I and TIF-IA remain constant even though the levels of specific transcription are dramatically decreased (Cavanaugh et al., 2002; Milkereit and Tschochner, 1998; Yuan et al., 2002). These results suggests that as a cell undergoes the transformation from actively dividing to stationary phase, there must be a modification in either pol I, TIF-IA or both that disrupts the formation of the pol I-TIF-IA complex.

The association of TIF-IA with pol I, required for specific transcription initiation, is brought about through the direct binding of TIF-IA to a specific pol I subunit. In *S. cerevisiae*, scTIF-IA has been shown to interact directly with the A43 subunit of pol I through affinity chromatography, and the suppression of a mutant A43 phenotype (Peyroche et al., 2000). In *S. pombe*, the overexpression of the A43 homologue, RPA21, has also been shown to suppress a mutant phenotype of spTIF-IA (Imazawa et al., 2002). Furthermore, in mammalian systems TIF-IA has been shown to directly interact with A43 through GST-pull down and affinity chromatography assays (Cavanaugh et al., 2002; Yuan et al., 2002). In addition to the direct physical interaction with A43 in mammalian systems, TIF-IA was shown to bind to the polymerase associated factor PAF67, found only associated with initiation-competent fractions of pol I (Seither et al., 2001; Yuan et al., 2002). TIF-IA has also been reported to make contacts to other factors within the pol I machinery.

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TIF-IA interacts directly with certain TBP associated factors (TAFs) of the fundamental transcription factor. In yeast, a physical interaction was observed between scTIF-IA and the Rrn6p subunit of core factor through yeast two-hybrid analysis and affinity chromatography (Peyroche et al., 2000). An additional interaction has been found in yeast between scTIF-IA and Rrn7p based on two-hybrid and GST-pull down analysis (C. Radebaugh et al., unpublished). Similarly, in humans, hsTIF-IA interacts with both TAF_I110 and TAF_I63 of SL1, as shown by co-immunoprecipitation, GST-pull down, and far-western blotting assays (Miller et al., 2001). Furthermore, in mouse, mmTIF-IA was shown to interact with TAF_I95 and TAF_I68 of TIF-IB by co-immunoprecipitation (Yuan et al., 2002). Therefore, both biochemical and genetic techniques confirm a direct interaction of the fundamental pol I transcription factor with the factor required for specific transcriptional initiation.

Given that TIF-IA is absolutely required for specific transcription and has been found to associate with both the fundamental transcription factor and pol I, one would be inclined to imagine a role for TIF-IA in the recruitment of pol I to the promoter. Reports from the human pol I transcription system provide support for this hypothesis. Affinity-purified antibodies made to peptides of hsTIF-IA are able to block the recruitment of pol I to a rRNA promoter containing bound SL1 (Miller et al., 2001). Additionally, the same antibodies inhibit specific transcription from the SL1 bound promoter complexes. The absence of pol I recruitment without TIF-IA combined with the inhibition of transcription upon antibody addition indicates a requirement for TIF-IA in recruitment. However, in the yeast system, binding experiments to promoter-DNA immobilized on beads have shown that although necessary for transcription initiation, scTIF-IA is not needed for

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recruiting pol I to the promoter. Bulk pol I can be recruited to a magnetic bead bound promoter without the presence of scTIF-IA. However, scTIF-IA must be associated with pol I prior to binding for the subsequent complex to be initiation competent (Aprikian et al., 2001). These results are similar to the murine system where preinitiation complexes containing pol I are capable of being formed in the absence of TIF-IA, with the factor being required at a subsequent step before transcriptional initiation (Schnapp and Grummt, 1991; Schnapp et al., 1993). In the latter species, unlike yeast, TIF-IA can be added after pol I recruitment.

There are also discrepancies concerning the exact requirements needed for pol I-TIF-IA association. Phosphorylation of specific transcriptional components has been shown to be important for cell cycle regulation of rRNA transcription. Specific phosphorylations/dephosphorylations are also important for growth dependent regulation. The subunits of pol I have been shown to possess multiple phosphorylation sites (Breant et al., 1983; Buhler et al., 1976). In yeast, the phosphorylation states of the A43 relative to the A190 subunits are significantly altered between free pol I and pol I-TIF-IA complex (Fath et al., 2001). The phosphorylation ratio A43/A190 is much higher in the pol I-TIF-IA complex than in free pol I. Furthermore, phosphatase treated pol I does not associate with scTIF-IA, while mock-treated pol I does. These results have prompted the authors to suggest that specific phosphorylations, such as that of A43, are required for the association of pol I with scTIF-IA. Interestingly, this work in yeast also demonstrated that scTIF-IA is capable of forming an initiation competent complex with pol I, independent of its specific phosphorylation state, including bacterially expressed scTIF-IA.

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In contrast, TIF-IA phosphorylation is required for pol I association in mammalian systems. The current model for mammalian pol I transcription indicates TIF-IA must be phosphorylated in order to bind to the A43 subunit of pol I. TIF-IA produced in Sf9 cells, and therefore believed to be correctly phosphorylated, was shown to physically interact with A43, whereas bacterially expressed TIF-IA could not (Cavanaugh et al., 2002). Interestingly, both TIF-IA preparations were able to bind to the TAF_I68 subunit of SL1, indicating a specific phosphorylation event is not necessary for interaction with the fundamental transcription factor. Additionally phosphatase treatment of FLAG-TIF-IA inhibited both transcriptional activity and the ability to associate with A43. Cycloheximide treatment also inhibited the phosphorylation of murine TIF-IA, and is associated with the loss of pol I-TIF-IA interaction and transcription (Cavanaugh et al., 2002). Recent results from the Grummt laboratory have indicated that two specific serine residues of mammalian TIF-IA (S633 and S649) are required to be phosphorylated for transcriptional activity (Zhao et al., 2003). The S633 and S649 residues were shown to be phosphorylated by ERK and RSK kinases, providing an activation of transcription upon growth factor stimulation. Substitutions of these serines with aspartic acid residues resulted in the ability of exogenously added TIF-IA to rescue extracts from density-arrested cells, whereas substitution with alanines did not lead to transcriptional rescue. These results indicate a requirement for a net negative charge, provided by an amino acid or a phosphate group, at these positions for both transcriptional activity and pol I binding. Unfortunately, the contradictory evidence presented from the different organisms makes it unclear which phosphorylation is required for the formation of pol I-TIF/IA complex. It seems that in lower eukaryotes, such as *S. cerevisiae*, a specific phosphorylation of a pol I

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subunit (A43) is required for the pol I-TIF-IA complex to form, whereas in mammals specific phosphorylation of TIF-IA itself is required.

The *A. castellanii* pol I transcription system has been well defined biochemically. TIF-IB, the fundamental transcription factor, is required for rRNA promoter driven transcription. TIF-IB has been shown to bind the rRNA core promoter from –67 to –17 relative to the transcription initiation site (*tis*) (Bateman et al., 1985). This binding is brought about by interactions with the minor groove in a structurally conserved and sequence tolerant manner (Geiss et al., 1997; Marilley et al., 2002). Partially purified TIF-IB is able to form a committed complex on the promoter that is stable through multiple rounds of transcription, whereas TIF-IB purified to near homogeneity cannot (Iida et al., 1985; Radebaugh et al., 1998; Radebaugh et al., 1994). An additional 141 kD factor, TIF-IE, has been shown to stabilize TIF-IB in the committed complex (Al-Khouri and Paule, 2002; Radebaugh et al., 1998). TIF-IE does not bind to the promoter and has been found to tightly associate with both TIF-IB and pol I through multiple chromatographic separations.

Pol I in *A. castellanii* has been purified to near homogeneity and consists of 12 subunits (Iida and Paule, 1992). It was first shown in *A. castellanii* that pol I is the component necessary for rRNA transcription that is regulated upon the switch from exponential growth to encystment (Bateman and Paule, 1986; Paule et al., 1984). Pol I has been isolated from both encysted (cyst) and trophozoite cells, and it was found that cyst pol I is deficient for specific transcription and promoter binding (Bateman and Paule, 1986; Paule et al., 1984). However, it is possible that the "pol I" activity our laboratory

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has reported as being the regulated component, likely refers to a pol I-TIF-IA complex. To date, no TIF-IA homologue has been isolated from *A. castellanii.*

We report here that an activity similar to TIF-IA has been characterized in the *A. castellanii* system. The yeast TIF-IA transcription factor has been shown to stimulate specific transcription in an *in vitro* reconstituted *A. castellanii* transcription assay. We also have identified an approximately 80 kD protein associated with transcription competent pol I fractions via cross-reactivity with an anti-TIF-IA polyclonal antibody made to the yeast factor (anti-scTIF-IA). We have found alkaline phosphatase treated pol I fractions possess similar biochemical properties as those purified from encysted cells, reduced specific transcription activity and an inability to bind promoters. This dephosphorylation was shown to cause a shift in electrophoretic mobility of TIF-IA that had been alkaline phosphatase treated in a pol I-TIF-IA complex. Furthermore we report here that the *A. castellanii* homologue to TIF-IA is required for pol I recruitment to the rRNA promoter.

3.3 MATERIALS AND METHODS

3.3.1 Purification of *A. castellanii* proteins

Purification of TIF-IB TIF-IB was purified from crude nuclear extract as described previously (Al-Khouri and Paule, 2002; Radebaugh et al., 1998), and was either from the first or second round of promoter affinity chromatography (1X or 2X DNA affinity TIF-IB).

Purification of Polymerase I Trophozoite RNA pol I was purified from whole cell extract as previously described (Spindler et al., 1978), with the following exceptions. All

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buffers used during purification contained 1 mM benzamidine. Cells pelleted in the continuous flow rotor were washed in 2.5 volumes of extraction buffer, instead of 0.15 M KCl. The lysed cell suspension was diluted in buffer as per protocol, with the addition of 12.5% glycerol. The polyethyleneimine (PEI) wash and elution buffer, Buffer A and Buffer B, contain 12.5% glycerol. The PEI supernatant was loaded onto a 400 ml cake of DEAE-cellulose (DE52) based on the starting cell mass, not based on specific protein concentration of PEI supernatant. The pol I fractions utilized in these experiments were purified through the heparin-Sepharose chromatography step.

RNA pol I was purified from encysted *A. castellanii* cells as described previously, (Paule et al., 1984), following the methods above with the following modification. After the initial disruption of the cell pellet using the Super Dispax Tissumizer, the unlysed cyst cells were broken open using a Yammato tissue homogenizer (10 rounds). This resulted in 68% lysis of encysted cells.

Purification of TIF-IE TIF-IE was purified as a side fraction of pol I by rate zonal sedimentation in a glycerol gradient as described previously (Al-Khouri and Paule, 2002; Radebaugh et al., 1998).

3.3.2 Plasmids and templates

For specific transcription assays, pEBH10 (Bateman et al., 1985) was linearized with *Nhe*I to produce a 420 nucleotide transcript. For promoter bead binding experiments the plasmid pJG310 was constructed to introduce a *Nhe*I site upstream of the rRNA promoter. pEBH10 was cleaved immediately upstream of the promoter region with *Eco*RI and *Bam*HI and the 377 bp *Eco*RI-*Bam*HI fragment from pBR322 was cloned into it. This creates a *Nhe*I site 238 bp upstream of the *tis* in pJG310. Plasmids pGG4C and

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pGG17C were used to prepare DNA fragments for DNase I footprinting assays as previously described (Geiss et al., 1997).

3.3.3 DNase I footprinting

The 150 bp *Bam*HI-*Sac*I fragment was isolated from pGG4C and radioactively labeled as previously described (Al-Khouri and Paule, 2002). A typical 40 µl DNase I footprinting reaction contained: 30 mM HEPES, pH 7.8, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40, 0.5 mg/ml bovine serum albumin, 10% glycerol, 100 mM KCl, and 15,000-20,000 CPM of the *Bam*HI-*Sac*I pGG4C fragment, with 0.25 ng/µl pBR322 linearized with *Nru*I as non-specific competitor. Unless otherwise stated, typical reactions contained 2-3 µl 1X DNA affinity TIF-IB, 0.5 µl TIF-IE, and 20-60 mU heparin-Sepharose pol I.

3.3.4 Immunoblotting

The presence of specific pol I subunits or TIF-IA was detected by immunoblotting. Protein samples were resolved by electrophoresis on either 10% or 11% SDS polyacrylamide gels and then transferred to PVDF membrane (Pall Life Science) using a Genie electrophoretic blotter as specified by the manufacturer (Idea Scientific). The membranes were blocked in TBS with 0.05% Tween 20 and 5% milk (TBST-milk) for 3 hours at room temperature then incubated overnight at 4°C with either anti-scTIF-IA polyclonal, anti ABC22.5 polyclonal, or anti AC39 monoclonal antibodies (see below) at a dilution of 1:1,000 in TBST-milk. The membranes were then washed twice briefly, once for 20 minutes, and twice for 5 minutes with TBST. The membranes were incubated with either anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase (Sigma) at a dilution of 1:30,000 in TBST-milk for 5 hours at room temperature. The membranes were washed as described above and antibody cross-reactivity was detected using ECF reagent

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(Amersham) on a STORM860 scanner (Molecular Dynamics). The anti-scTIF-IA polyclonal antibody was made to full-length recombinant scTIF-IA. The anit-ABC22.5 polyclonal antibody and the anti-ABC39 monoclonal antibody were made using purified *A. castellanii* ABC22.5 and AC39 subunits, respectively.

3.3.5 Specific Transcription Run-off Assay

The standard specific transcription assays for the *A. castellanii* system were carried out as previously described (Radebaugh et al., 1998), with the following exceptions. Typically 1 μl of 2X DNA affinity or 2 μl of 1X DNA affinity TIF-IB were used in the transcription reactions with 30 mU of pol I. Nucleic acids were precipitated by addition of 75 μl of 4.68 M ammonium acetate, 0.33 mg/ml linear polyacrylamide, and 450 μl of 95 % ethanol. Nucleic acids were then pelleted for 30 minutes at 14,000 RPM in an Eppendorf centrifuge. Pellets were washed with 500 μl of 70% ethanol, dried under vacuum, resuspended in 5 μl formamide dye, and electrophoresed as previously described (Radebaugh et al., 1998). Specific transcriptions assays for the *S. cerevisiae* system were conducted as previously described (Keener et al., 1998), and in Chapter 2.

3.3.6 Non-Specific Transcription Assays

The non-specific transcriptional activity of pol I was measured by a RNA polymerase assay as previously described (Spindler et al., 1978).

3.3.7 Factor binding to promoter bound SA-PMPs

The pJG310 plasmid was cleaved with *Nhe*I and *Pvu*II, and the 461 bp fragment containing the promoter element was purified as follows. The DNA cleavage reactions were electrophoresed through a 1.5% agarose gel, and the 461 bp fragment was excised and purified with a QIAEX II Gel Extraction Kit (Qiagen). The *Nhe*I cleavage left a 5'

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overhang that was labeled with biotin-dCTP in a fill-in reaction. A typical 50 μl fill-in reaction contained 0.1 mM dNTPs (A, T, and G), 5 Units of Klenow fragment exo- (Fermentas), 0.5 mM biotin-dCTP, and 580 ng of the 461 bp fragment in a 1X concentration of Klenow buffer (Fermentas). The reactions were incubated at 37°C for 30 minutes, followed by an incubation at 70°C for 10 minutes to heat kill the enzyme. Free NTPs and enzyme were removed by size exclusion chromatography in a Chroma Spin+TE-100 column (BD Biosciences) at 700 x g for 5 minutes. Biotin labeled promoter fragments were bound to a 40 μl suspension of Streptavadin MagneSphere® Paramagnetic Particles, SA-PMPs, (Promega) according to manufactures protocol. The bead-DNA mixture was then equilibrated with protein binding buffer, 30 mM HEPES pH 7.9, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40, 0.5 mg/ml insulin, 10% glycerol, 100 mM KCl, and either 2.5 ng/μl linearized (*Nru*I) or 3 ng/μl pBR322 non-specific competitor DNA. Typical binding reactions contained the equilibrated beads, 2-10 μl of 1X DNA affinity purified TIF-IB, and 100-800 mU heparin-Sepharose purified pol I in binding buffer in a 50-100 μl reaction volume. The binding reactions were incubated for 30 minutes at 25°C. The protein bound beads were then collected using a magnetic separator. Non-specifically bound or trapped proteins were removed by washing the beads one or two times with 50-100 μl of binding buffer. Bound proteins were then eluted with 50-100 μl binding buffer containing 1 M KCl or by suspension in 1X SDS-PAGE sample buffer. If necessary, proteins were chloroform-methanol precipitated as previously described (Wessel and Flugge, 1984). Precipitated proteins were suspended in 1X SDS-PAGE sample buffer. The protein samples were then analyzed by immunoblotting.

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To assay for the presence of factors bound to the promoter-beads following transcription, TIF-IB and pol I were bound to promoter-beads as above. Following the 30 minute incubation, the protein bound beads were collected as above and washed once. To PIC bound beads, 50-100 µl of binding buffer containing 0.5 mM NTPs was added and transcription was allowed to proceed for 30 minutes at 25°C. Released proteins were extracted using a magnetic separator, the beads were washed once more, and proteins remaining bound were eluted as above.

3.3.8 Alkaline phosphatase treatment of *A. castellanii* pol I fractions

Calf intestinal alkaline phosphatase covalently attached to agarose beads were purchased from Sigma. The alkaline phosphatase beads were washed twice with two volumes distilled H_2O and then twice with pol I dialysis buffer: 50 mM HEPES pH 7.8, 10 % glycerol, 0.1 mM EDTA, 0.2 % NP-40, 100 mM KCl, and 0.2 mg/ml BSA. Pol I (300-800 mU) was incubated with 5 U alkaline phosphatase at 30-37°C for the times indicated. The beads were collected by centrifugation at 14,000 x g for 2 minutes and the supernatants containing alkaline phosphatase treated pol I were removed. The fractions were then used in transcription, footprinting, promoter bead-binding, and immunoblotting assays.

3.4 RESULTS

3.4.1 Yeast scTIF-IA stimulates transcription in an *in vitro* reconstituted transcription assay containing purified *A. castellanii* factors.

TIF-IA is required for specific rDNA transcription from several species. Additionally, this factor possesses a high level of conservation, whereby the human

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protein is able to function in a yeast strain containing a lethal deletion of scTIF-IA, and TIF-IA purified from mouse and human cells is able to complement transcriptionally deficient extracts from the reciprocal species (Moorefield et al., 2000; Schnapp et al., 1993). Based on these results, we asked whether the yeast transcription factor scTIF-IA would have an effect on the *A. castellanii* transcription system. We incubated pol I fractions with either purified scTIF-IA or a control protein, BSA, for two hours at 4°C and 30°C, before using this pol I in a reconstituted *in vitro* transcription reaction. At each preincubation temperature, transcription from pol I that had been preincubated with the yeast transcription factor was greater than with the pol I that had been preincubated with the control protein (Figure 3.1, compare lane 1 with 2 and 3 with 4). Additionally, in both *A. castellanii* and *S. cerevisiae* reconstituted transcription systems, incubating pol I with increasing amounts of scTIF-IA resulted in a linear increase in the amount of specific transcript produced (Figure 3.2). The fact that yeast TIF-IA is able to stimulate transcription in a reconstituted *A. castellanii* system indicates that not only may there be a homologue to scTIF-IA in *A. castellanii*, but also that the conservation between the two species must be fairly high.

3.4.2 A putative acTIF-IA is associated with *A. castellanii* pol I.

Pol I fractions were analyzed in a western blot assay for the presence of TIF-IA by cross-reactivity to a polyclonal antibody produced against cloned scTIF-IA expressed in *E. coli*. A cross-reacting polypeptide paralleled transcription activity in the pol I fractions, with an apparent molecular weight of approximately 80,000, similar to the size reported for TIF-IA homologues in other species (Bodem et al., 2000; Moorefield et al., 2000; Schnapp et al., 1993; Yamamoto et al., 1996). Pol I fractions were analyzed for

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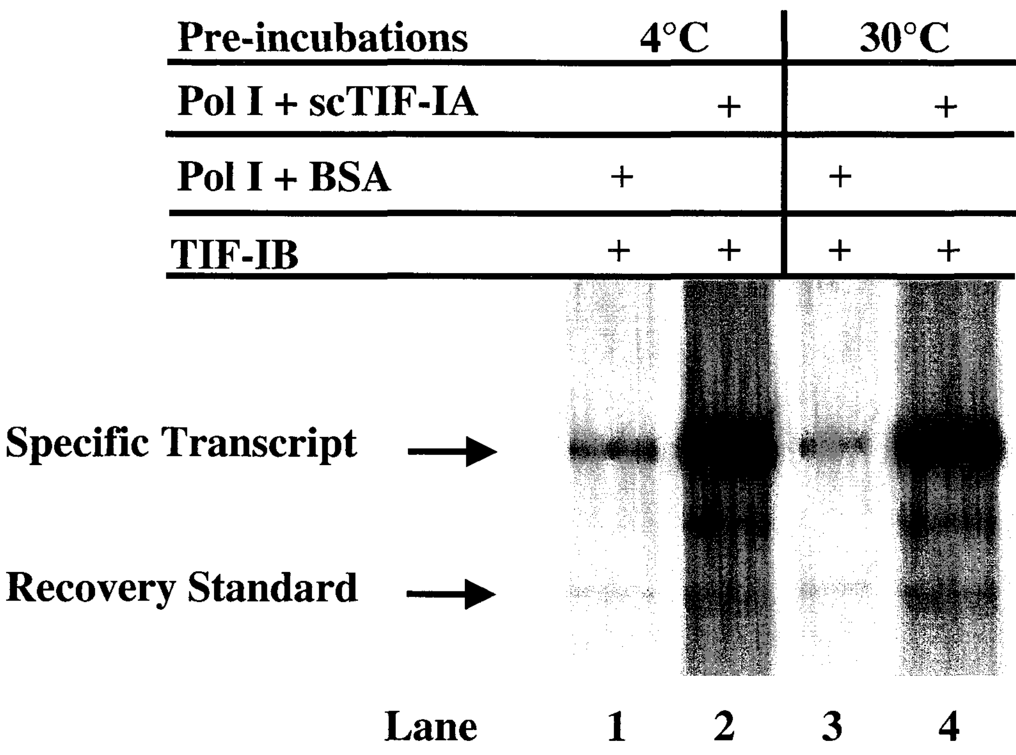

Figure 3.1. Yeast TIF-IA stimulates transcription in reconstituted *A. castellanii* transcription system. Pol I heparin-Sepharose fractions (80 mU) were preincubated with 0.175 μg of either MonoQ purified scTIF-IA or BSA for two hours at 4°C or 30°C. Pol I (30 mU) was then added to a transcription assay containing 1 μl of 2X DNA affinity TIF-IB. PhosphorImage file jg022001.

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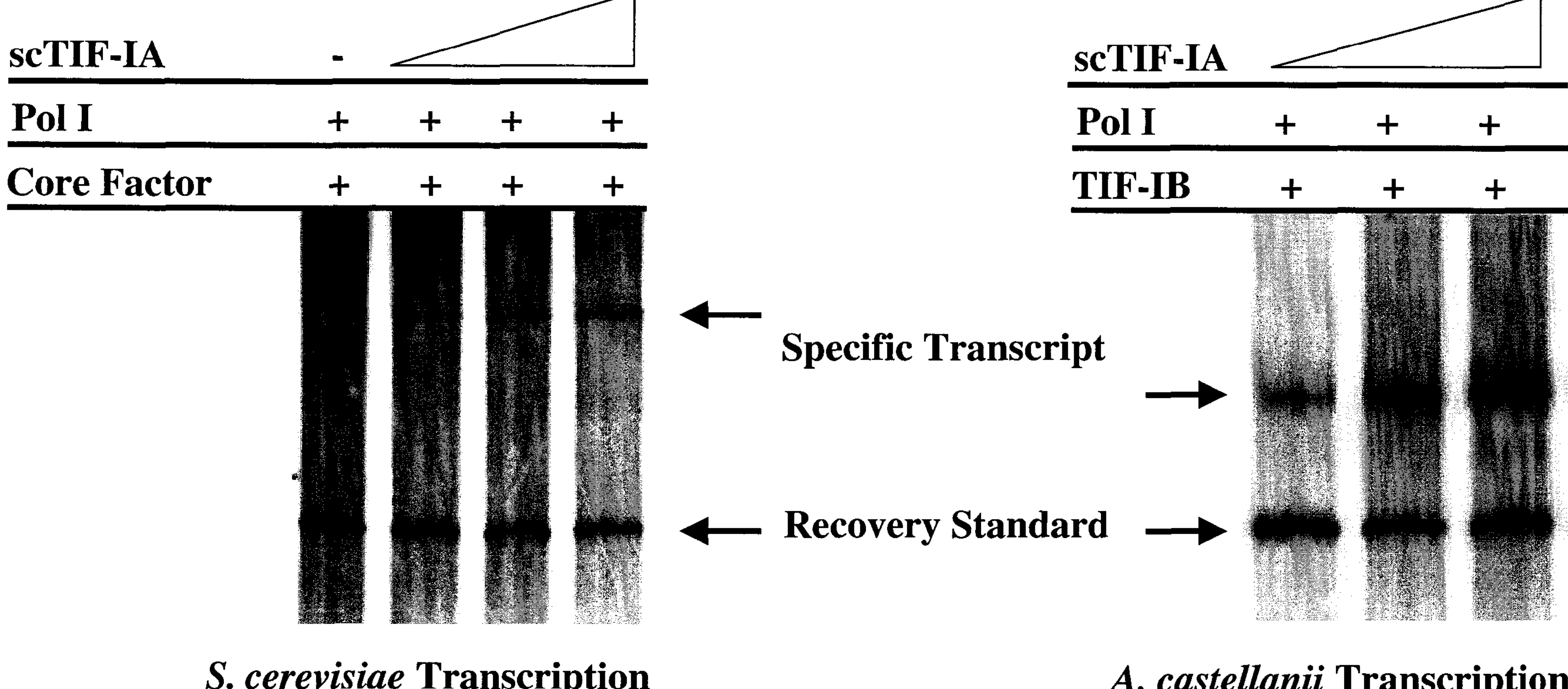

Figure 3.2. Increasing amounts of yeast TIF-IA correspond to increasing amounts of specific transcript. 15.6, 46.7, and 140 ng of yeast MonoQ purified scTIF-IA were preincubated with 8 μl *S. cerevisiae* heparin-Sepharose pool pol I and 80 mU *A. castellanii* heparin-Sepharose pol I for two hours at 30°C. The pol I-TIF-IA complexes were then used in their respective *in vitro* transcription assays containing 0.75 μl Q-Sepharose core factor or 1 μl of 2X DNA affinity TIF-IB. PhosphorImage files jg021801 and jg012401.

both specific and non-specific transcription activity, as well as for antibody cross-reactivity. Figure 3.3 shows the peak of TIF-IA protein corresponds with the peaks of both specific and non-specific transcription activities. These results are similar to those in yeast, where the presence of scTIF-IA corresponded to specific transcription. However, in yeast, the peaks of specific and nonspecific transcription activity are not aligned; scTIF-IA cross-reactivity parallels specific but not nonspecific transcription, presumably revealing the pol I-TIF-IA complex (Milkereit and Tschochner, 1998). Given that non-specific and specific transcriptional activities are coincidental during the purification of pol I from *A. castellanii*, it has proven impossible to separate the pol I-TIF-IA complex from free pol I in the *A. castellanii* system.

3.4.3 Pol I fractions purified from encysted *A. castellanii* cells contain minimal amounts of TIF-IA resulting in reduced promoter binding and transcription activities.

Pol I was purified from both trophozoite and cyst *A. castellanii* cells, and their activities were measured in transcription, footprinting and promoter-bead binding assays. When *A. castellanii* cells are starved of nutrients in an encystment medium, there is a cessation of cellular growth and division (Stevens and Pachler, 1973) that coincides with a down-regulation of rRNA transcription (Bateman and Paule, 1986). Pol I purified from encysted cells possesses the ability to transcribe DNA non-specifically in a manner equivalent to that of trophozoite pol I; however, cyst pol I is down regulated in both specific transcription and promoter binding as assayed by DNase I footprinting (Bateman and Paule, 1986). We asked whether cyst pol I could bind to bead bound promoter DNA under conditions similar to those used for promoter binding in a footprinting assay. When

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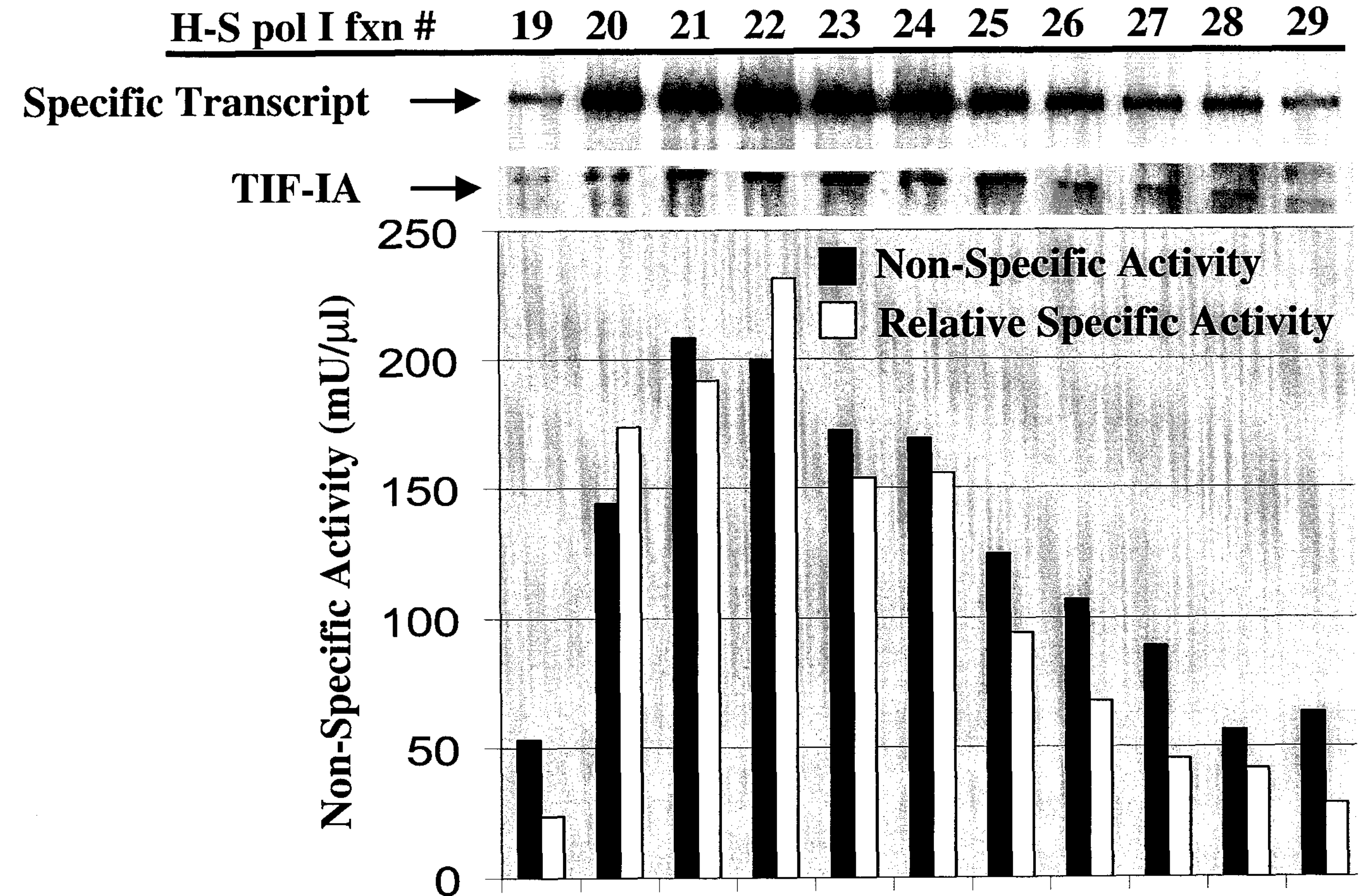

Figure 3.3. TIF-IA corresponds to the peaks of specific and non-specific transcriptional activities. Heparin-Sepharose pol I fractions were assayed for specific (0.15 μl) and non-specific (1.5 μl) transcription activity as well as cross-reactivity with the anit-scTIF-IA pAb in an immunoblot (3 μl). PhosphorImage files jg080502 and jg082102b.

trophozoite and cyst pol I are assayed in a promoter-bead binding assay with equal amounts of non-specific transcription activity, cyst pol I exhibits a reduced ability to bind to the promoter (Figure 3.4A; 80% of trophozoite binds; 48% of cyst binds). Additionally, when fractions containing equal amounts of cyst and trophozoite pol I, based on non-specific transcription activity and cross-reactivity to anti-ABC22.5 (Figure 3.4B), are assayed on a western blot for the presence of TIF-IA, the cyst polymerase possesses much less TIF-IA than the trophozoite enzyme (Figure 3.4B). The lack of pol I-TIF-IA complex is believed to be responsible for the reduced ability of pol I to bind to the promoter and transcribe the rDNA gene.

3.4.4 Alkaline phosphatase treatment reduces the specific transcription and promoter binding activities of pol I.

Specific phosphorylations are reported to be necessary for the formation of the specific transcription competent pol I-TIF-IA complex (Cavanaugh et al., 2002; Fath et al., 2001; Zhao et al., 2003). In *S. cerevisiae*, phosphorylation of pol I specific subunits is required for the assembly of the transcriptionally active pol I-TIF-IA complex, regardless of the phosphorylation state of TIF-IA (Fath et al., 2001). Alternatively, it has been shown in mammalian cells that the phosphorylation of two serine residues of TIF-IA (S649 and S633) is essential for rDNA transcription to occur (Zhao et al., 2003). We analyzed how phosphatase treatment of *A. castellanii* pol I affected the ability of the enzyme, or more specifically the pol I-TIF-IA complex, to participate in specific transcription and rRNA promoter binding. First, pol I fractions were incubated with increasing amounts of agarose bead-bound calf intestinal alkaline phosphatase. As a fixed amount of pol I was incubated with increasing amounts of alkaline phosphatase, the level

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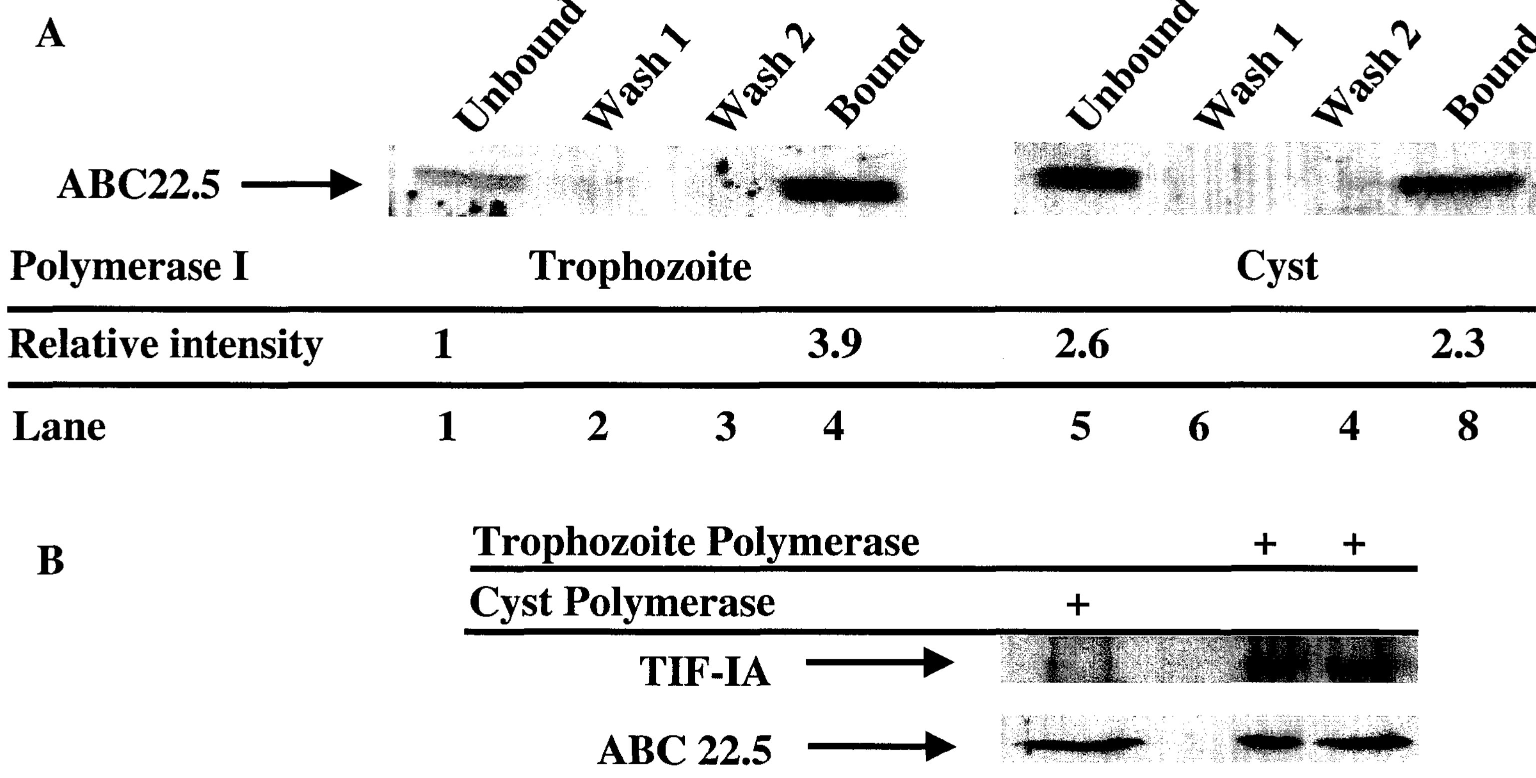

Figure 3.4. Cyst pol I is deficient for promoter binding and acTIF-IA association. A) Equal amounts of trophozoite and cyst pol I (150 mU) were added with 6 μl 1X DNA affinity TIF-IB to bead binding reactions. Samples were subjected to immunoblotting and the presence of pol I was detected by cross-reactivity to the anti-ABC22.5 pAb. PhosphorImage file jg091403. **B)** Equal amounts of trophozoite and cyst pol I (710 mU) were subjected to immunoblotting. The presence of TIF-IA was detected by cross-reactivity to an anti-scTIF-IA pAb, while pol I was detected by cross-reactivity with an anti-ABC22.5 pAb. PhosphorImage files jg110702 and jg111102.

of specific transcription decreased in a dose-dependent fashion (Figure 3.5, white bars). When the same pol I fractions were assayed for their ability to transcribe DNA non-specifically from calf thymus DNA, polymerase activity was not affected as severely (Figure 3.5, black bars). Although there is a slight decrease initially in non-specific activity, increased units of alkaline phosphatase do not cause additional reduction in activity as with specific transcription. Additionally, the amount of pol I recovered from the phosphatase beads was measured in a western blot by cross-reactivity with a monoclonal antibody specific to the AC39 pol I subunit. It is clear from the western blot that the reduction in non-specific activity is due to a slight loss of total pol I protein, while the loss in specific activity is significantly greater, demonstrating a targeted effect on promoter-dependent transcription (Figure 3.5).

The effect of phosphatase treatment of pol I on its ability to bind to the promoter was evaluated by two methods: DNase I footprinting and promoter bead-binding assays. For the footprinting assay, pol I was treated with or without alkaline phosphatase for 30 minutes at 30°C, then assayed for non-specific transcriptional activity. Equal amounts of pol I activity were then assayed for promoter binding by DNase I footprinting. Pol I that had not been treated with phosphatase provided the additional protection downstream of the TIF-IB footprint characteristic of pol I (Bateman et al., 1985) (Figure 3.6, lane 5), whereas the phosphatase treated pol I fraction failed to provide the additional protection (Figure 3.6, lane 6). Alkaline phosphatase treated pol I fractions were then used in a promoter bead binding assay to assess their DNA binding activity. Pol I was incubated at 30°C with or without alkaline phosphatase for the times shown, equalized for non-specific transcription activity and added to the binding reaction. Incubation of pol I with

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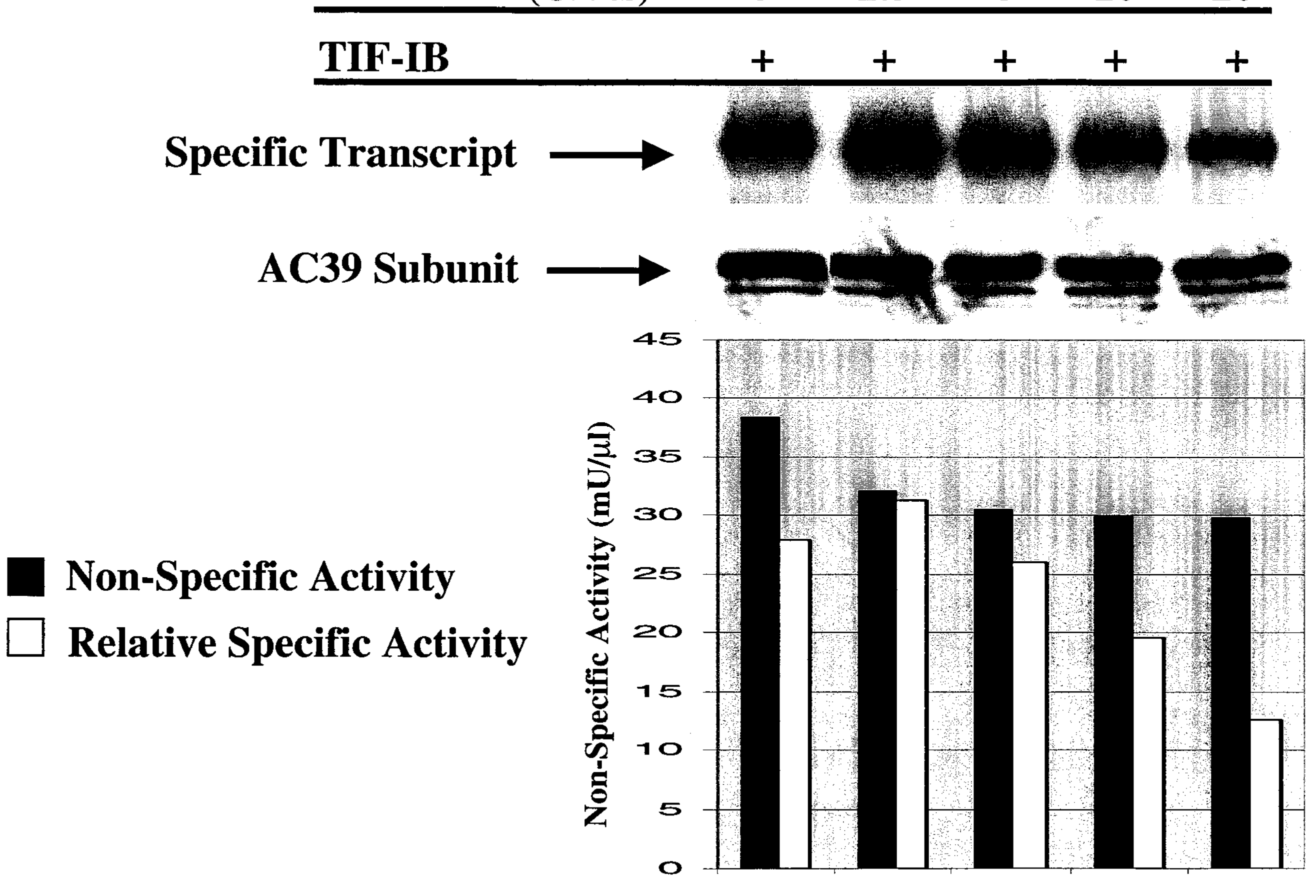

Figure 3.5. Alkaline phosphatase treatment of pol I causes a decrease in specific transcription. Heparin-Sepharose pol I (1500 mU) was incubated with indicated amounts of alkaline phosphatase then used in specific (34 mU), non-specific (408 mU) and an immunoblot (612 mU) assays. Pol I recovery from the agarose beads was measured by cross-reactivity to the anti-AC39 mAb. PhosphorImage files jg031402 and jg031402b.

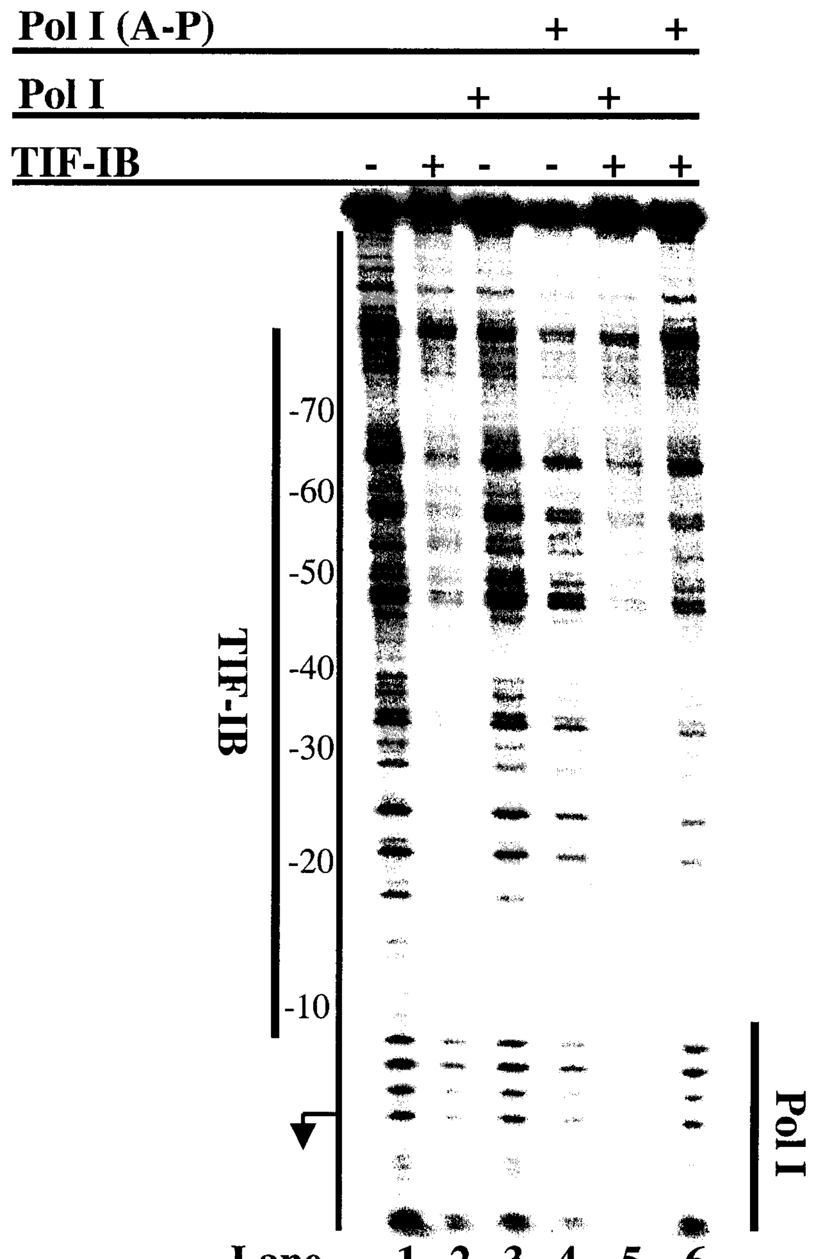

Figure 3.6. Alkaline phosphatase treatment eliminates the ablility of pol I to bind to the promoter. Heparin-Sepharose pol I (630 mU) was incubated with or without 5 U of alkaline phosphatase for 30 min at 30°C and subsequently added (52.5 mU) to a DNase I footprinting reaction with 0.5 μl 2X DNA affinity TIF-IB and 1 μl of TIF-IE. PhosphorImage file jg050502a.

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alkaline phosphatase inhibited the ability of pol I to bind to the promoter beads in a time-dependent manner (Figure 3.7). To show that TIF-IB (TBP) binding was not affected by the ability of pol I to bind the promoter, fractions from the bead binding reaction were immunoblotted and probed with anti-TBP polyclonal antibodies. As can be seen in Figure 3.7, the decrease in pol I binding to the beads is not due to a decrease in TIF-IB binding. Together, these results suggest that phosphatase treatment inhibits specific transcription, by possibly initiating a disruption of the pol I-TIF-IA complex. This reduces the ability of pol I to be specifically recruited to the promoter element. These characteristics of the phosphatase treated enzyme are similar to the pol I isolated from encysted cells and clearly imply a role for phosphorylation/dephosphorylation in the regulation of rRNA transcription in *A. castellanii.*

3.4.5 Alkaline phosphatase treatment of the pol I-TIF-IA complex causes an electrophoretic mobility shift of TIF-IA.

Mammalian TIF-IA has been shown to possess different electrophoretic mobilities based on its state of phosphorylation (Zhao et al., 2003). To examine the effects of alkaline phosphatase treatment on acTIF-IA, we assayed phosphatase treated pol I fractions in an immunoblot. Pol I fractions from the heparin-Sepharose column were incubated with or without alkaline phosphatase for 30 minutes at 37°C. Upon alkaline phosphatase treatment, the fractions were then subjected to SDS-PAGE and immunoblotting using anti-scTIF-IA polyclonal antibody. Figure 3.8 shows that when treated with alkaline phosphatase, the electrophoretic mobility of the TIF-IA found in complex with pol I is altered, resulting in a faster migrating band. The alteration in

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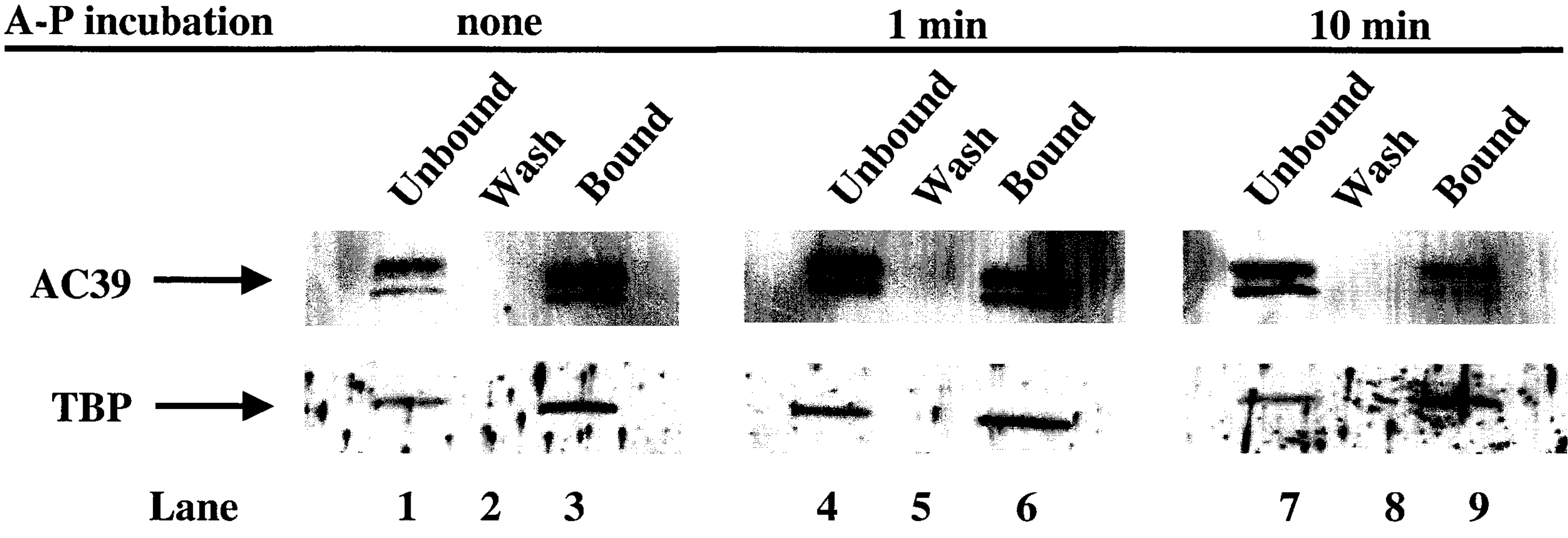

Figure 3.7. Alkaline Phosphatase inhibits the ability of pol I to bind to the promoter. Heparin-Sepharose pol I (610 mU) was incubated without alkaline phosphatase for 10 min, or with 5 U of alkaline phosphatase for 1 and 10 minutes at 30°C. Pol I fractions (180 original mU) were then added with 12 μl 1X DNA affinity TIF-IB to promoter beads in a bead-binding reaction. Samples were subjected to immunoblotting and probed for the presence of pol I (AC39) or TIF-IB (TBP) with anti-AC39 mAb, and anti-TBP mAb, respectively. PhosphorImage files jg071203b, jg071203c, and jg071503c.

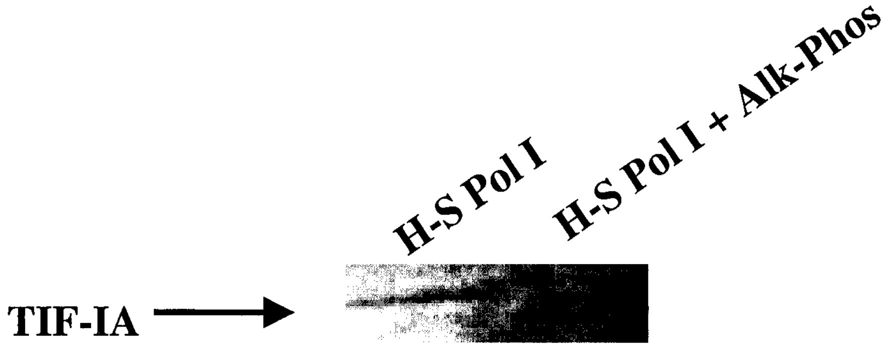

Figure 3.8. Alkaline-phosphatase treatment of the pol I-TIF-IA complex leads to a shift in the molecular weight of TIF-IA. Heparin-Sepharose pol I (858 mU) was incubated with and without alkaline phosphatase at 37°C for 30 min, then 650 mU were subjected to western blotting and TIF-IA was detected by cross-reactivity with an anti-scTIF-IA pAb. PhosphorImage file jg021302.

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migration due to the dephosphorylation of TIF-IA is believed to be responsible for the loss of specific transcription ability as well as the loss of promoter binding.

3.4.6 TIF-IA is required for pol I promoter binding.

Whether pol I recruitment to the promoter requires TIF-IA is controversial (Aprikian et al., 2001; Miller et al., 2001; Schnapp et al., 1993). In yeast, both free pol I and the pol I-TIF-IA complex are recruited to the promoter. This results in the formation of preinitiation complexes (PICs) that are transcriptionally inactive or active, respectively (Aprikian et al., 2001). Upon the addition of nucleotide triphosphates, PICs containing transcriptionally active pol I are able to initiate transcription resulting in the release of pol I and scTIF-IA from bead-bound template DNA. Pol I from inactive PICs remains bound. In contrast, past studies from our laboratory have shown that nearly all of the pol I stably bound to the *A. castellnaii* rRNA promoter is competent for promoter clearance and specific transcription. DNase I and MPE footprinting assays have shown that the stable pol I footprint is translocated downstream of the start site upon the addition of nucleotide triphosphates (Bateman and Paule, 1988; Bateman and Paule, 1986). Additionally potassium permanganate, MPE·Fe(II), and DEPC footprinting show the presence of a pol I footprint as the pol I is walked down the promoter by the specific addition of required nucleotides (Kahl et al., 2000). This suggests all components required for initiation are associated with pol I. We tested whether this actively recruited *A. castellanii* pol I is associated with acTIF-IA. Pol I was incubated with TIF-IB-bound promoter DNA immobilized on paramagnetic beads, in the presence of excess nonspecific competitor DNA. Protein not bound to the promoter DNA was washed from the beads and the template bound PICs eluted with SDS. The samples were then subjected to

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immunoblotting and probed for the presence of pol I and TIF-IA. TIF-IA was present in the bound sample (Figure 3.9, lane 5). The fraction of pol I found not to be recruited to the promoter consisted of pol I not associated with TIF-IA. Pol I bound to immobilized DNA was shown to be initiation competent and was released upon addition of nucleotide triphosphates (Figure 3.10). These results show that in contrast to yeast pol I transcription, all the pol I bound to the *A. castellanii* promoter is associated with TIF-IA and competent for specific transcription initiation.

3.5 DISCUSSION

The transcription of the ribosomal genes *in vitro* is species specific (Grummt et al., 1982); however, not all of the pol I transcription factors exhibit this strict species specificity. The fundamental transcription factor, TIF-IB/SL1, has been shown to confer species specificity to the pol I transcriptional apparatus (Eberhard and Grummt, 1996; Rudloff et al., 1994). TIF-IA, on the other hand, is promiscuous. The human TIF-IA gene can functionally substitute for the yeast gene *in vivo* (Moorefield et al., 2000). Additionally, purified TIF-IA from both the mouse and human systems can function in transcription in the reciprocal species (Schnapp et al., 1993). We have taken advantage of the functional conservation of the TIF-IA factor to assay for TIF-IA related activity in *A. castellanii*. We report here that purified yeast TIF-IA is capable of stimulating transcription in a reconstituted *in vitro* transcription assay from *A. castellanii* (Figure 3.1). Furthermore, antibodies to the yeast protein cross-react with a 80 kD protein found tightly associated with *A. castellanii* pol I active in both specific transcription (Figure 3.3) and promoter binding (Figure 3.9).

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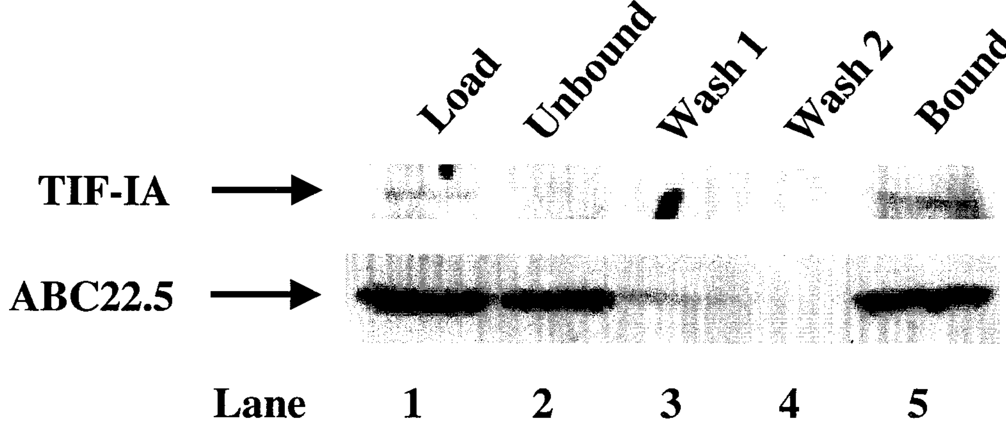

Figure 3.9. TIF-IA is required for pol I recruitment to the rRNA promoter. Heparin-Sepharose pol I (680 mU) and 10 μl 1X DNA affinity TIF-IB were added to promoter bound beads in a bead binding reaction. Samples were subjected to immunoblotting and probed for the presence of pol I (ABC22.5) and TIF-IA with anti-ABC22.5 and anti-scTIF-IA polyclonal antibodies. PhosphorImage files jg101703a and jg101803c.

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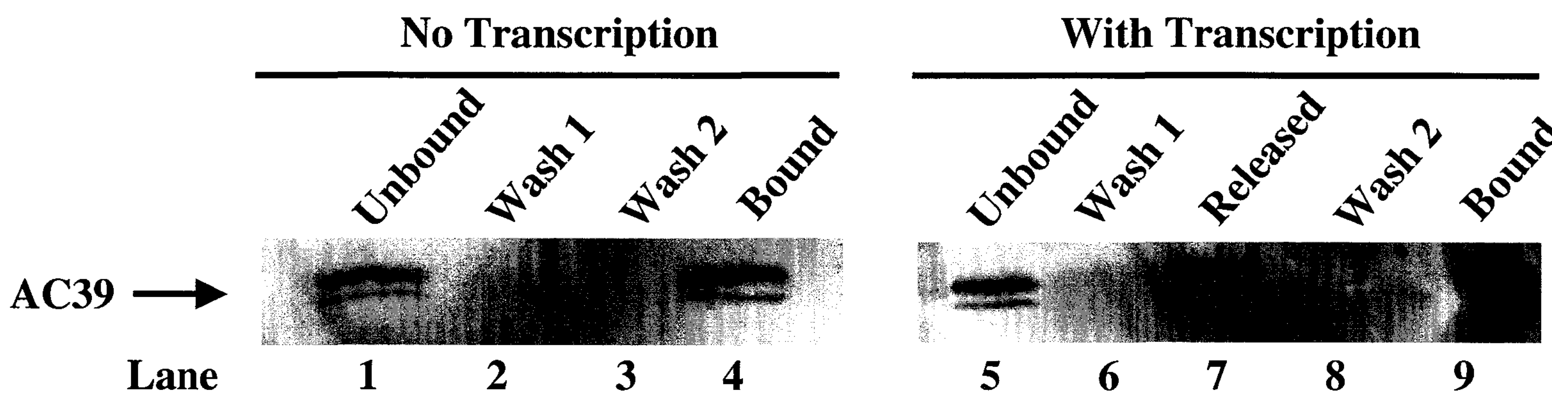

Figure 3.10. All the pol I bound to the promoter beads is competent for specific transcription. Heparin-Sepharose pol I (210 mU) and 6 μl 1X DNA affinity TIF-IB were added to promoter bound beads in two separate bead binding reactions. After a 30 minute incubation the reactions were washed and eluted as per protocol, the second reaction was washed then incubated in binding buffer containing NTPs. Transcription was allowed to occur for 30 min at 25°C. The released proteins were removed and the beads were washed and eluted as per protocol. Samples were subjected to immunoblotting and probed for the presence of pol I (AC39) with an anti-AC39 mAb. PhosphorImage files jg061303b and jg061303c.

Ribosomal RNA transcription is regulated by numerous mechanisms. In the mammalian cell cycle, rRNA synthesis is shut down during mitosis by modification of both the fundamental transcription factor, SL1/TIF-IB, and the activating factor, UBF (for review see Grummt, 2003). However, many studies have shown that the activity of the fundamental transcription factor is not the target of modification in growth related rRNA transcriptional repression (Buttgereit et al., 1985; Milkereit and Tschochner, 1998; Paule et al., 1984). Depending on the particular system, the target for this regulatory mechanism has been shown to be either pol I or TIF-IA (Bateman and Paule, 1986; Cavanaugh et al., 2002; Tower and Sollner-Webb, 1987; Yuan et al., 2002). As a cell encounters unfavorable growth conditions, either pol I itself or its essential transcription factor, TIF-IA, must be modified. We find that phosphatase treatment of the pol I-TIF-IA complex results in the loss of transcriptional initiation and DNA binding activity (Figure 3.5, Figure 3.6, and Figure 3.7). This loss of activity corresponds to an altered electrophoretic mobility of TIF-IA, indicating that a dephosphorylation of TIF-IA correlates with the disruption of the pol I-TIF-IA complex (Figure 3.8). An alteration of the electrophoretic mobility of TIF-IA has similarly been shown to correspond to a decrease in transcriptional activity in the mouse system (Zhao et al., 2003). Additionally, cross-reactivity with an anti-scTIF-IA polyclonal antibody has been observed in purification fractions not associated with pol I, possibly identifying a large pool of free TIF-IA (data not shown). This free TIF-IA also possesses the faster electrophoretic mobility characteristic of dephosphorylated TIF-IA, suggesting the modification is necessary for association with pol I. These results do not rule out the possibility that *A.*

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castellanii pol I could also be a target of a growth-dependent regulatory modification, as in yeast (Fath et al., 2001).

In *A. castellanii* the switch from exponential growth to encystment correlates with the inactivation of rRNA transcription. Pol I isolated from cyst cells transcribes DNA non-specifically as efficiently as pol I isolated from trophozoite cells, but exhibits reduced specific transcriptional activity as well as reduced promoter binding (Bateman and Paule, 1986; Paule et al., 1984) (Figure 3.4A). Cyst pol I is shown here to contain less TIF-IA than trophozoite pol I (Figure 3.4B). Additionally, trophozoite pol I treated with alkaline phosphatase has reduced specific transcription and promoter binding ability (Figure 3.5, Figure 3.6, and Figure 3.7), similar to cyst pol I. We conclude that as *A. castellanii* cells undergo encystment, dephosphorylation of TIF-IA or pol I is probably responsible for the decrease in transcriptional activity.

The central role of TIF-IA phosphorylation in *A. castellanii* is open to some doubt because, scTIF-IA expressed in *E. coli* stimulates *A. castellanii* pol I even though it is not phosphorylated. Reports examining the ability of recombinant TIF-IA to function in pol I subunit binding, pol I recruitment, and/or transcriptional initiation have been contradictory. Cavanaugh et al., (2002), reported the loss of interaction between human TIF-IA and mouse A43 when recombinant hsTIF-IA was purified from *E. coli.* However, hsTIF-IA expressed in insect, Sf9, cells does bind to the mouse A43 subunit. This suggests a specific post-translational modification must occur in order to facilitate the association between pol I and hsTIF-IA. Contradictory to this data, Yuan et al., (2002), has shown human TIF-IA expressed in *E. coli* binds to GST-tagged rpa43, even though they later reported specific phosphorylation of S633 and S649 is necessary for

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transcription (Zhao et al., 2003). Moreover, recombinant yeast TIF-IA expressed in *E. coli* is capable of associating with yeast pol I, via its A43 subunit, to yield transcriptionally active complex, (Keener et al., 1998; Fath et al., 2001; Peyroche et al., 2000), similar to the *A. castellanii* results. In yeast, it is the polymerase that must be phosphorylated for association with TIF-IA (Fath et al., 2001). It is possible that the strict requirement for TIF-IA phosphorylation could have diverged through phylogeny. Alternatively, these discrepancies may arise because of contaminating kinases or phosphatases in the polymerase or factor preparations used by the various laboratories, or to differences in conditions used in interaction assays. Additionally, the yeast protein may have evolved in a manner such that a negatively charged residue/epitope is present, resulting in a relaxed requirement for a specific phosphorylation event for binding to pol I. This may explain why a change in electrophoretic mobility of the *A. castellanii* TIF-IA homologue, associated with dephosphorylation of the pol I-TIF-IA complex, corresponded to a loss in transcriptional activity. This despite the fact that bacterially expressed yeast TIF-IA functions in the *A. castellanii* system without specific phosphorylation.

There also are differences between laboratories concerning the TIF-IA requirement for pol I recruitment. In both yeast and mouse, pol I can be recruited to the promoter without TIF-IA (Aprikian et al., 2001; Schnapp et al., 1993). In contrast, human pol I does not bind to the PIC without hsTIF-IA (Miller et al., 2001). *A. castellanii* pol I bound to the promoter is competent for specific transcription and, therefore, in parallel with other species, must be associated with TIF-IA (Bateman and Paule, 1988; Bateman and Paule, 1986; Kahl et al., 2000). By direct measurement, we show here that the

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transcriptionally competent pol I (Figure 3.10) that binds to the rRNA promoter, is associated with TIF-IA (Figure 3.9). TIF-IA is not found associated with the pol I fraction that is incapable of promoter binding.

Regardless of what step in transcriptional initiation TIF-IA is required, a functional pol I-TIF-IA complex is necessary for specific transcription. Numerous studies have shown that the active complex is used stoichiometrically in crude systems (Brun et al., 1994; Hirschler-Laszkiewicz et al., 2003; Milkereit and Tschochner, 1998), suggesting a modification of one of its components by contaminants in the systems, presumably by a phosphatase. In highly purified systems, multiple rounds of transcription are more the norm, in the absence of the phosphatase (Schnapp et al., 1993; C. Terpening, unpublished). In vivo, following each round of transcription, TIF-IA and/or pol I needs to be re-phosphorylated to allow replenishment of the active complex. In rapidly growing cells, the rate of rRNA transcription is so high that conversion of the pool of active complex into de-phosphorylated components can occur extremely rapidly. In growing yeast for example, complexes are deactivated at a rate of 2000 per minute (Warner, 1999); the entire complement of 50,000 pol I molecules could be inactivated in 25 minutes, or if the steady state estimate of only 2-10% are in active form (Milkereit and Tschochner, 1998), only 2.5 minutes would be needed to shut down rRNA transcription by this mechanism. Clearly, discerning the target(s) and enzymes that mediate these interconversions is important to fully understand the mechanisms responsible for rRNA transcriptional regulation.

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CHAPTER 4

MULTIPLE PROTEIN-PROTEIN INTERACTIONS MEDIATE RNA POLYMERASE I RECRUITMENT

This chapter describes the identification of protein-protein interactions between the yeast pol I transcription factors involved in pol I recruitment. This work was completed by Dr. Cathy Radebaugh, Brice McConnell, and me. I performed the transcription reactions shown in Figure 4.4. These reactions were conducted with H_6-Rrn7p and control extract prepared by Dr. Radebaugh, using basal yeast transcription factors I had prepared. This work will be submitted to the *Proceedings of the National Academy of Sciences* for publication.

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MULTIPLE PROTEIN-PROTEIN INTERACTIONS MEDIATE RNA POLYMERASE I RECRUITMENT

4.1 ABSTRACT

The recruitment of RNA polymerase I (pol I) to the rRNA promoter is a critical step in the regulation of rRNA transcription. In the yeast *Saccharomyces cerevisiae*, pol I is thought to be recruited to the promoter via a protein-protein interaction between Rrn3p and the Rrn6p subunit of CF. However, pol I not associated with Rrn3p is specifically recruited to bead-bound DNA templates and therefore, an uncharacterized interaction(s) between pol I and CF must be occurring. We utilized the yeast two-hybrid and GST pull-down assays to identify additional interactions between CF and pol I that had not been previously seen. We found that the Rrn7p subunit of CF interacts with the A190 subunit of pol I and with Rrn3p. The former interaction could be responsible for the recruitment of pol I in the absence of Rrn3p while the latter interaction is homologous to the TAF_I68/TIF-IA interaction identified in mammalian cells.

4.2 INTRODUCTION

Actively growing cells require high levels of protein synthesis and therefore high levels of ribosomes and ribosome biosynthesis. In eukaryotic cells, the rRNAs are transcribed by RNA polymerase I (pol I) and their transcription can constitute up to 60% of total cellular transcription (Warner, 1999). The recruitment of pol I to the rRNA genes (rDNA) and the regulation of pol I transcription are critical steps in the regulation of

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normal cellular growth. Understanding how rRNA transcription is regulated requires a through knowledge of the proteins involved and their interactions with each other.

In the yeast *Saccharomyces cerevisiae*, rRNA transcription requires pol I and at least four general transcription factors: Upstream Activation Factor (UAF), TATA binding protein (TBP), Core Factor (CF) and Rrn3p (reviewed in Nomura, 2001). The binding of UAF to the upstream promoter element (UPE, -146 to about –51) is the first step in the initiation of rRNA transcription. UAF is a multi-protein complex comprised of Rrn5p, Rrn9p, Rrn10p, UAF30p and the histones H3 and H4 (Keener et al., 1997; Keys et al., 1996). While it is not required for basal levels of transcription *in vitro*, UAF is required along with the UPE for activated levels of transcription. When bound to the UPE, UAF forms a stable complex that commits the bound rDNA template to transcription and also suppresses rRNA transcription by RNA polymerase II (Keys et al., 1996; Oakes et al., 1999; Vu et al., 1999).

After it has bound the rRNA promoter, UAF recruits TBP to the preinitiation complex through a protein-protein interaction between Rrn9p and TBP (Steffan et al., 1996). In contrast to most other eukaryotes, in yeast TBP is not found tightly associated with any of the other pol I specific transcription factors. TBP is required for rRNA transcription *in vivo* (Cormack and Struhl, 1992; Schultz et al., 1992) and for activated, but not basal levels of transcription *in vitro* (Keener et al., 1998). Together, the template bound UAF and TBP recruit CF and pol I to the preinitiation complex (Aprikian et al., 2001).

CF is the fundamental pol I transcription factor in yeast and is thought to bind the core promoter element (-38 to +5), although its binding is not stable (Aprikian et al.,

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2001; Keys et al., 1996). CF is a multi-protein complex composed of three subunits: Rrn6p, Rrn7p and Rrn11p (Keys et al., 1994; Lalo et al., 1996; Lin et al., 1996). Recently, the Rrn7p and Rrn11p subunits of CF have been shown to share sequence similarity with the TAF_I68 and TAF_I48 subunits of the mammalian homologue to CF, TIF-IB/SL1 (Boukhgalter et al., 2002). The recruitment of CF to the rDNA template is mediated by protein-protein interactions between the Rrn7p and Rrn6p subunits of CF, and the Rrn9p subunit of UAF, and TBP, respectively (Lin et al., 2002; Steffan et al., 1996). However, using bead-bound templates, Aprikian and co-workers found that CF was not stably recruited to the template in the absence of pol I (Aprikian et al., 2001). Thus, although UAF and TBP are capable of recruiting CF to the rDNA template, the stable association of CF with the template requires pol I.

RNA polymerase I from *S. cerevisiae* has been extensively characterized and shown to be comprised of 14 polypeptides (reviewed in Carles et al., 1998). Seven of the pol I subunits, A190, A135, A49, A43, A34.5, A14 and A12 are specific for the pol I enzyme while the other seven are shared with RNA polymerase II and or III. Rrn3p is a single subunit transcription factor that associates with pol I and is required for the specific initiation of rRNA transcription by pol I (Keener et al., 1998; Yamamoto et al., 1996). Proteins with homologous functions and sequences to yeast Rrn3p have been identified in both human (hRRN3) and mouse (TIF-IA) cells (Bodem et al., 2000; Moorefield et al., 2000; Schnapp et al., 1993). In extracts prepared from yeast and mammalian cells, only a small fraction of the total Rrn3p and pol I are found together in a stable complex (Milkereit and Tschochner, 1998; Miller et al., 2001; Schnapp et al., 1990; Tower and Sollner-Webb, 1987). Yeast Rrn3p associates with pol I via the A43

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subunit and is thought to bridge the interaction of pol I with CF by also interacting with Rrn6p (Peyroche et al., 2000).

The association/dissociation of Rrn3p and pol I is thought to be a regulatory target for pol I transcription in yeast as well as mammalian cells. Rrn3p/TIF-IA has been shown to dissociate from pol I during a single round of transcription and is incapable of reassociating, possibly due to a transcription-dependent post-translational modification of the factor and /or pol I (Hirschler-Laszkiewicz et al., 2003; Milkereit and Tschochner, 1998). Extracts from yeast grown to stationary phase are inactive for pol I transcription and have reduced levels of Rrn3p-pol I complexes while the total cellular levels of Rrn3p and pol I remain essentially unchanged. It has also been shown that yeast pol I has to be phosphorylated to associate with Rrn3p and that, although free Rrn3p is phosphorylated, it does not need to be to form a functional complex with pol I (Fath et al., 2001). Pol I from yeast is phosphorylated on the A190, A43, A34.5, A23 and A19 subunits (Breant et al., 1983; Buhler et al., 1976). However, it currently is not clear how the phosphorylation state of any of these subunits pre- or post- transcriptionally affects the association of Rrn3p with pol I.

To completely elucidate the mechanisms by which pol I is recruited to the rRNA promoter and its transcription regulated, all of the potential interactions between pol I and its transcription factors must be identified. The interactions between Rrn3p and Rrn6p, and Rrn3p and the A43 subunit of pol I are the only interactions between yeast transcription factors and pol I that have currently been identified. This contrasts with mammalian systems where TIF-IA/hRRN3 has been shown to interact with two subunits of TIF-IB/SL1 (TAF_I68 and TAF_I95) as well as Polymerase Associated Factor (PAF) 67

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and the A43 subunit of pol I (reviewed in Grummt, 2003). In addition, in the mouse system Upstream Binding Factor (UBF) was shown to interact with the 180, 114 and 44 kDa pol I subunits and to PAF53 (Hanada et al., 1996).

To determine whether additional interactions are also occurring in yeast, we have completed a yeast two-hybrid analysis of each of the CF subunits with Rrn3p and the subunits specific to pol I. We found that the Rrn7p subunit of CF interacts with the A190 subunit of pol I. This interaction and an interaction between Rrn7p and Rrn3p were also evident in a glutathione S-transferase (GST) pull-down assay. To further demonstrate the importance of Rrn7p in pol I transcription, we used bacterially expressed Rrn7p to inhibit basal transcription in an in vitro assay.

4.3 MATERIALS AND METHODS

4.3.1 Strains and plasmids

The strains and select plasmids used in this study are listed in Table 1. All of the plasmids except pNOY3263 and pBS/35Sp were prepared using the GATEWAY Cloning System. Genomic DNA was isolated from BJ926 cells according to the method in (Davis et al., 1980). The *RRN6*, *RRN7*, *RRN11*, *RPA190*, *RPA135*, *RPA49*, *RPA43*, *RPA34*, *RPA14* and *RPA12* genes were amplified from genomic DNA by PCR using Elongase Polymerase Mix and the manufacturer's (Invitrogen Life Technologies) suggested protocol. Primer pairs used in the PCR amplification were designed to anneal to the 5' and 3'-ends of the gene and to incorporate an *Eco*RI, *Xba*I or *Xho*I site at the 3'-end. Entry clones for each gene were prepared by ligating the PCR products to pENTR1A plasmid DNA between the *Dra*I and *Eco*R1, *Xba*I or *Xho*I sites. The entry clones were

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Yeast strains used in this study

Strain	Description
MaV203	*Matα*, *leu*2-3, 112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::*lacZ*, *HIS*3$_{UAS\ GAL1}$::*HIS*3@*LYS*2, *can*1^R, *cyh*2^R
BJ926	*Matα/Matα*, *prb*1-1122/*prb*1-1122, *prc*1-126/*prc*1-126, *pep*4-3/*pep*4-3 *can*1/*can*1, *gal*2/*gal*2, *his*1/+, +/*trp1*
NOY797	*Matα*, *ade*2-1, *ura*3-1, *leu*2-3, 112, *trp*1-1, *his*3-11, *can*1-100 *rrn*7::*LEU2*, pNOY403[*TRP1*, *RRN*7-(HA1)$_3$-(His)$_6$]

Plasmids used in this study

Yeast plasmids	Description	Vector
pCR2	*CEN, TRP*1, *GAL4*[768-881]-*RPA1*90	pDEST22
pCR38	*CEN, TRP*1, *GAL4*[768-881]-*RRN6*	pDEST22
pCR46	*CEN, TRP*1, *GAL4*[768-881]-*RPA43*	pDEST22
pCR52	*CEN, LEU*2, *GAL4*[1-147]-*RRN7*	pDEST32

E. coli plasmids	Description	Vector
pNOY3263	A derivative of pET15b carrying the *RRN3* gene with a -(HA1)$_3$-(His)$_6$ tag	pET15b
pCR68	*GST-RPA190* under control of the T7 promoter	pDEST3
pCR69	*GST-RRN7* under control of the T7 promoter	pDEST3
pCR71	(His)$_6$-*RRN7* under control of the T7 promoter	pDEST2
pBS/35Sp	A derivative of pBS that contains rDNA from -210 to +535 cloned into the SmaI and HindIII sites	pBS(-)

Table 1. Yeast strains and plasmids used in study.

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recombined with pDEST32, pDEST22, pDEST3 or pDEST2 using LR Clonase Enzyme mix. The pBS/35Sp plasmid contains a 745 bp *Sma*I-*Hind*III fragment from rDNA (-210 to +535) cloned into the *Sma*I and *Hind*III sites of pBS- (Stratagene).

4.3.2 Yeast Two-Hybrid Assays

The yeast strain MaV203 was used in all of the two-hybrid assays. Plasmids containing CF and pol I genes fused to the *GAL4* DNA Binding (DB) and Activation domains (AD) were introduced in pairs into MaV203 cells using a standard lithium acetate transformation. The pDEST22 plasmid was included with each DB-fusion construct as a control for artificial recruitment. The ability of each transformation pair to activate transcription was assessed by streaking two colonies from each transformation onto glucose synthetic media lacking leu, trp and his, and containing 20-30 mM 3-aminotriazole (AT). Cells were grown at 30°C for 3-7 days. Plasmid pairs that were capable of supporting growth on AT were used to retransform MaV203 cells. Cells from two colonies of each transformation were grown to log phase in glucose synthetic media lacking leu and trp, and ten-fold serial dilutions prepared. 8 μl aliquots of each dilution were spotted onto selective media containing 20-30 mM AT. The cells were grown at 30°C for 7 days.

4.3.3 Protein Expression of Yeast Two-Hybrid Constructs

Protein extracts were prepared from cells containing the indicated DB or AD fusion construct as described in (Cormack and Struhl, 1992). Approximately 50 μg of protein was electrophoresed through SDS-10% or 7.5% polyacrylamide gels by standard methods. Protein was transferred from the gel to PVDF membrane (Pall Life Science) using a Genie electrophoretic blotter as specified by the manufacturer (Idea Scientific).

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The membranes were blocked in TBS with 0.05% Tween 20 and 3% milk (TBST-milk) for two hours at room temperature and then incubated overnight at 4°C with either monoclonal anti-AD or anti-DB antibodies (Santa Cruz) at a dilution of 1:1000 in TBST-milk. The membranes were washed two times briefly, once for 25 minutes and two times for 5 minutes with TBST. The membranes were incubated with anti-mouse IgG conjugated to alkaline phosphatase (Sigma) at a dilution of 1:30,000 in TBST-milk for 4 hours at room temperature. The membranes were washed as noted above and bound antibodies detected using ECF reagent (Amersham) and a STORM860 scanner.

4.3.4 Preparation of Recombinant A190, Rrn3 and Rrn7 Proteins

BL21-Codon Plus RIL cells (Stratagene) containing pCR68, pCR69 and pCR71 were grown at 30°C with agitation to an OD_{600} of 0.80. Isopropyl β-D-thiogalactoside was added to a final concentration of 0.4 mM and the incubation at 30°C continued an additional hour. The cells were harvested, frozen in liquid nitrogen and stored at -80°C.

GST-Rrn7p and GST-A190 purification. Cell pellets were suspended in 20 mls PBS/0.1 mM phenylmethylsulfonyl fluoride (PMSF)/1 mM DTT. The cells were lysed by sonnication and the lysates spun at 10,000 x g and 4°C for 10 minutes. The supernatants were decanted, combined with 1 ml of glutathione-agarose (Sigma) equilibrated with PBS and incubated at 4°C with gentle rocking for 30 minutes. The resins were collected and washed three times with 20 mls of PBS. GST-Rrn7p was eluted off the resin by suspending the resin in 1 ml of 10 mM Tris-HCl, pH 8.0/10 mM reduced glutathione and incubating at 4°C with gentle rocking for 2 minutes. The resin was collected and the supernatant containing GST-Rrn7p removed. The elution was repeated a total of five times. Fractions containing GST-Rrn7p were combined and dialyzed against 20 mM Tris-

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HCl, pH 7.5/100 mM KCl/0.2 mM EDTA/0.1% NP-40/10% glycerol/0.1 mM PMSF/1 mM DTT.

H_6-Rrn7p purification. The cell pellet was suspended in 15 mls of 100 mM Tris-HCl, pH 7.9/300 mM KCl/0.1 mM PMSF. The cells were lysed by sonication and the lysate spun at 27,000 x g and 4°C for 15 minutes. The supernatant was decanted and the Tris-HCl concentration reduced by adding an equal volume of 300 mM KCl/20% glycerol. The adjusted supernatant was filtered through a 0.45 μm filter and loaded onto a 1.5 ml His Bind (Novagen) column equilibrated with binding buffer (50 mM Tris-HCl, pH 7.9/ 300 mM KCl/10% glycerol/0.1 mM PMSF/5 mM β-mercaptoethanol). The column was washed with 15 ml of binding buffer and 9 ml of wash buffer (binding buffer plus 5 mM imidazole). Protein was eluted off of the column with 9 ml of elution buffer (binding buffer plus 250 mM imidazole). 0.5 ml fractions were collected. Fractions containing H_6-Rrn7p were combined and dialyzed as noted above.

H_6-HA1-Rrn3p purification. H_6-Rrn3p was prepared from BL21-Codon Plus RIL cells containing pNOY3263 as detailed in (Keener et al., 1998) with the following modifications. The S100 fraction was loaded onto a 200 ml Biorex 70 column equilibrated with breaking buffer. The flow through from this column was loaded directly onto a 20 ml Q-Sepharose Fast Flow column equilibrated with Q buffer.

4.3.5 Purification of RNA Polymerase I and Core Factor

Pol I was purified from BJ926 cells according to the protocol in (Keener et al., 1998). CF was purified from NOY797 cells which contain a triple HA1 and a hexahistidine tag on the amino and carboxy-termini of Rrn7p, respectively. The purification was completed as detailed in (Keener et al., 1998) with the following

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modifications. The anti-HA1 column was omitted and following the heparin-Sepharose column, fractions containing CF were combined and the KCl concentration reduced to 240 mM by adding gradient buffer plus 10 mM $MgCl_2$/0.05% Tween 20. The adjusted CF pool was then fractionated on a 2 ml Q-Sepharose Fast Flow column as described in Chapter 2 of this dissertation.

4.3.6 In Vitro Transcription

The plasmid pBS/35Sp linearized at the *Eco*RV site was used as the template for the in vitro transcription reactions. The conditions were optimized using a basal transcription assay and varying amounts of CF, pol I, and Rrn3p. The reactions were carried out at 25°C in a final volume of 20 μl and contained 20 mM Tris-acetate pH 7.9, 4 mM magnesium acetate, 8% glycerol, 2 mM DTT, 100 mM K^+ glutamate, 0.25 mg/ml acetylated BSA, 0.025 units/μl RNasin, 10 ng DNA template, 200 μM ATP, CTP, GTP, 6μM UTP and 2.5 μCi [α-32P]UTP (Perkin-Elmer, 3000 Ci/mmol). Partially purified H_6-Rrn7p and extract from mock-induced cells were normalized for total protein and diluted 1:20 and 1:40 with dialysis buffer (see above). All of the reaction components except the NTPs and proteins were combined on ice. CF and H_6-Rrn7p or mock extract were then added followed by pol I with Rrn3p and NTPs to start transcription. The reactions were allowed to proceed for 30 minutes and were then terminated by adding 80 μl of 0.625 mg/ml proteinase K/0.625% SDS and incubating at 50°C for 60 minutes. The transcripts were then processed and detected as described in (Radebaugh et al., 1998).

4.3.7 GST Protein Binding Assays

Glutathione-agarose was equilibrated with reaction buffer (20 mM Tris-HCl, pH 7.9/10 mM $MgCl_2$/1 mM DTT/10% glycerol plus 75 or 100 mM KCl as indicated). GST

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or GST-Rrn7p and 20 µl of equilibrated glutathione resin were brought to 400 µl with reaction buffer and incubated on a rotator at 4°C for one hour. The resin samples were collected and washed three times with 400 µl of reaction buffer with 100 mM KCl. Approximately 25 pmoles of bead bound GST, GST-Rrn7p or GST-A190 and 15 pmoles of H_6-Rrn3p or H_6-Rrn7p were brought to 400 µl with 75 or 100 mM KCl reaction buffer and incubated on a rotator at 4°C for one hour. The resin samples were collected and washed as noted above. After the final wash the resins were suspended in an equal volume of 2X SDS loading dye, incubated at 95°C for four minutes and electrophoresed through SDS-10% or 7.5% polyacrylamide gels. A control sample containing 10% of the amount of H_6-fusion protein used in the reactions was included on each gel. Proteins were transferred to PVDF membrane and detected as noted above except that monoclonal anti-His antibody (Sigma) was used at a dilution of 1:2,500.

4.4 RESULTS

The Rrn7p subunit of CF interacts with Rrn3p in a yeast two-hybrid assay. When compared to mammals, fewer interactions between the yeast pol I specific transcription factors and pol I have currently been reported. To test for additional interactions between CF and Rrn3p-pol I we completed a yeast two-hybrid analysis of each of the CF subunits (Rrn6p, Rrn7p and Rrn11p) with Rrn3p and the subunits specific to pol I (A190, A135, A49, A43, A34.5, A14 and A12). The subunits unique to pol I were chosen for use in this assay because it seems unlikely that an interaction specific for pol I transcription would occur with a polymerase subunit shared with either of the other two RNA polymerases.

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We utilized the Gateway technology (Invitrogen) for the facile generation of Gal4p DB (amino acids 1-147) and Gal4p AD (amino acids 768-881) fusion proteins. Using this system, the three CF subunits, Rrn3p, and the seven pol I subunits were fused to both the Gal4p DB and AD proteins. All of the DB-fusion proteins were then tested for artificial recruitment of pol II in the yeast strain MaV203. This strain of yeast contains the *GAL1* promoter (with four Gal4p binding sites) fused to the *HIS3* TATA element and coding sequence. Activation of *HIS3* gene expression can be monitored by cell growth on 3-aminotriazole, a competitive inhibitor of the *HIS3* gene product. The DB-Rrn6p and DB-A12 fusion proteins were both able to activate transcription of the *HIS3* gene in the absence of any Gal4p AD-fusion protein as evidenced by growth on plates containing 3AT. For this reason, Rrn6p and A12 could not be tested for interaction in our two-hybrid analysis.

To verify that all of the fusion proteins were being expressed correctly, we completed an immunoblot analysis of protein extracts from yeast cells containing each individual fusion protein construct. Monoclonal antibodies to either the Gal4p DB or AD were used to detect fusion protein expression. The results of the western analysis of the extracts from cells containing the DB-fusion constructs are shown in Figure 4.1. Similar expression levels were seen for all of the Gal4p AD-fusion proteins (data not shown). All of the fusion constructs, except perhaps DB-*RRN11*, were clearly expressed, resulting in the detection of proteins of the expected mass (i.e., Gal4p DB plus test protein mass). To further ensure that the DB and AD-fusion constructs were expressing correctly, we

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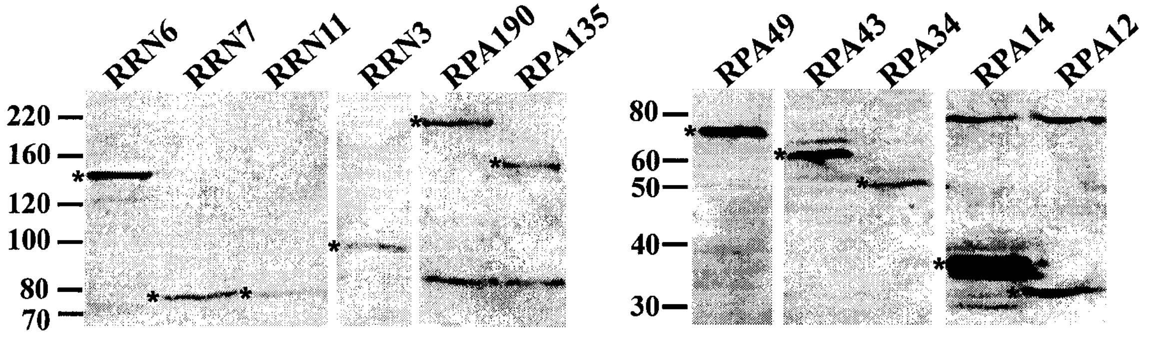

Figure 4.1. The Gal4p DB-fusion constructs were expressed in vivo. Protein extracts were prepared from strains harboring each DB-fusion construct as indicated above each lane. Approximately 50 mg of total protein from each extract were electrophoresed through SDS-7.5% (left panel) or 10% (right panel) polyacrylamide gels. Fusion protein expression was detected by immunoblotting with antibodies to the Gal4p DB. The DB-fusion proteins are marked by an asterisk to the left of each protein band. The relative electrophoretic mobility and mass of protein size standards electrophoresed in parallel with the protein extracts are given to the left of each panel.

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reproduced any applicable two-hybrid interactions that had been previously published (i.e., Rrn11p with Rrn6p or A14 with A43).

Once it was demonstrated that the Gal4p DB and AD-fusion proteins were being correctly expressed, the two-hybrid analysis was completed as follows. MaV203 cells expressing DB-Rrn7p or DB-Rrn11p were transformed individually with AD-*RRN3* or each of the seven AD-pol I subunit fusion constructs. MaV203 cells expressing AD-Rrn6p were transformed with the DB-*RRN3* or each of the seven DB-pol I subunit fusion constructs. Cells containing each of the DB-fusion proteins were also transformed with AD vector as a control for background cell growth. Two colonies from each of the transformations were tested for the ability to activate *HIS3* gene expression. Of the 23 combinations of DB and AD-fusion proteins tested, only DB-Rrn3p with AD-Rrn6p and DB-Rrn7p with AD-A190 produced growth that was clearly above background with AD vector. DB-*RRN7* in combination with AD-*RRN3* yielded growth that was slightly, although not convincingly above background (data not shown).

The Rrn3p/Rrn6p interaction was previously shown (Peyroche et al., 2000) and we will not discus it further here. To verify the Rrn7p/A190 interaction, MaV203 cells were again transformed sequentially with DB-*RRN7* and AD-*RPA190* or DB-*RRN7* and the AD vector. Ten-fold serial dilutions of log-phase cells from each transformation were screened for growth on 3AT. Cells containing the DB-Rrn7p and AD-A190 fusion proteins were again clearly able to grow on plates containing 3AT (Figure 4.2). The activation of *HIS3* transcription due to the interaction between Rrn7p and A190 is even more significant when the affect of simply having the AD-A190 fusion protein in the cell is taken into account. Cells containing DB-*RRN7* and AD-*RPA190* grow at a slower rate

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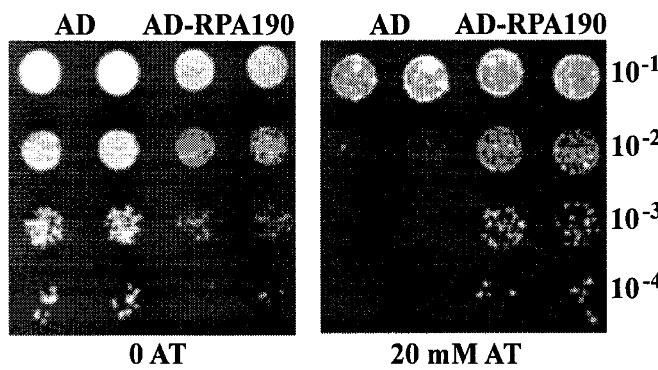

Figure 4.2. Rrn7p interacts with the A190 subunit of pol I in a yeast two-hybrid assay. Ten-fold serial dilutions of cells containing the DB-*RRN7* fusion construct and either the AD vector (AD) or AD-*RPA190* fusion construct were spotted onto plates either without or with 20 mM AT and the cells allowed to grow for seven days at 30°C.

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than cells containing DB-*RRN7* and the AD vector, resulting in smaller colonies on the plate without 3AT. Thus, the cells containing DB-*RRN7* and AD-*RPA190* that are growing on the plate with 3AT have overcome both the growth inhibition due to 3AT and their inherent slower growth rates as a result of the presence of AD-*RPA190.*

The Rrn7p subunit of CF interacts with the A190 subunit of pol I in a GST pull-down assay. We carried out a GST pull-down assay to confirm the Rrn7p/A190 interaction seen in the yeast two-hybrid analysis. GST-A190 and H_6Rrn7p fusion proteins were purified from *E. coli* extracts using the appropriate affinity chromatography. GST or GST-A190 fusion protein were bound to glutathione-agarose beads and incubated with the purified H_6Rrn7p fusion protein. Non-specifically bound proteins were removed from the agarose beads by washing. Specifically bound and interacting proteins were removed by denaturation and the presence of H_6Rrn7p analyzed by immunoblotting using monoclonal antibodies to the H_6-tag. H_6Rrn7p bound the GST-A190 beads (Figure 4.3A, lane 2) but not the control GST beads (Figure 4.3A, lane 3). Thus, the Rrn7p/A190 interaction seen in the two-hybrid analysis is also seen in a GST pull-down assay. The data from these two assays clearly support a direct interaction between Rrn7p and the A190 subunit of pol I.

The Rrn7p subunit of CF interacts with Rrn3p in a GST pull down assay. As noted above, we saw a weak and not totally convincing interaction between Rrn7p and Rrn3p in the two-hybrid analysis. An interaction between these two polypeptides would not be unexpected. The mammalian homologue of yeast Rrn7p is TAF_I68 and, in humans, it has been shown to interact with TIF-IA/hRRN3 in a far western analysis and in several protein pull-down assays (Miller et al., 2001). However, an interaction between mouse

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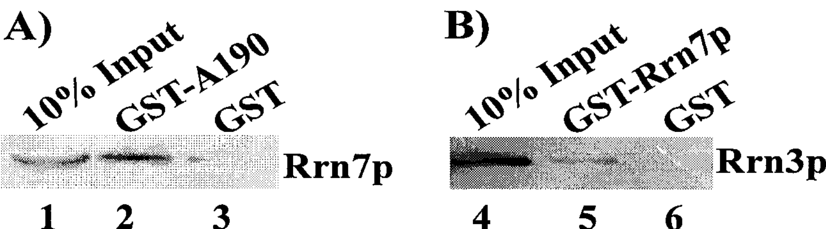

Figure 4.3. Rrn7p interacts with the A190 subunit of pol I and Rrn3p in GST pull-down assays. Panel (A) Recombinant GST-A190 or GST was bound to glutathione agarose beads and incubated with purified recombinant H_6-Rrn7p (lanes 2 and 3). Panel (B) Recombinant GST-Rrn7p or GST was bound to glutathione agarose beads and incubated with purified recombinant H_6-Rrn3p (lanes 5 and 6). After washing, bound proteins were removed from the beads by heat denaturation in SDS. The proteins were then electrophoresed through SDS-10% polyacrylamide gels and the presence of H_6-fusion proteins detected by immunoblotting with anti-H_6 antibodies. Lanes 1 and 4 contain 10% of the amount of each H_6-fusion protein used in the incubation with the glutathione agarose beads.

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TIF-IA and TAF_I68 was not evident in a yeast two-hybrid assay (L. Rothblum, personal communication). For this reason, we decided to evaluate the potential interaction between yeast Rrn3p and Rrn7p in a GST pull-down assay. The assay was completed essentially as described above except that GST-Rrn7p and H_6-Rrn3p purified from *E. coli* extracts were used., H_6-Rrn3p bound the GST-Rrn7p beads (Figure 4.3B, lane 5) but not the control beads bound with GST (Figure 4.3B, lane 6). Thus, it appears that as with mammals, in yeast the Rrn3p transcription factor interacts with two subunits (Rrn6p and Rrn7p) of the fundamental transcription factor.

Free Rrn7p can squelch pol I basal transcription. We reasoned that if the interaction of Rrn7p with Rrn3p and/or the A190 subunit of pol I is a critical interaction for pol I transcription then an excess of free Rrn7p could suppress transcription. We added partially purified H_6-Rrn7p to a basal transcription reaction to investigate this possibility. We chose basal transcription and not activated transcription so that any reduction in transcription levels could be attributed to blocking the interaction of Rrn7p with the Rrn3p-pol I complex and not to interfering with the UAF-CF interaction (i.e., Rrn7p with Rrn9p). As can be seen in Figure 4.4A we found that, indeed, partially purified H_6-Rrn7p (lane 2) completely inhibited pol I transcription.

Since the H_6-Rrn7p we used to inhibit transcription was not homogeneous, it was possible that a contaminating protein was responsible for the inhibition. To account for this possibility, a control extract was prepared from induced *E. coli* cells that did not contain the H_6-Rrn7p fusion construct. The control extract was fractionated on affinity resin exactly as was done for the extract containing H_6-Rrn7p. Fractions from the column that contained the peak of protein were combined and the resulting pool (control pool)

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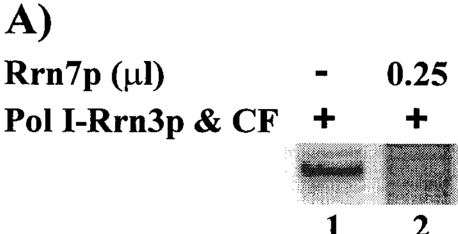

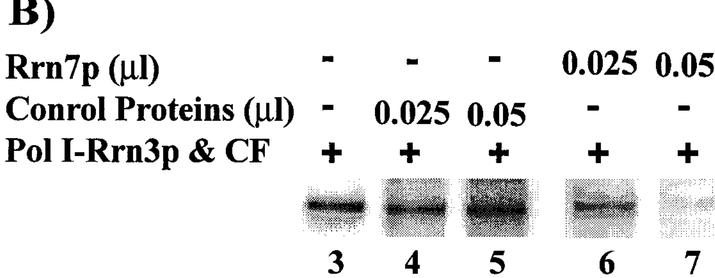

Figure 4.4. Recombinant H_6-Rrn7p can suppress basal transcription in a dose dependant manner. Panel (A) Partially purified H_6-Rrn7p or buffer were added to optimized basal transcription reactions prior to incubation at 25°C for 30 minutes. The transcription products for the buffer control reaction (lane 1) or the reaction with H_6-Rrn7p (lane 2) are shown. Panel (B) A control protein extract was prepared from *E. coli* cells and fractionated on Ni-affinity resin. The peak protein fractions from the column were combined (control proteins). The control protein pool and partially purified H_6-Rrn7p were normalized for total protein and either 0.025 or 0.05 μl of each added to basal transcription reactions as noted above. The transcription products for the reactions with the control proteins (lanes 4 and 5) or the reactions with H_6-Rrn7p (lanes 6 and 7) are shown.

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analyzed by SDS-PAGE. The distribution of proteins in the control pool was essentially the same as the contaminating proteins in our H_6-Rrn7p preparation (data not shown).

We normalized the control pool and H_6-Rrn7p preparations to the same total protein concentration and then repeated the basal transcription reactions. In addition, to show that the degree of pol I transcription inhibition is dependent on the amount of H_6-Rrn7p added, we reduced the starting amount of H_6-Rrn7p by ten-fold and did a small titration. In this assay, Figure 4.4B, the control pool (lanes 4 and 5) did not inhibit pol I transcription at either of the amounts tested while the H_6-Rrn7p inhibited transcription in a dose-dependent manner (lanes 6 and 7). Clearly, the interaction(s) between Rrn7p and the Rrn3p-pol I complex is essential for basal levels of transcription *in vitro*, and this interaction can be competed by Rrn7p not associated with CF.

4.5 DISCUSSION

We utilized the yeast two-hybrid and GST pull-down assays to identify interactions between CF and the Rrn3p-pol I complex that had not been seen previously. We focused our analysis on the CF subunits, Rrn3p, and the subunits unique to pol I as we felt these polypeptides were the most likely to play a role in the specific recruitment of pol I. The results of this study clearly show that the Rrn7p subunit of CF interacts with Rrn3p and the A190 subunit of pol I.

The Rrn7p/A190 interaction is the first to be shown between a subunit of a fundamental pol I transcription factor and a subunit of pol I. However, this interaction was not unexpected. Recently, A. Bric et al., (submitted) used site-specific photo-cross-linking to map the subunits of TIF-IB and pol I from *Acanthamoeba castellanii* along the

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rDNA. The TAF_I96 subunit of *A. castellanii* TIF-IB is homologous to Rrn7p and was the only subunit of the factor that was cross-linked directly upstream of the *tis* (between –11 and –1), the same region that is included in the pol I DNase I footprint (Bateman et al., 1985). That this subunit overlaps the pol I binding site supports the possibility that it interacts with the Rrn3p-pol I complex and assists in recruiting pol I to the rDNA. Additionally, when pol I was added to the preinitiation complex TAF_I96 and the A185 and A133 subunits of *A. castellanii* pol I were cross-linked at +10. The A185 subunit of *A. castellanii* pol I is the homologue to the yeast A190 subunit. Thus, the two proteins that are homologous to Rrn7p and A190 are clearly positioned close enough in the initiation complex to interact with each other.

During our evaluation of the photo-cross-linking results of the *A. castellanii* proteins it became apparent to us that Rrn7p and the A135 subunit of pol I might also interact with each other. TAF_I96 and the A133 pol I subunit (homologous to the *S. cerevisiae* A135 pol I subunit) cross-link at all of the sites evaluated between –11 and –1, and as noted above, at +10. However, an interaction between Rrn7p and the A135 subunit of pol I was not detected in our two-hybrid assay. False negatives are not uncommon in the two-hybrid assay and it is possible that these two polypeptides do interact and we simply could not detect it in our assay. For example, the interaction between Rrn7p and Rrn3p was also not detected in our two-hybrid analysis, but was in the GST pull-down assay. An interaction between Rrn7p and the A135 subunit of pol I might also be detected if these two proteins were analyzed using a different methodology. Alternatively, it is also feasible that in spite of their apparent proximity in the preinitiation complex that in fact Rrn7p and the A135 subunit of pol I do not interact.

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The interaction between Rrn7p and the A190 subunit could serve to strengthen the association of CF with pol I and enhance the recruitment of polymerase to the rRNA promoter. The protein requirements for the specific recruitment of pol I have been extensively studied in several systems with differing results. Using purified proteins from human cells, Miller et al., (2001) demonstrated that in addition to UBF and SL1, hRRN3 is required for specific promoter binding by pol I. In contrast, pol I isolated from yeast and mouse cells was capable of being recruited to the template without the presence of Rrn3p/TIF-IA (Aprikian et al., 2000; Schnapp and Grummt, 1991). In the absence of Rrn3p and its interactions with CF, the Rrn7p/A190 interaction might be sufficient for the recruitment of pol I *in vitro*. However, it is unlikely that pol I without Rrn3p is recruited to the rDNA *in vivo*. The majority of pol I (>90%) in *S. cerevisiae* is not associated with Rrn3p and purified Rrn3p cannot activate pol I bound to the promoter in its absence (Aprikian et al., 2001; Milkereit and Tschochner, 1998). If polymerase alone could be specifically recruited to the promoter, eventually all of the preinitiation complexes formed would be transcriptionally inactive or dead-end complexes. This clearly could not happen in actively growing cells where rRNA needs to be rapidly synthesized. Thus, although the Rrn7p/A190 interaction might be sufficient to recruit pol I *in vitro*, it is most likely the combination of this interaction with the CF/Rrn3p interactions that leads to the recruitment and stable binding of the Rrn3p-pol I complex *in vivo*.

The Rrn7p/A190 and Rrn7p/Rrn3p interactions are most likely not involved in the growth-dependent regulation of rRNA transcription. However, they could be targets for other regulatory pathways. In mammalian cells, multiple pathways regulate rRNA transcription and both UBF and TIF-IB/SL1 subunits have been shown to be modified by

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reversible acetylation and phosphorylation (reviewed in Grummt, 2003). In *S. cerevisiae*, rRNA transcription is regulated in response to environmental stress and nutrient availability, although little is known about the mechanism through which this regulation occurs (Warner, 1999; Clarke et. al., 1996). The association of Rrn3p with pol I might also regulate transcription in these pathways but, it is also possible that modification of CF, UAF and/or pol I subunits(s) plays a role. Currently it is not known whether or not the subunits of CF or UAF are post-translationally modified *in vivo*. Phosphatase treatment of crude protein fractions containing CF and UAF did not inhibit rRNA transcription in an *in vitro* reaction. It is possible, however, that a kinase was present in the crude sample and critical sites on the factors were rephosphorylated under the conditions used for transcription. The Rrn7p/Rrn3p protein-protein interaction is evolutionarily conserved, frequently an indication of an essential interaction and a possible site for regulation. Additionally, both Rrn3p and the A190 subunit of pol I are phosphorylated *in vivo*, which could lead to a regulation of their interaction with other proteins (Breant et al., 1983; Buhler et al., 1976; Fath et al., 2001).

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CHAPTER 5

ADDITIONAL OBSERVATIONS AND FUTURE DIRECTIONS

5.1 Purifying the *A. castellanii* TIF-IA homologue.

Immunoblot analysis of crude fractions prepared during the purification of pol I has identified large pools of "free" TIF-IA. Cross-reactivity of the anti-yRrn3p polyclonal antibody with an approximately 80 kD band is robust in the PEI supernatant fraction (data not shown). This "pool" of TIF-IA possesses a faster electrophoretic mobility than TIF-IA associated with pol I (Figure 5.1, compare lanes 1 and 2), consistent with its being unphosphorylated. Additionally, no pol I was found in the PEI supernatant fraction, as assayed by cross-reactivity to an anti-AC39 monoclonal antibody. The identification of a pool of free TIF-IA provides a starting point for the purification of *A. castellanii* TIF-IA. John Anderson has begun to fractionate this cross-reacting protein. Once the factor has been purified closer to homogeneity, activity of the factor may be tested in specific transcription assays. It is possible that this free pool of TIF-IA is not properly modified (phosphorylated), and therefore incapable of participating in specific transcription. However, it may be possible to phosphorylate this protein using cellular kinases such as ERK and RSK. Zhao et al., (2003) have shown ERK and RSK kinases are responsible for the phosphorylation of mammalian TIF-IA upon mitogen stimulation. If *A. castellanii* TIF-IA is not capable of being phosphorylated by kinases such as ERK and RSK, it may be possible to assay for activity in the yeast transcription system. Studies have shown that

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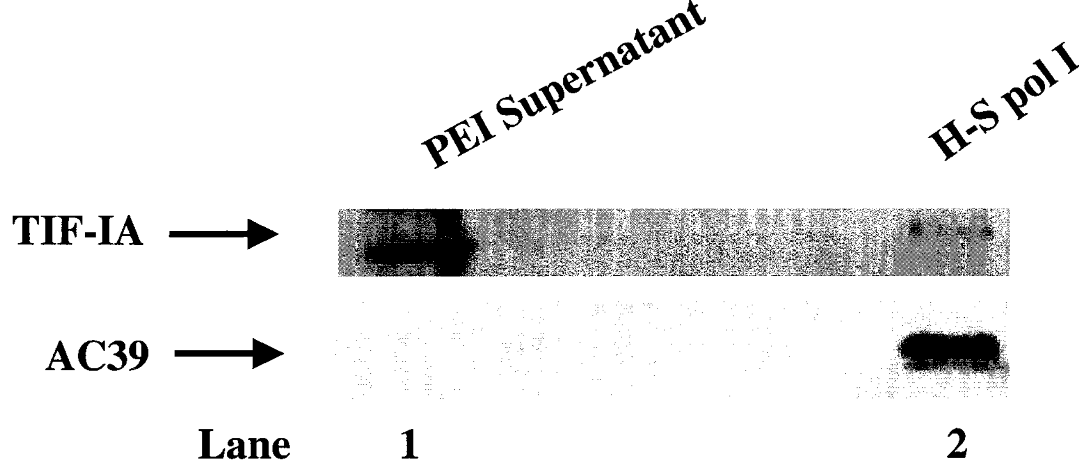

Figure 5.1. Free TIF-IA has a faster electrophoretic mobility than pol I associated TIF-IA. 10 μl of PEI supernatant and 5 μl of heparin-Sepharose pol I were subjected to immunoblotting. The presence of TIF-IA and pol I were detected with an anti-yRrn3p pAb and an anti-AC39 mAb, respectively. PhosphorImage files jg111802 and jg111902.

TIF-IA/Rrn3p possesses functional conservation between the species (Moorefield et al., 2000; Schnapp et al., 1993). Additionally, recombinant yeast Rrn3p is capable of functioning in both the yeast transcription system and the *A. castellanii* transcription system (Chapter 3 of this dissertation). Thus it may be possible that unmodified (unphosphorylated) *A. castellanii* TIF-IA could function in an *in vitro* yeast transcription assay.

The purification of the proposed *A. castellanii* TIF-IA homologue also may be sufficient to obtain protein sequence data. This may provide further evidence that our cross-reacting band is truly an *A. castellanii* TIF-IA/Rrn3p homologue.

5.2 Producing an antibody to *A. castellanii* TIF-IA.

The immunoblotting experiments performed in Chapter 3 used an anti-yRrn3p polyclonal antibody made against full-length recombinant yeast Rrn3p. Although the TIF-IA/Rrn3p transcription factor has been shown to be functionally conserved, the sequence conservation between the various cloned factors is not that high, with 40-43% sequence similarity (21-27% identity) (Bodem et al., 2000; Moorefield et al., 2000). Therefore, it is likely that the polyclonal antibody we have prepared does not recognize a significant number of epitopes in the *A. castellanii* TIF-IA homologue. Thus, it would be advantageous to prepare an antibody to the actual *A. castellanii* factor.

With the production of a better polyclonal antibody to *A. castellanii* TIF-IA, it may be possible to detect if TIF-IA is released from the transcribing polymerase upon promoter clearance. Studies conducted previously in the laboratory have mapped where pol I is contacting the DNA as it elongates down the rRNA genes (Kahl et al., 2000). By the selective omission of specific nucleotides, pol I may be paused at different sites on

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modified templates. Using a modification of these experiments on bead-bound rDNA, it may be possible to detect when/if TIF-IA dissociates with pol I. Using highly purified transcription components, it has been shown that TIF-IA recycles, providing multiple rounds of transcription (Schnapp et al., 1993; C. Terpening, unpublished). Therefore, when using pure transcription components, it is possible the TIF-IA will not dissociate from pol I. However when crude preparations are used to form functional PICs on the promoter, TIF-IA/Rrn3p activity is lost, resulting in a dissociation of TIF-IA/Rrn3p from pol I (Hirschler-Laszkiewicz et al., 2003; Milkereit and Tschochner, 1998). When using crude fractions, it may then be possible to detect when/if TIF-IA is released from pol I. Furthermore, if TIF-IA is released using a certain combination of "crude" fractions in an *A. castellanii* transcription system, it may be possible to isolate a phosphatase activity from one of the fractions. Additionally, using an already established yeast transcription system in the laboratory, it may be possible to look for phosphatase activity in that system as well.

5.3 Is there a specific transcription stimulating activity in pol I glycerol gradient fraction 13?

In initial attempts to identify/locate an activity in *A. castellanii* homologous to TIF-IA/Rrn3p, glycerol gradient fractions were found to stimulate rRNA transcription. The glycerol gradient fractionation of pol I separates a pool of TIF-IE from pol I (Al-Khouri and Paule, 2002; Radebaugh et al., 1998). The peak of TIF-IE sediments in fractions 5-8, while pol I sediments in fraction 14-16 (Al-Khouri and Paule, 2002). Cross-reactivity of a protein in the glycerol gradient fractions with our anti-yRrn3p polyclonal antibody peaked in fraction 13. Fraction 13 does not contain pol I activity and possesses minimal

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TIF-IE activity (Al-Khouri and Paule, 2002). In order to determine if fraction 13 contained any TIF-IA related activity, fraction 13 was tested in specific transcription assays. Fraction 13 was preincubated with pol I fractions from the glycerol gradient and assayed for activity in a reconstituted specific transcription assay. The addition of fraction 13 to pol I containing, specific transcription competent, glycerol gradient fractions, resulted in an increase of transcription (Figure 5.2, compare lanes 2, 3, 4, and 5, with 6, 7, 8, and 9, respectively). Fraction 13 itself was shown to not possess any specific transcription activity (Figure 5.2, lane 1). It is not believed that this increase in transcription activity is due to TIF-IA. The initial cross-reactivity with the anti-yRrn3p polyclonal antibody to a protein in fraction 13 was found to be non-specific. Additionally, fraction 13 was not capable of functionally replacing yeast Rrn3p in a yeast *in vitro* transcription assay.

Therefore, it is unclear what, if any, function glycerol gradient fraction 13 has in rRNA transcription. Several factors from a variety of species have been shown to affect the activity of rRNA transcription (see Chapter 1 of this dissertation). It is possible that fraction 13 could be a homologue to any one of these factors. Further characterization of this fraction may identify a functional homologue to a factor known to regulate rRNA transcription, or it may uncover a novel factor that stimulates rRNA transcription in *A. castellanii.*

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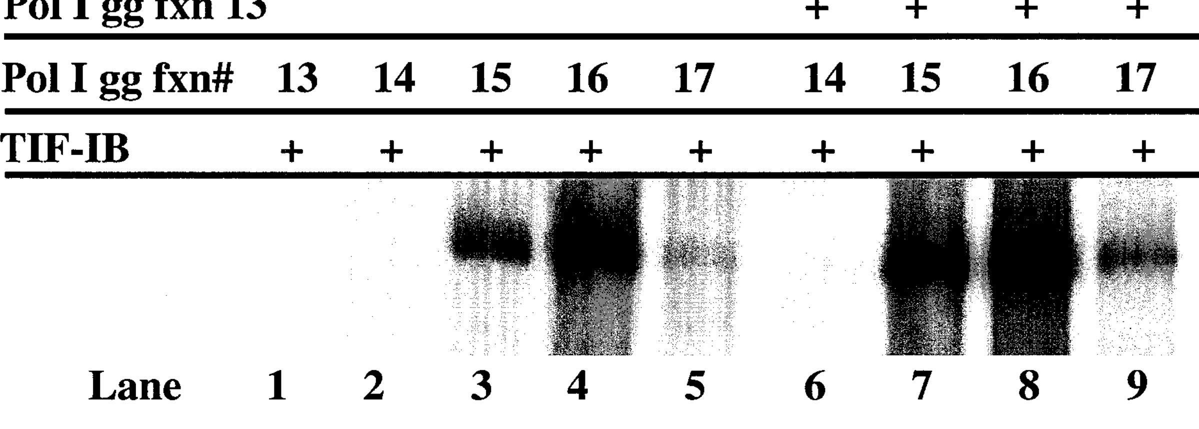

Figure 5.2. Glycerol gradient fraction 13 stimulates rRNA transcription. Glycerol gradient pol I (1.5 μl) was preincubated with 1.5 μl pol I gradient buffer or 1.5 μl glycerol gradient fraction 13 for one hour at 30°C. 2 μl preincubation mix was combined with 1 μl of TIF-IB 2X DNA affinity in a specific transcription reaction. PhosphorImage file jg042601.

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