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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

A STUDY OF THE REGULATION OF TRANSCRIPTION IN *SACCHAROMYCES CEREVISIAE* AND *ESCHERICHIA COLI*

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

EILEEN MARY CLARKE

Norman, Oklahoma

1999

A STUDY OF THE REGULATION OF TRANSCRIPTION IN *SACCHAROMYCES CEREVISIAE* AND *ESCHERICHIA COLI*

A Dissertation

APPROVED FOR THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

By

Daniel L. Nigg

Ann West

Acknowledgments

My accomplishments over the past five years would not have been possible without the support of many people. First I would like to thank Dr. Dan Riggs for his mentoring and patient guidance throughout my studies. He has not only greatly influenced my scientific learning but also my personal growth. I truly admire Dr. Riggs and hope that I can be as positive a role model to others.

Secondly, I would like to thank my family, especially my mom and dad. They have been a constant source of love and encouragement my entire life. From them I have learned the skills needed to persevere during my graduate studies.

Next, I want to acknowledge all of my friends and labmates. Thanks to all of those who made the last five years more enjoyable. The antics of the Spaniards Gustavo and Celina, the laughs from the Frenchies, Valerie, Isabelle, Sandrine, the float trips with Bill, Paul, Lea, and the fun I have had with my adopted Hanna lab family, will always be with me.

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Table of Contents

List of Figures

Part 1

Regulation of Transcription in

Saccharomyces cerevisiae

Chapter 1

Literature Review

Introduction

I. Ribosomal RNA transcription

Ribosome biogenesis is a complicated process which has intrigued researchers for decades. Since protein synthesis consumes considerable cellular resources, it is not surprising that this process is tightly regulated. In order to survive, organisms are required to detect changes in the environment and respond to these signals by changing the rate of ribosome production to meet the translational needs of the cell. By mass, ribosomes are composed of two-thirds ribosomal RNA (rRNA) and one-third ribosomal proteins (r-proteins). In prokaryotes, only one RNA polymerase (RNAP), is responsible for synthesizing the rRNA and the r-protein mRNA, thus allowing for a single target of the regulatory signals. In eukaryotes, the process is more complex and requires the orchestration of three nuclear RNA polymerases to produce the ribosomal constituents.

In eukaryotes, ribosome biogenesis requires the coordinated synthesis of four ribosomal RNA species transcribed by RNAP I (17-18S, 5.8S, and 25-28S rRNA) and RNAP III (5S rRNA), and approximately 80 ribosomal proteins translated from mRNAs synthesized by RNAP II (Jacob, 1995) (Figure 1). These four rRNA transcripts are then assembled into the 40S and 60S ribosomal subunits which are in the nucleus. Once assembled, the immature subunits are then transported out of the nucleus and into the cytoplasm to form complete ribosomes. In all cells, from prokaryotes to vertebrates, rRNA gene expression is regulated in order to maintain a constant number of ribosomes in the cell.

Various approaches have been developed in order to elucidate the role of gene promoters and other cis-acting elements as well as trans-acting protein factors which cooperate to direct the specific RNA polymerases in controlling gene expression. The study of the ribosome biogenesis offers a unique system by which the coordination of gene expression as well as growth rate dependent transcriptional regulation can be studied.

This chapter will focus on what is currently known about transcription by RNA polymerase I (RNAP I) in eucaryotic organisms with an emphasis on the yeast *Saccharomyces cerevisiae*. The DNA elements and basal transcription factors essential for RNAP I transcription are described.

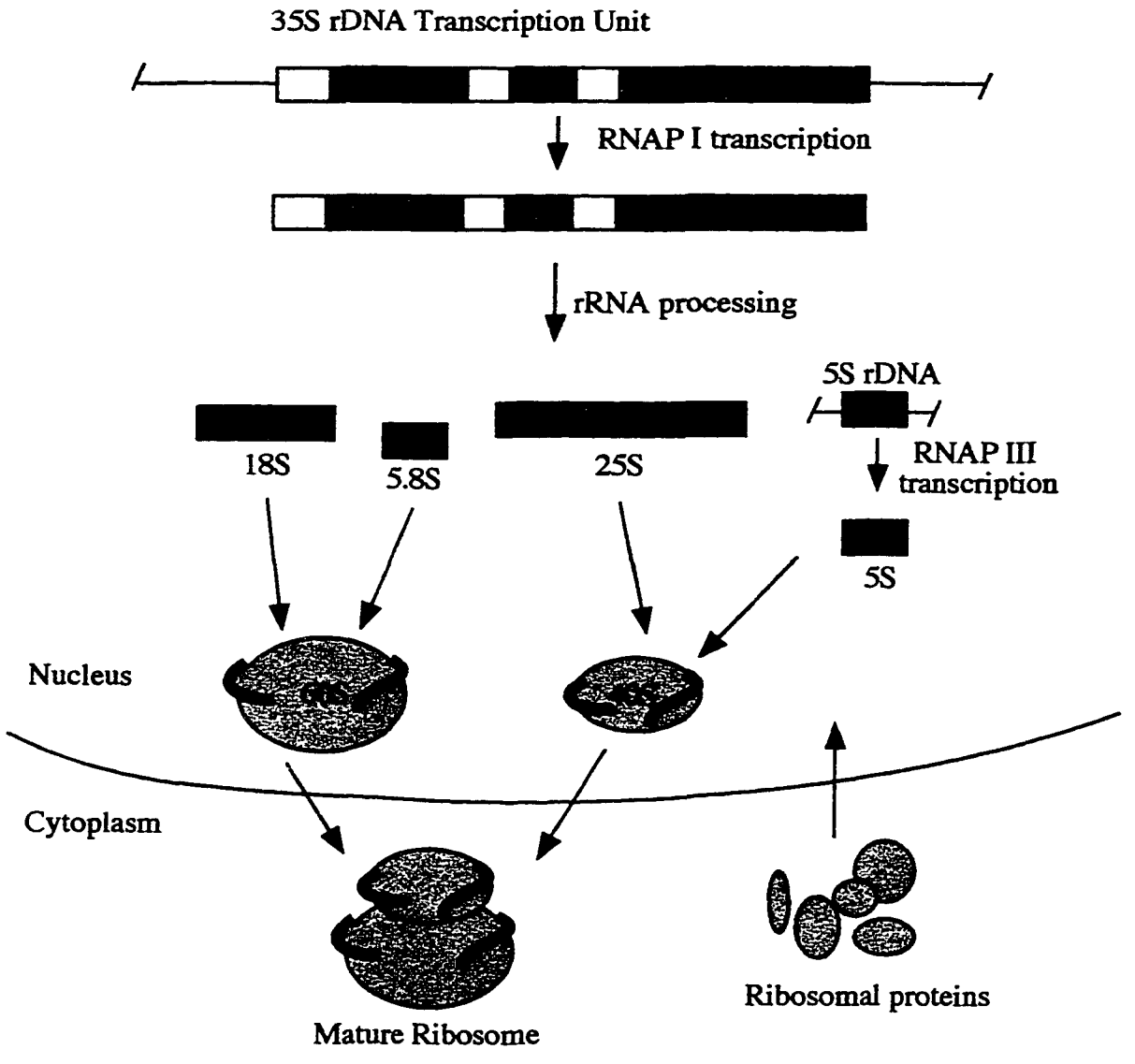


Figure 1: Ribosome biogenesis in yeast. RNAP I synthesizes a 35 S transcript which is processed into the three largest rRNAs (18 S, 5.8 S and 25 S) while RNAP III produces the 5 S transcript. The ribosomal proteins are synthesized in the cytoplasm and transported into the nucleus and assembled with the rRNAs to form the immature ribosomal subunits. The immature subunits are then tranported to the cytoplasm for assembly of the mature ribosome.

II. Cis-acting elements

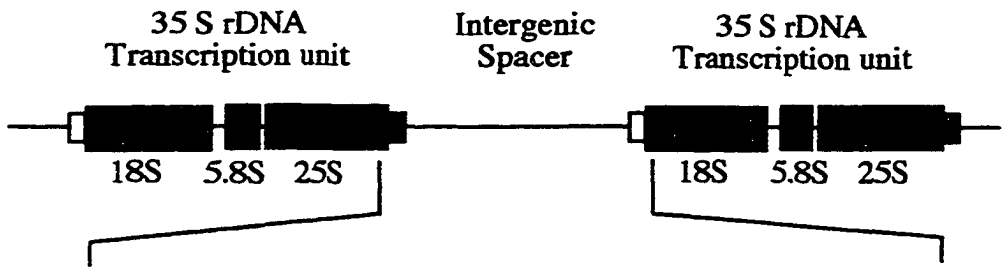
Ribosomal RNA gene organization in eukaryotes—Ribosomal RNA is a major structural component of ribosomes and multiple copies of the rRNA genes are required to meet the demand. The rRNA gene (rDNA) is highly reiterated and is arranged in a tandem array in clusters of head-to-tail repeats separated from the next unit by an intergenic spacer that ranges in size from 2 kb to over 30 kb, depending on the organism (Jacob, 1995). In most species the number of copies of rDNA ranges from 100 to 5000, which are located at one or more chromosomal loci. In yeast, there are approximately 150 copies of the 9.1 kb rDNA unit located on chromosome XII (Petes, 1979). A yeast strain has been engineered in which all of the rDNA was deleted from chromosome XII and replaced by multiple extrachromosomal plasmids, each bearing a single rDNA repeat (Nierras *et al.*, 1997). This strain grew reasonably well and responded to physiological stimuli, which indicated that neither chromosomal location nor repetition is important for RNAP I regulation and ribosome assembly. Although organisms contain multiple copies of the rDNA gene, only a fraction of these genes are actively transcribed. In order to measure the number of active ribosomal genes, the adenosine analog, 5,6-dichloro-1-β-D-ribofuranosylbenximidazole (DRB) was added to a culture of growing cells (Scheer *et al.*, 1984). DRB does not inhibit RNAP I transcription, but causes nucleoli to disperse into beaded structures. Each bead was then stained with anti-RNAP I antibodies and represented an active rDNA transcription unit which could be counted. This technique, which was used to study mammals, marsupials, birds, and amphibians, showed that the number of genes being actively transcribed was considerably less than the number of rDNA repeats (Haaf *et al.*, 1991). In a separate study, in which a crosslinking agent psoralen was used to measure the fraction of active ribosome genes accessible to the reagent, it was estimated that for yeast growing in a rich media, only 46% of the genes were active. These studies suggest that RNAP I is not the limiting factor, as the active genes appear fully loaded with elongating polymerase, but

rather that the rate-limiting step is most likely to be the formation of a stable preinitiation complex at the rDNA gene promoter. This also suggest that some component of the preinitiation complex is probably limiting so that only a fraction of the rDNA gene promoters are able to initiate transcription.

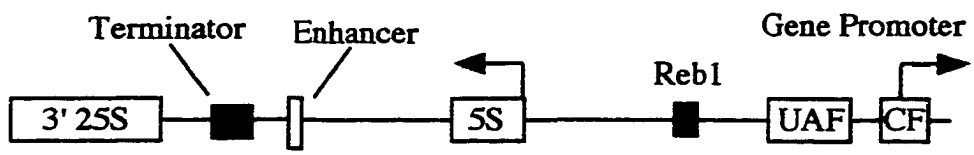
The gene promoter—Despite the fact that RNAP I promoters have evolved rapidly and lack sequence conservation, RNAP I promoters in eukaryotes all appear to be organized with similar domain structure. Figure 2 shows a comparison of the yeast rDNA organization with that of a typical vertebrate. The gene promoter of most eukaryotes spans approximately 150 bp and consists of two regions, a core region and an upstream control element (UCE) or upstream promoter element (UPE), which are separated by a central region whose spacing is critical but precise sequence is variable. The core promoter generally extends from about 40 bp upstream to a few nucleotide pairs downstream from the initiation site of transcription (Paule, 1993). For *in vitro* transcription with high concentrations of template, the core element is sufficient to direct accurate initiation which suggests that this is the primary element involved in recruiting the RNAP I to the promoter. *In vivo,* faithful initiation of transcription can occur in the absence of other cis-elements although the upstream binding domain heavily stimulates transcription (Choe *et al.*, 1992). The spacing between these two domains is critical in some vertebrate promoters suggesting that proteins bound to both elements must interact in a precise fashion (Haltiner *et al.*, 1986; Pape *et al.*, 1990; Xie & Rothblum, 1992).

In yeast, the promoter domain structure is similar to other eucaryotic RNAP I promoters. The yeast promoter contains a core element, which encompasses approximately 50 nucleotides including the transcriptional start site and extends to –40, and an upstream element, which extends from ~ –50 to –150. The functional necessity of these two domains

is similar to higher eukaryotes as the core element is essential for transcription both *in vivo* and *in vitro* while the upstream element is stimulatory (Vogelauer *et al.*, 1998,).



Yeast rDNA



Vertebrate rDNA

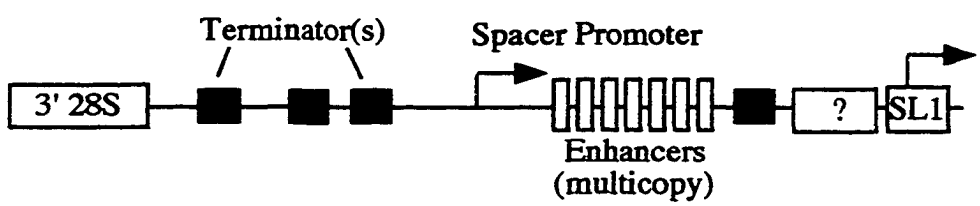


Figure 2: Comparison of the intergenic spacers of yeast rDNA and a typical vertebrate. The diagram shows a segment of tandemly repeated rDNA. The gene regions are separated by the intergenic spacer which contains all of the transcriptional regulatory elements. The diagram is not drawn to scale.

The enhancer elements—The intergenic spacers of rDNA contain sequences that have a strong positive influence on transcription. These segments of DNA, called enhancers, can stimulate transcription from the promoter and generally function independent of orientation or distance. They were first located within the intergenic spacers from frog DNA and characterized as 60 bp to 80 bp repetitive elements which occurred in blocks of 6-12 units (Moss, 1983). In general, each repeat block is preceded by a "spacer promoter" which itself is recognized by RNAP I and, under ill-defined conditions, is subject to transcription by RNAP I. The function of the spacer promoter remains unclear. It has been shown that enhancers act *in cis* to stimulate adjacent promoters but compete against promoters located *in trans*. Furthermore, promoters *in cis* to multiple enhancers are preferentially transcribed when in competition with promoters that contain fewer repeats. It is thought that enhancers increase transcription by increasing the formation of stable preinitiation complexes at the gene promoter. Although the mechanism for this is not well understood, it has been observed that enhancers appear to compete for binding some limiting factors essential for preinitiation complex formation (Pikaard *et al.*, 1990).

In *Saccharomyces cerevisiae*, a single copy 190 bp enhancer element is required for maximal transcription of the rDNA. The enhancer is located 2 kb upstream of the initiation site and just downstream of the rRNA precursor and does not contain a spacer promoter. Binding sites for ABF 1 and Reb 1, two nuclear proteins, have been identified near the yeast rDNA enhancer (Ju *et al.*, 1990; Kang *et al.*, 1995; Morrow *et al.*, 1989; Morrow *et al.*, 1990). Reb 1, bound at this site, has been implicated in termination of transcription by RNAP I (Lang *et al.*, 1994) while the function of ABF 1 remains unclear. Neither of these proteins have been shown to affect enhancer function or interaction with the enhancer element.

III. Trans-acting Factors

Work done in higher eukaryotes to identify trans-acting factors involved in rDNA transcription—Specific transcription by all three nuclear RNA polymerases requires a concerted effort between multiple protein factors and the RNA polymerase. This complex process requires that the transcription factors assemble with the distinct promoter to form a preinitiation complex. The recruitment of the polymerase to the promoter creates the formation of an initiation competent complex. After the formation of the first several phosphodiester bonds of the RNA, the polymerase enters the elongation phase where it moves along the DNA extending the growing RNA chain. Once the gene is transcribed, both the polymerase complex and the RNA are released at the terminator.

Major efforts have been made in many laboratories to elucidate the components of the RNAP I transcription machinery in an attempt to better understand this entire process. Three *trans*-acting factors, which are essential in rDNA specific transcription initiation, have been identified and characterized in the human system. The activities of these three factors are conserved throughout various eucaryotic systems. Other systems have identified additional factors which associate closely with the polymerase and may be involved in the transcription of rDNA. These factors however are not considered to be subunits of the polymerase. Below is a brief summary of the various *trans*-acting factors involved in rDNA transcription in eukaryotes with special emphasis on the components of the yeast RNA polymerase I transcription system.

RNA Polymerase I—Three nuclear RNA polymerases have been extensively purified from a variety of eucaryotic organisms. Transcription analysis has confirmed that RNAP I only catalyses the synthesis of ribosomal RNA in the nucleus (Nogi *et al.*, 1991). The exact composition of this multi-subunit enzyme is rather conjectural in higher eukaryotes although for most eucaryotic the composition of the RNAP I enzyme are similar

in structure composed of two large subunits (homologous to the β and β' of *E. coli* RNAP) as well as several other smaller subunits some of which are shared with RNAP II and III. The core mammalian RNAP I is a large complex enzyme with an approximate molecular weight of 500-600,000 Da. Recent studies report that mammalian RNA polymerase I is composed of at least 12 subunits with 3 associated factors (PAFs) (Hanada *et al.*, 1996; Hannan *et al.*, 1998). Four of the mammalian RNAP I subunits have been cloned including the largest subunits A190 and A127 which are analogous to the β and β' subunits of *E. coli* (Hannan *et al.*, 1998; Sentenac *et al.*, 1992). The other cloned subunits, AC40 and AC19 (Gadal *et al.*, 1997; Song *et al.*, 1994) are common to both RNA polymerase I and III. Comparison of the polypeptide pattern of homologous RNA polymerases from different organisms has found certain subunits to be functional analogs (Sentenac, 1985).

Upstream Binding Factor (UBF)— UBF, the first well-characterized RNAP I *trans*-acting factor, has been purified to homogeneity from human, mouse, frog and rat cells. It exists as a dimer with each subunit having a molecular weight ranging from 85 to 97 kDa. UBF has been found to bind promiscuously to regions of the core promoter, the upstream control element and the enhancer elements. A consensus sequence has not been identified within these various binding regions, rather it is thought that UBF recognizes a general DNA conformation. UBF is a member of a family of transcription factors that contain a common DNA binding motif, the high mobility group (HMG) domain. HMG boxes are highly basic domains of about 80 amino acids in length that were first recognized as having significant similarity to elements present in the small chromosomal proteins HMG1 and HMG2. The number of HMG boxes differ among species, for example in human UBF there are six HMG boxes while in *Xenopus* there exist only five, and is one of the determinants of species specificity (even though the xUBF and hUBF give identical

footprints on both the *Xenopus* and human promoters). It has been suggested that each HMG-box within the UBF has a specific function. These functions may include DNA sequence selection and provision of specific interfaces involved in protein-protein interactions with other transcription factors (Grummt, 1999). The N-terminal region of mammalian UBF makes up the dimerization domain of the protein while the highly acidic C-terminal region is involved in transcription interaction (Hu *et al.*, 1994). This C-terminal domain has also been shown to be subject to regulatory modifications. It appears, that in order for UBF to activate transcription, serine residues within the acidic C-terminus must be phosphorylated (O'Mahony *et al.*, 1992a).

HMG-box proteins are able to bend DNA (Ferrari *et al.*, 1992; Giese *et al.*, 1992). In one study, *Xenopus* UBF was shown to loop DNA fragments as small as 150 bp (Putnam *et al.*, 1994). In another study, electron micrograph image analysis demonstrated that a single dimer of UBF could organize 180 bp of DNA into a loop of ~360° (Bazett-Jones *et al.*, 1994). A possible hypothesis is that a UBF dimer, that has the promoter region of the DNA wrapped around it, could bring the core region and the UCE into close proximity which would allow for physical interaction of transcription factors with both promoter elements. This is consistent with the extended footprint seen when hUBF and SL-1 (selectivity factor) were footprinted on human rDNA promoters (Jantzen *et al.*, 1992). Thus, UBF-DNA complexes may provide productive interactions between transcription factors bound at these two recognition sites that are separated by 120 bp. This model is further supported by experiments demonstrating spacing changes between core and the UCE affect transcription. Specifically, a one-half helix turn change abolishes activation while a full helix turn does not affect activation (Clos *et al.*, 1986; Windle & Sollner-Webb, 1986; Xie & Rothblum, 1992).

Promoter Selectivity Factor 1 (SL-1)—An intrinsic property of gene transcription by RNAP I is the species specificity of the initiation reaction. Studies have shown that the factor SL-1 in humans (Rib 1 in *Xenopus* (McStay *et al.*, 1991), TIF-IB or Factor D in mouse (Grummt *et al.* 1990), and rSL1 in rat (Smith *et al.*, 1990) interacts with the ribosomal gene promoter and is responsible for conferring the species-specific transcription of rDNA (Bell *et al.*, 1990). Human SL-1 cannot replace mouse TIF-IB in transcription assays as well as TIF-IB cannot reprogram human polymerase machinery to initiate transcription on the mouse promoter. SL-1 is a multiprotein complex which functions to interact with the basal element of the rDNA promoters forming a preinitiation complex which recruits RNAP I to the template. SL-1 is composed of the TATA binding protein (TBP) and TBP associated factors (TAFs) (Comai *et al.*, 1992). TBP is a universal transcription factor which is also involved in class II and III gene transcription (Rigby, 1993; Sharp, 1992). Evidence has been presented in which a chimeric complex of hTBP and mouse TAF's exhibited specificity for the mouse promoter. This suggests that TBP can be exchanged between human and mouse factors and that the TAFs associated with SL-1 provide polymerase specificity of transcription. Furthermore, UV crosslinking experiments have also shown that the largest TAFs bind to the rDNA promoter supporting the direct role of these proteins in species specificity (Rudloff *et al.*, 1994). In the human reconstituted system, SL-1 requires the initial binding of UBF to the promoter in order to form a stable preinitiation complex (Bell *et al.*, 1990; Bell *et al.*, 1989). In contrast, the mouse factor (TIF-IB) does not require the presence of UBF to interact with the promoter although UBF stabilizes this interaction. The formation of a preinitiation complex by SL-1 is capable of directing multiple rounds of RNA polymerase recruitment to the promoter.

Polymerase I Associated Factors—Additional factors have been identified as copurifying with RNAP I, but are not tightly bound subunits. TIF-IC has been identified as

a constitutive factor with a native mass of 65 kDa (Schnapp *et al.*, 1994a). It has been suggested that TIF-IC is required for assembly of detergent resistant initiation complexes and formation of the first phosphodiester bond. These studies also indicate that TIF-IC acts to stimulate overall transcription elongation rate by possibly reducing the time polymerase spends in pause sites (Schnapp *et al.*, 1994b). The activity of this factor closely resembles that of TFIIF (RAP30/74) in class II gene transcription. TFIIF has two subunits which have separable functions, one of which plays a role in initiation and the other plays a role in elongation (Conaway, 1991; Conaway, 1990). There are several functional properties which TIF-IC and TFIIF have in common. Both TIF-IC and TFIIF interact with the polymerase and this interaction suppresses nonspecific initiations. They are both required for stable association of RNA polymerase with the respective promoters for the formation of productive initiation complexes. Finally, both play a role in initiation and elongation (Conaway & Conaway, 1991; Schnapp *et al.*, 1994a). The fact that other laboratories have not identified or characterized factors which participate in elongation can be attributed to the association of this factor with the polymerase. Therefore, this factor could be a component of their crude polymerase fraction. Alternatively, differences in assay conditions could also be a contributing factor in the dispensability of this factor in their reconstituted systems.

Besides TIF-IC, several laboratories have identified factors in the mouse reconstituted system which are required to convert a stable preinitiation complex into a productive initiation complex. This activity, which has been called TIF-IA, TFIC (Mahajan *et al.*, 1990; Mahajan & Thompson, 1990), and factor C* (Brun *et al.*, 1994), may represent the same biochemical component isolated under various conditions. This conclusion remains to be verified since there are no antibodies to these factors and none have been cloned. TIF-IA factor associates very tightly with the polymerase and conveys a specific initiation competence to RNAP I. It is thought that after initiation, TIF-IA is liberated from the initiation complex and facilitates transcription from templates bearing preinitiation

complexes lacking TIF-IA. Moreover, the rate of reinitiation is increased by TIF-IA activity (Schnapp *et al.*, 1990). In this regard, TIF-IA may be considered a functional homologue to the bacterial σ^{70} factor. TIF-IA has also been implicated in growth-dependent regulation of rDNA transcription which will be discussed later in further detail. Figure 3 illustrates a model for the formation of the RNAP I initiation complex in mouse.

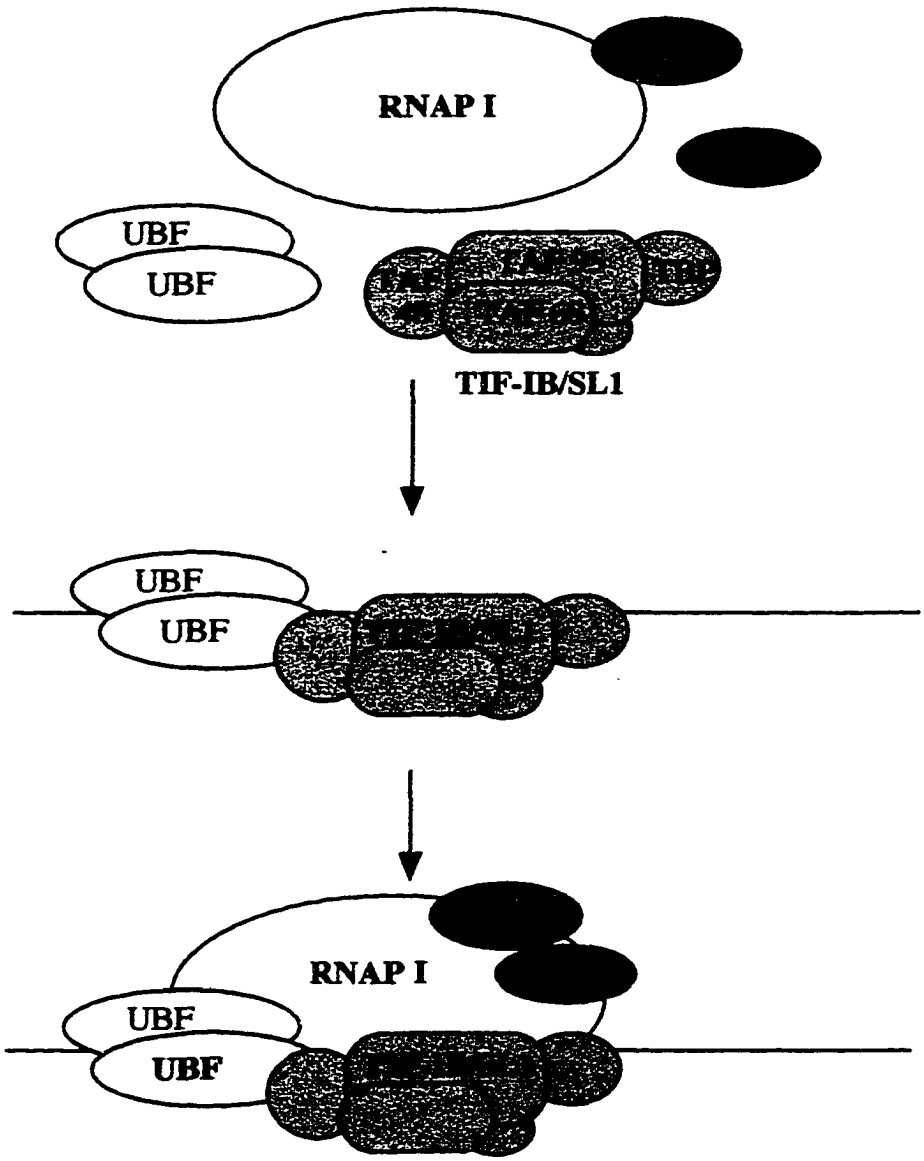


Figure 3: Model for the formation of the RNAP I initiation complex in mouse. The TBP-TAF complex, TIF-IB, and UBF, first bind to the core element of the rDNA promoter. Next, RNAP I and the two associated factors, TIF-IA and TIF-IC, are recruited via protein-protein interactions.

Yeast RNAP I transcription complex—The RNA polymerase I enzyme from yeast has been purified and all of the subunits have been cloned. Yeast RNAP I is composed of 14 distinct subunits each of which has been genetically tested for its function in rRNA transcription (Thuriaux *et al.*, 1995). The subunits of this enzyme can be classified into three groups: i) four core subunits: β' like (A190), β-like (A135) and two similar to the bacterial α subunits (AC40 and AC19); ii) five subunits common to all three RNA polymerases: ABC27, 23, 14.5, 10a, 10b; and iii) five RNAP I specific subunits: A40, 43, 34.5, 14, 12.2 (Sentenac *et al.*, 1992).

The majority of the RNA polymerase I subunits are essential for growth except for the A34.5 and A49 subunits which are not strictly required. Experimental evidence has demonstrated that the two largest subunits, which contain zinc fingers, cross-link to nascent chain RNA (Sentenac *et al.*, 1992). The A135 subunit contains a putative nucleotide binding domain suggestive of a role in elongation (Riva *et al.*, 1987; Sentenac *et al.*, 1992). The overall structure of yeast RNAP I has been determined by examination of the two-dimensional crystals by electron microscopy (Schultz *et al.*, 1993). The location of the major subunits within this structure have been determined by immunoelectron microscopy (Klinger *et al.*, 1996).

The laboratory of Masayasu Nomura has taken advantage of the ease with which yeast can be genetically altered and used genetic techniques to identify transcription factors specifically required for RNAP I transcription. Their screen involves the use of strains carrying the 35S rRNA gene fused to the GAL7 promoter on a plasmid. Mutants were isolated which were specifically defective in transcription of the chromosomal rDNA by RNAP I, but whose growth could be rescued by the synthesis of the rRNA by RNAP II. This screen has identified a variety of protein complexes which have similarities to the transcription factors in the higher eukaryotes.

One complex known as the core factor (CF) was found to be composed of three proteins encoded by RRN6, RRN7, and RRN11 genes (Keys *et al.*, 1994). This complex is essential for forming a transcription-competent preinitiation complex although stable binding of this factor with the promoter is dependent on the initial binding of other factors. Additionally, CF has been shown to interact weakly with TBP *in vitro*. Nomura and co-workers have hypothesized that CF is the functional homologue to the metazoan SL-1 factor (Figure 4).

Another factor identified by this screen, UAF (upstream activating factor), has also been characterized (Keys *et al.*, 1996). UAF is a multiprotein complex containing five proteins, the Rrn5, Rrn9, Rrn10 proteins and histone 3 and 4. This factor interacts with the upstream element of the promoter and greatly stimulates *in vitro* rDNA transcription but unlike the CF it is not essential. Template commitment experiments demonstrated that UAF is apparently bound stably to the template and is necessary and sufficient for template commitment. Nomura's group also studied the interaction of TBP with these two complexes and reported that TBP interacts most strongly with UAF. Moreover, their results indicate that TBP together with UAF participates in the recruitment of CF to the rDNA promoter to form a stable preinitiation complex.

The third transcription factor identified and characterized by this genetic screen is encoded by the RRN3 gene (Yamamoto *et al.*, 1996). This 72 kDa protein is specifically required for rDNA transcription by RNAP I. It does not appear to be involved in the formation of stable preinitiation complexes. Rather, it interacts directly with RNA polymerase I stimulating its recruitment to the promoter. More specifically, the rrn3p interacts *in vitro* with the A49 and A34.5 subunits which are unique to RNAP I.

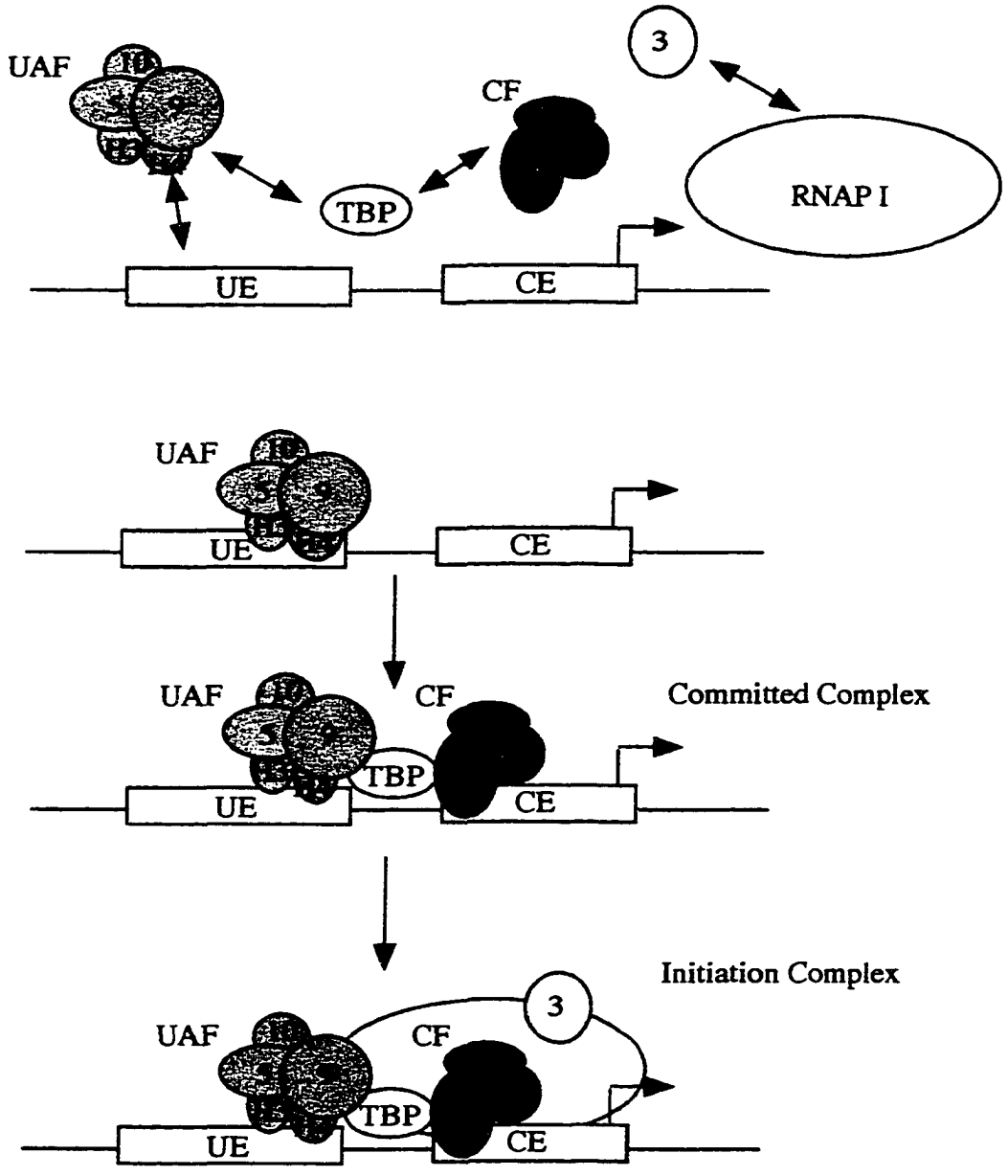


Figure 4: Model for the formation of the RNAP I initiation complex in yeast. The two elements of the yeast rDNA promoter, the upstream element (UE) and the core element (CE) are shown as open boxes. The upstream activating factor (UAF), which is composed of the Rrn5p, Rrn9p, and Rrn10p proteins and histones 3 and 4, binds to the UE. The TATA-binding protein (TBP) and the core factor (CF) join the UAF and bind to the CE to form a committed complex. The RNAP I and the Rrn3p protein are recruited to the promoter to form the initiation complex. Double-headed arrows indicate observed interactions between UE, UAF, CF, and TBP (reviewed in Milkereit *et al.*).

Our laboratory has designed a protocol to begin the biochemical dissection of the components of the rDNA transcription complex allowing for *in vitro* experiments to determine the molecular mechanisms regulating rRNA synthesis (Riggs *et al.*, 1995). We have identified three biochemical fractions required for the specific transcription of the rDNA. A high Q column developed with a KCl gradient is used to fractionate the transcriptional machinery from a crude cell extract into three biochemical fractions Q-A, Q-B, and Q-C. Characterization of these components by non-specific transcription assays has revealed that all of the RNA polymerase activity is present in the Q-B fraction. This activity was due to the presence of RNAP I as it was not affected by α-amanitin or tagetin which are inhibitors of RNAP II and RNAP III activity respectively. Gel retardation and template commitment assays identified fraction Q-A to contain the factor(s) necessary for the formation of the preinitiation complex which commits the template to transcription. In addition, DNase footprinting indicates that this fraction interacts with two domains of the promoter which have been shown to be critical for stable transcription complex formation.

The objective of part one of this dissertation is two fold: i) Chapter Two examines the environmental conditions under which rRNA synthesis is regulated in yeast and ii) Chapter Three examines which component of the transcription machinery is the target of the regulatory signals.

Chapter 2
Regulation of the RNA polymerase I and III transcription systems in response to growth conditions

Eileen M. Clarke, Cheryl L. Peterson, Aaron V. Brainard and Daniel L. Riggs*

Abstract

To better understand the mechanisms that regulate stable RNA synthesis, we have analyzed the RNA polymerase I and III transcriptional activities of extracts isolated from cells propagated under a variety of conditions. Under balanced growth conditions the levels of both RNA polymerase I and III specific transcription increased proportionally with growth rate. Upon nutritional starvation, RNA polymerase I transcription rapidly declined, followed by 5S rDNA, and eventually tDNA transcription. Transcriptional activities in extracts were restored when the non-growing cultures were resuspended in fresh medium, even though growth did not resume. The differential expression of 5S rDNA and tDNA genes in extracts prepared from cells subjected to partial starvation was traced to a 5S rDNA-specific inhibitor, and not to a defect in any RNA polymerase III transcription factor. Characterization of this inhibitor indicated that it was not 5S rRNA. It was sensitive to phenol extraction, resistant to RNase, and its target did not appear to be TFIIIA. Not all treatments that slowed or stopped growth down-regulated the stable RNA transcription apparatus. Cells which have been subjected to either energy starvation or cycloheximide treatment still retain the ability to synthesize stable RNA *in vitro*, suggesting the presence of alternative regulatory mechanisms.

Introduction

It has been appreciated for a number of years that organisms adjust their translational capacity to meet, but not exceed, the need for protein synthesis. A central aspect of this regulation is the control of stable RNA (tRNA and rRNA) production. In prokaryotes the three rRNA genes are cotranscribed with a number of tRNA genes by the same RNA polymerase, providing a simple target of regulation, initiation of transcription. In eukaryotes three RNA polymerase complexes are responsible for stable RNA synthesis. RNA polymerase I (RNAP I)[1] produces the 35S rRNA molecule which is processed into the three largest rRNAs, while the smallest rRNA and tRNAs are produced by RNAP III. *In vivo* analyses of *Saccharomyces cerevisiae* under a variety of treatments clearly establish a direct link between translational load, stable RNA synthesis and ultimately ribosome biogenesis. Under some conditions the coordinate synthesis of both rRNA and tRNA is observed. For example, cells with slower balanced (constant) growth rates, have decreased levels of both rRNA and tRNA synthesis, although tRNA synthesis is decreased to a lesser extent (Waldron, 1977). Similar coordinated regulation is observed during some unbalanced, transitory, growth conditions. Upon nitrogen starvation, both rRNA and tRNA synthesis are quickly shut off (Oliver & McLaughlin, 1977). Likewise, in response to a nutritional upshift, the synthesis of both rRNA and tRNA rapidly increases, although rRNA at a faster rate (Kief & Warner, 1981; Ludwig, 1977; Waldron, 1977). In some cases the rates of rRNA and tRNA synthesis are uncoupled. Upon amino acid starvation, rRNA synthesis is diminished by about 80%, while tRNA synthesis is only modestly affected (Oliver & McLaughlin, 1977; Shulman *et al.*, 1977). Regulation of rRNA or tRNA synthesis has also been observed in higher eukaryotes in response to a variety of additional treatments. These include hormones (Cavanaugh & Thompson, 1983), the tumor-

promoting phorbol ester TPA (Garber *et al.*, 1991; Vallett *et al.*, 1993), and entry into the encystment phase in *Acanthameoba* (Paule *et al.*, 1984).

The molecular basis of the regulation of rRNA synthesis by RNAP I has been examined in several organisms under a rather limited spectrum of conditions (reviewed in references (Paule, 1993; Reeder, 1992; Sollner-Webb & Tower, 1986). Because of technical considerations, studies in higher eukaryotes have been largely confined to the examination of cells in unbalanced growth (Bateman & Paule, 1986; Grummt, 1981; Tower & Sollner-Webb, 1987). In these cases, this response is due to the inactivation of a factor found associated with RNAP I. This factor, known as C*, TIFI-A, or TFIC (Brun *et al.*, 1994; Buttgereit *et al.*, 1985; Mahajan *et al.*, 1990; Tower & Sollner-Webb, 1987) is necessary for formation of the initiation complex, and is inactivated early in the transcription cycle (Brun *et al.*, 1994; Mahajan *et al.*, 1990; Schnapp *et al.*, 1990). Although the modification of a factor associated with RNAP I has been the best studied regulatory response, several lines of evidence suggest the presence of other regulatory mechanisms, including the modification of an RNAP I transcription factor (Larson *et al.*, 1993; O'Mahony *et al.*, 1992b; Voit *et al.*, 1992), or the accumulation of specific inhibitors (Kermekchiev & Muramatsu, 1993; Kuhn, 1995). Less is known about the molecular basis of RNAP III regulation. The transcription factor TFIIIB is inactivated during cessation of growth (Dieci *et al.*, 1995; Gokal *et al.*, 1986; Sethy *et al.*, 1995; Tower & Sollner-Webb, 1988) and mitosis (Gottesfeld *et al.*, 1994; White *et al.*, 1995). A transcriptional inhibitor which interacts with the TATA-binding protein in TFIIIB has been identified, although it function in regulation is not clear (White *et al.*, 1994). In contrast, viral infection and serum factors have been shown to alter the activity of the TFIIIC fraction (Fradkin *et al.*, 1987; Hoeffler *et al.*, 1988). Recently the differential expression of the 5S rRNA and tRNA genes during encystment in *Acanthamoeba castellanii* has been attributed to the disappearance of the 5S rRNA specific transcription factor TFIIIA.

Despite this progress, very little is known about the overall picture of stable RNA synthesis in any one organism, as few studies have examined both the RNAP I and III transcription complexes under a variety of conditions. There are compelling reasons to address these questions using *Saccharomyces cerevisiae*. The ease with which yeast are cultivated in defined media and the availability of a number of genetic backgrounds facilitate the manipulation of balanced and unbalanced growth rate by altering the growth media. Despite these advantages, virtually all of the work in yeast has been restricted to *in vivo* analysis, largely due to the technical difficulties of isolating RNAP I and III transcription extracts from small quantities of cells. To facilitate the *in vitro* analysis of stable RNA transcription, we recently developed a method for the preparation of both RNAP I and RNAP III (5S rDNA and tDNA) transcription extracts from less than one gram of cells (Riggs *et al.*, 1995). This protocol minimizes the chance of inactivation due to trivial reasons, as no column chromatography is involved, and only at the last step is the RNAP I extract separated from the RNAP III extract. Here we describe the analysis of stable RNA synthesis in extracts prepared from cells which have been subjected to a variety of different growth conditions.

Materials and Methods

Plasmids— The plasmid pDR10 linearized with *Eco*RV was used to assay for 35S rRNA synthesis by RNAP I (Riggs *et al.*, 1995). The 5S rDNA gene used in transcription and footprinting experiments was contained on plasmid pBB111R (Braun *et al.*, 1989). The plasmid pTZ1 (Kassavetis *et al.*, 1989), which contains the *SUP4* $tRNA^{Tyr}$ gene with a G62 to C promoter up mutation, was used for tDNA transcription assays.

DNase footprinting—The probe used for footprinting was the 5 S rDNA-containing *Eco*RI - *Hin*dIII fragment from pBB111R. The *Eco*RI site was labeled by filling in the 3' recessive end with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I. Chromatographic fractions were incubated for 20 m at 30°C with 2 fmol of probe in reaction containing 20 mM Tris acetate pH 7.5, 200 mM potassium glutamate, 10 mM magnesium acetate, 10 mM B-mercaptoethanol, 10% (v/v) glycerol, 0.5% (w/v) polyvinyl alcohol, and 100 ng of vector DNA (pBSKSII-) in a total volume of 20 µl. Samples were digested with 0.05 to 0.1 units of DNase I (RNase-free; Boehringer-Mannheim) for 0.5 to 2 m at 30°C. Digestion was terminated with the addition of 10 µl of stop mix which contained 75 mM EDTA, 0.5 mg/ml sheared salmon sperm DNA and 1.7 M potassium acetate. Samples were extracted with phenol-chloroform and precipitated with ethanol. The pellets were resuspended in formamide load buffer and run on 10% polyacrylamide (37.5:1, acrylamide:bisacrylamide) containing 8 M urea.

Growth of cultures—The yeast strain O22 (*MATa his2-1*) was used for the steady state growth, as well as the histidine starvation experiments (Figs. 1 and 4, respectively). This strain was selected because of its genetic background and high growth rate in minimal medium. In these experiments strain O22 was cultivated in yeast carbon base (Difco) containing 2% w/v glucose and 20 µg/ml histidine (YCB+his). This medium was supplemented with various nitrogen sources (8 mM): ammonium sulfate, glutamine, valine,

or tyrosine. For the balanced growth experiments, an overnight culture grown in YCB+his+ammonium sulfate medium was used to inoculate YCB+his medium containing the appropriate nitrogen source. These cultures were incubated for 2-3 generations after the new balanced growth rate was achieved before they were harvested. To elicit histidine or nitrogen starvation, a low density (<1.0 A_{595} unit) exponential phase culture growing in YCB+his+ammonium sulfate was diluted with approximately ten volumes of fresh warm media lacking either histidine or ammonium sulfate. Further dilutions into fresh, warm medium were made to keep cell density less than 1.0 A_{595} unit at all times. In all cases supplementation of the starved cultures with the missing nutrient restored growth. Strain JHRY20-2CΔ1 grown in YEP (1% w/v yeast extract, 2% w/v peptone, adjusted to pH 5.5 with HCl) supplemented with glucose (2% w/v, unless otherwise noted) was used in the remaining experiments. Growth into stationary phase (Fig. 2) has been described in ref. (Riggs *et al.*, 1995).

Chromatography of the transcription extracts and characterization of the fractions—The protocols for cell breakage, extract preparation, Q chromatography, and transcription assays have been previously described (Riggs *et al.*, 1995). For the chromatography of the RNAP III factors the "low salt supernatant" was chromatographed on a Q column developed with a 50 to 700 mM KCl gradient. The inhibitor was removed from 5S $rRNA^-/tRNA^+$ extracts by adjusting the extract to 500 mM KCl and loading on a Q column (Macro-prep® high load, Bio-Rad; 10 mg protein load per ml resin), and the flow through was collected and assayed. Fractions were treated with RNase by incubation in the presence of RNase A immobilized on acrylic beads (Sigma, catalog number R-7005) which had been prepared in the following manner. First, approximately 3 mg of RNase beads was extensively washed with 1 ml volumes of water three times. Protein binding sites on the beads were blocked by incubation in the presence of 50 μg BSA in a volume of about 100 μl at room temperature for 30 min followed by another extensive water wash. One hundred

μl of the Q-550 fraction was added to the moist beads and incubated at room temperature for 30 min with occasional gentle mixing. The supernatant was withdrawn and passed through a small empty chromatography column to remove the residual beads. Digestion of the RNA was verified by denaturing polyacrylamide gel electrophoresis of the treated sample.

Results

In the experiments described below, we examined the RNAP I and III transcriptional capacity of extracts prepared from cells in balanced and unbalanced growth. In exponential phase, cells are in balanced growth, that is all cellular constituents are synthesized at a constant rate. In contrast, changes in environmental conditions provoke unbalanced growth conditions where the cellular components are differentially expressed, which enables the cell to adapt to the altered environment. If the new conditions permit growth, this transient phase of unbalanced growth yields to a new balanced growth phase, at a growth rate determined by the new growth conditions.

Balanced growth rate regulation of stable RNA synthesis—We examined cultures growing at decreasing growth rates under steady state, balanced growth conditions. In these experiments the cell density was kept low (less than 1.0 A595 unit) by diluting the culture into fresh, warm medium. The strain O22 was cultured in a minimal medium with glucose as the carbon/energy source and either ammonium sulfate, glutamine, valine or tyrosine as the sole nitrogen source. These cultures had generation times of 1.5, 3, 5 and 8 hours, respectively. The cells were harvested, and RNAP I and III transcription extracts (low salt pellets and supernatants) were prepared as previously described (Riggs *et al.*, 1995). The levels of specific RNAP I and III transcription were analyzed *in vitro* using either a 35S rDNA (to assay RNAP I), 5S rDNA, or tDNA template. Extracts prepared from the cells having a reduced balanced growth rate supported reduced levels of both RNAP I and III transcription (Fig. 1), although RNAP I transcription was the most sensitive to the decreased growth rate. We have also observed similar results in response to changes in growth rate brought about by the substitution of different carbon/energy sources in a rich medium (for example see Fig. 6*C*). This adjustment of the RNAP I and III transcriptional

activities in response to a range of balanced growth rates appears to be sufficient to account for the regulation of stable RNA synthesis observed under balanced growth condition *in vivo*.

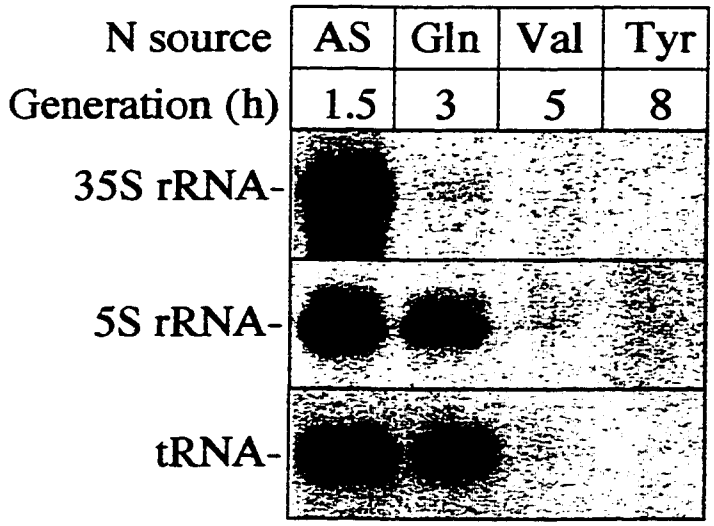


Figure 1: Stable RNA synthesis in extracts prepared from cultures having decreasing steady state growth rates. Extracts were prepared from cultures grown in defined media containing either ammonium sulfate (AS), glutamate (Gln), valine (Val) or tyrosine (Tyr) as the sole nitrogen source with the indicated generation (doubling) times. The synthesis of the 35S rRNA transcript by RNAP I was assayed in the low salt pellets, while 5S rRNA or tRNA synthesis by RNAP III was determined in the corresponding supernatants, as described in materials and methods.

Differential regulation of stable RNA synthesis during entry into stationary phase—We have also examined the cellular response to the imposition of unfavorable growth conditions. Previously, we characterized inactivation of RNAP I transcription during the transition between exponential phase, where glucose is fermented and the cells grow with a generation time of 1.5 h, and stationary phase (Riggs *et al.*, 1995). In this study we have extended this analysis by characterizing the 5S rDNA and tDNA transcriptional activities of extracts isolated from cells during the transition phase. Three sequential samples were taken from a transition phase culture (samples A, B, and C in Fig. 2, *top*). RNAP III transcription extracts prepared from these samples were assayed for tDNA and 5S rDNA transcription. While in early transition phase the RNAP III complexes were equally active on both templates, as the culture progressed further into the transition phase, a striking decease in 5S rDNA transcription was observed (*bottom*). Extracts prepared from the culture in mid-transition phase (such as sample B) were slightly decreased in tDNA transcriptional activity, while 5S rDNA activity was almost totally abolished. We have observed this differential expression in all extracts prepared from high density cultures. The persistent tRNA synthetic capacity in these slowly growing cells (generation times of greater than 24 h) in unbalanced growth is in sharp contrast to the lack of significant tRNA synthesis in extracts made from slowly growing cells in balanced growth (8 h generation time, Fig. 1).

It has been reported recently that several characteristics of stationary cells can be reversed by incubation in the presence of glucose (Granot & Snyder, 1993). To determine if stable RNA synthesis can be restored, we replaced the spent growth medium (in which all of the glucose has been consumed) in transition phase cultures (like culture B in Fig. 2) with fresh growth medium. RNAP I transcription and 5S rRNA synthesis in extracts, which had been turned off completely, were activated by this treatment, and tRNA synthesis

was further stimulated (Fig. 3*A*). When cycloheximide was in the recovery medium no activation occurred (lane 3). This activation was transient, as extracts prepared from cultures which had been incubated for longer than 1.5 h had significantly reduced levels of stable RNA synthesis (Fig. 3B). No significant growth (cell division) was observed, presumably because of the high cell density, during the incubation period in fresh growth medium, and the only visible change in cell morphology was the appearance of buds which correlated with the peak of activation. Unlike other characteristics of stationary phase cells, resuspension in a glucose solution was not sufficient to activate stable RNA synthesis. Only in the presence of glucose in a complete medium (either fresh or spent) were RNAP I and III transcription activated.

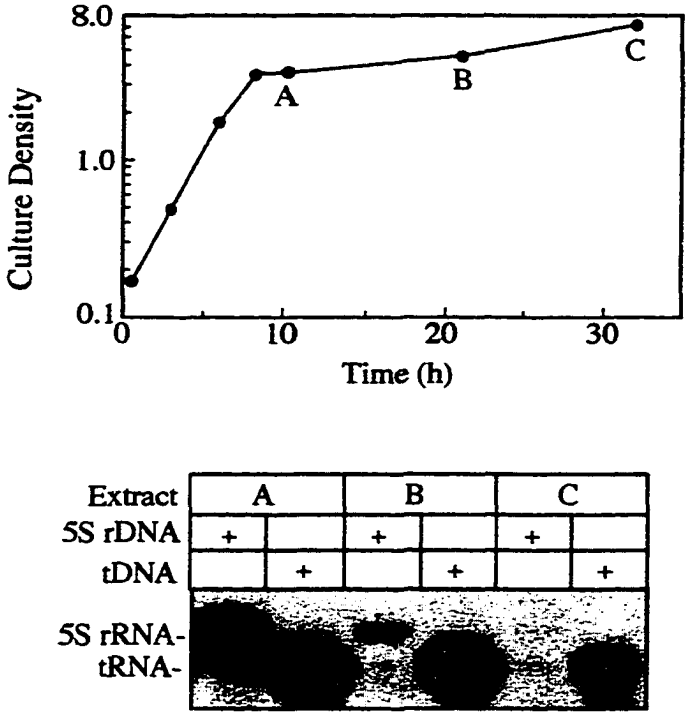


Figure 2: Stable RNA synthesis in extracts prepared from transition phase cultures. Three samples of a culture growing in YEP glucose (2% w/v) were collected at the indicated times during the transition phase (the culture density was measured in A595 units). RNAP III extracts were prepared from each sample (A, B and C) and assayed with either a 5S rDNA or tDNA template (bottom). None of the three extracts contained RNAP I activity (not shown).

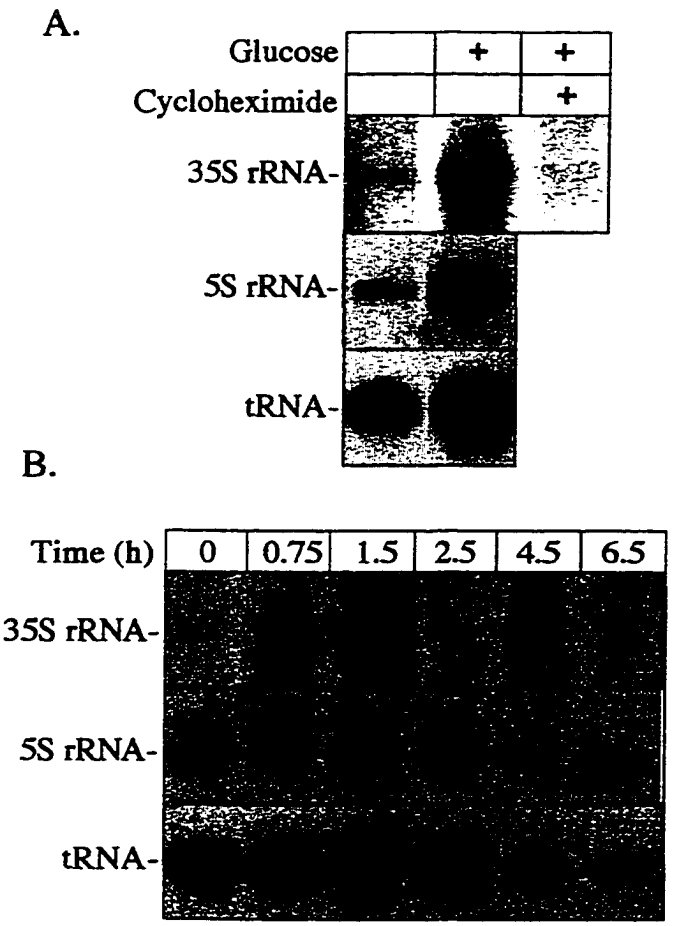


Figure 3: Resuspension of transition phase cultures in fresh growth medium activated RNAP I and III transcription. A) A portion of a high density cell culture was resuspended in fresh YEP containing 2% (w/v) glucose, which was in one case supplemented with cycloheximide. After 1.5 h incubation the cells were harvested and RNAP I and III transcription extracts were prepared. The extracts were analyzed for RNAP I activity with a 35S rDNA template, or RNAP III activity on either a 5S rDNA or a tDNA template. B) Time course of activation and subsequent inactivation. Extracts were prepared from cultures which has been resuspended in fresh medium and incubated for the times indicated.

Starvation for essential nutrients regulates stable RNA transcription —One of the classical downshift conditions which has been extensively studied in prokaryotes is starvation for an essential amino acid. The collective change in gene expression, turning off rRNA and tRNA synthesis and turning on amino acid biosynthetic genes, is termed the stringent response. To examine this response in yeast, we shifted a culture of a histidine auxotroph from minimal medium containing histidine into one lacking histidine. Under these conditions, the culture continues to grow at a 1.5 h doubling time as internal histidine pools are utilized, then it gradually stops growing (Fig. 4, *top*). An extract prepared from a culture having a reduced growth rate (extract A) did not support RNAP I or 5S rDNA transcription while tRNA synthesis continued (Fig. 4, *bottom*). An extract prepared from the culture after growth had ceased (extract B) was totally defective in stable RNA transcription. This response is specifically due to starvation for histidine, as supplementation of the nongrowing culture with histidine restores growth. The inactivation of the transcription we observed is sufficient to account for the noncoordinated synthesis of rRNA and tRNA in response to amino acid starvation *in vivo* (Oliver & McLaughlin, 1977; Shulman *et al.*, 1977). Using a similar approach we also examined the effect of starvation for nitrogen on RNAP I and III transcription in extracts (not shown). Within two hours after the growth rate changed, RNAP I transcription was turned off. Once again, when growth had ceased, all stable RNA synthesis was eliminated, paralleling what has been observed *in vivo* (Oliver & McLaughlin, 1977).

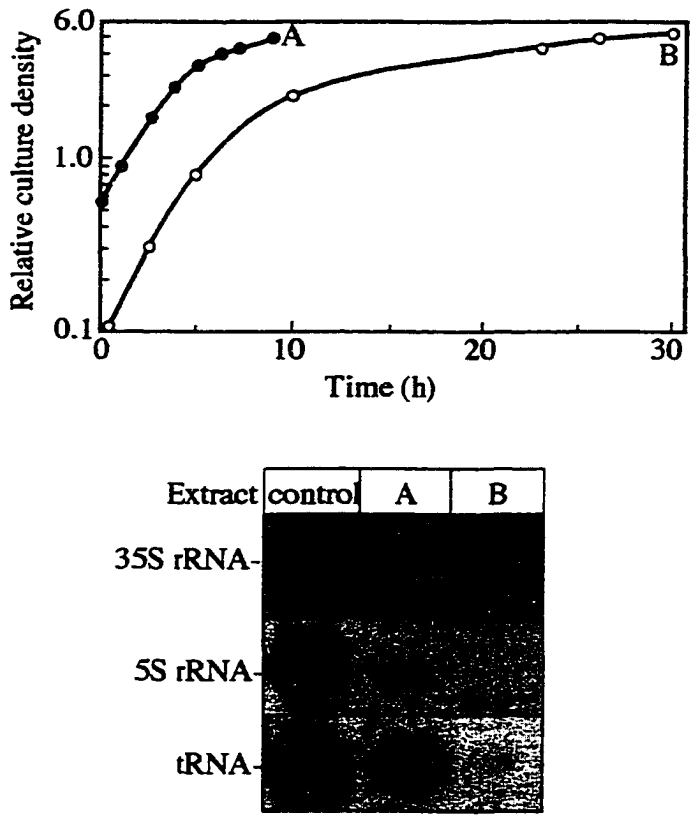


Figure 4: Stable RNA synthesis in cultures subjected to histidine starvation. Cultures of a histidine auxotroph grown in minimal media were deprived of histidine by dilution with fresh warm media lacking histidine. At all times the cultures were kept at a low cell density (less than 1.0 A595 unit); the growth curve (*top*) is the *relative* cell density (corrected for dilutions) plotted against time. RNAP I and III extracts prepared from two independent cultures (A and B) were analyzed for the synthesis of 35S rRNA, 5S rRNA and tRNA (bottom). The control extracts were prepared from exponential phase cells collected from a culture grown in minimal medium containing histidine.

Growth rate can be can be altered without affecting the activity of components of the stable RNA transcription systems—Numerous studies suggest the activities of the RNAP I and III transcription systems are directly regulated by growth rate. We have identified several conditions under which the growth rate significantly decreases without altering the integrity of any RNAP I or III transcription factors required for specific transcription *in vitro*.

Addition of the protein synthesis inhibitor cycloheximide to a culture in exponential phase results in the eventual cessation of cell growth. Within several hours after addition, growth ceased at a cell density considerably lower than that of untreated cultures (Fig. 5, *top*). To our surprise RNAP I and III extracts prepared from these cycloheximide treated cells were very active, even when protein synthesis had been inhibited for as long as 15 hours (Fig. 5, *bottom*). Numerous extracts have been prepared from cycloheximide treated cultures, and as long as the addition was made to the cells while they were in mid-exponential phase (several generations before leaving exponential phase), the extracts were all very active. We have observed that the cycloheximide treatment for long periods of time made the cells much easier to break open. To preserve the transcriptional activities, the breakage with glass beads had to be carefully monitored to avoid excessive cell lysis, which inactivates extracts. These results with cycloheximide appear to be at odds with those of Dieci *et al.* (Dieci *et al.*, 1995), who observed specific inactivation of two components of the RNAP III factor TFIIIB in response to cycloheximide treatment. This discrepancy may be due to the cell density at which the cycloheximide was added, or differences between strains.

A second approach to examining the relationship between growth rate and stable RNA transcription was the manipulation of the energy source. When energy is derived from glucose fermentation in a rich medium, cultures grow at the same rate regardless of the extent of aeration. But in nonaerated cultures which contained limiting amounts of glucose (1% w/v), growth immediately ceased when the glucose was exhausted (Fig. 5, *top*), as the

remaining carbon sources were not fermentable and there was insufficient oxygen present for respiration. These nongrowing cells were essentially energy starved. Growth immediately resumed if glucose was added to these cultures or if the cultures were aerated. Extracts prepared from cultures which had been energy starved for as long as fifteen h retained significant specific RNAP I and III transcriptional activities (Fig. 5, *bottom*). A second approach we used to elicit energy starvation was to supplement a culture growing in a rich medium containing glucose with the non-metabolizable glucose analog glucosamine. Glucosamine inhibits the intracellular accumulation of glucose *in vivo*, possibly by acting as a competitive inhibitor of hexokinase, which is associated with the high affinity glucose uptake system (McGoldrick & Wheals, 1989). Glucosamine at low concentrations in the presence of glucose does not significantly alter glucose mediated catabolite repression, thus minimizing the changes in cellular metabolism which might be encountered when changing from glucose to a nonfermentable carbon source. When glucosamine was added to an exponential phase culture growing in YEP glucose (2% w/v), the growth rate was decreased to a doubling time of about 10 hours (Fig. 5, *top insert*). Despite this slow growth rate, significant RNAP I and III activities were observed (*bottom*). When glucosamine was added to a higher concentration and incubation was continued until cell growth ceased, RNAP I activity was turned off, while RNAP III transcription persisted (not shown).

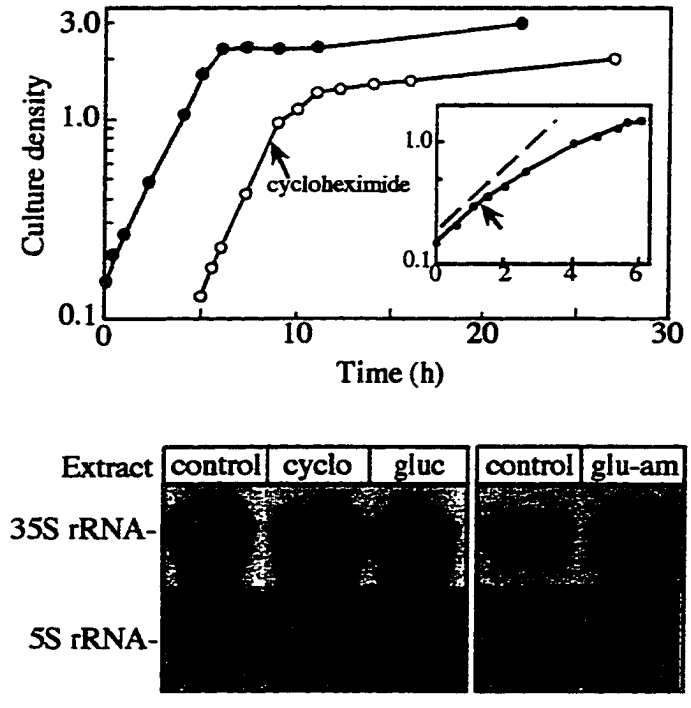


Figure 5: The RNAP I and III transcription systems were not regulated in response to all treatments that inhibit growth. Energy starvation was elicited by growing a culture without aeration in YEP medium containing limiting (1%) glucose (*top*, closed circles) or by the addition of glucosamine to an exponential phase culture growing in YEP containing 2% glucose (*top graph inset*). Glucosamine (1.5% w/v final concentration) was added at the time indicated by the arrow, and the dashed line represents the 1.5 h generation time of an exponential phase culture. Inhibition of protein synthesis was achieved by the addition of cycloheximide to an exponential phase culture (open circles). Cycloheximide was added to a final concentration of 10 μg/mL at a cell density corresponding to 0.8 A595 units (arrow). Extracts prepared from the samples taken at the last data point were assayed for RNAP I and III (5S rRNA) activities (bottom: lanes 1 and 4, control extracts from exponential phase cells; *2*, cyclohexamide treated cells; *3*, glucose starved cells; and *5*, glucosamine treated cells).

Restoration of RNAP I and III transcription in inactive extracts—Based upon our previous results (Riggs *et al.*, 1995), and by analogy to other systems examined, one might predict that specific RNAP I and III transcription are regulated by inactivating one of the essential transcription factors. We sought to identify the target of these responses to different environmental conditions by restoring transcription in inactive extracts with chromatographic fractions prepared from active extracts. One goal of these experiments was to determine if the responses to the different environmental insults shared a common target in the transcription apparatus. For example, do balanced growth rate control (such as slow growth on a poor nitrogen source) and the yeast "stringent response" both regulate the same component of the RNAP I or III transcription complexes?

Extracts prepared from transition phase cells, which do not contain RNAP I transcriptional activity, can be restored by the addition of the RNAP I B fraction, which is one of the three chromatographic fractions required to reconstitute specific RNAP I transcription (Riggs *et al.*, 1995). The B fraction, which is inactive alone, fully restored activity to all of the inactive RNAP I extracts that we have examined (Fig. 6*A-C*). The B activity has been purified over several different columns, and in each case, the B activity (defined as the activity which restores specific RNAP I transcription in the presence of the RNAP I A and C activities), the RNAP I non-specific transcriptional activity, and the ability to restore inactive extracts, have co-purified. Yeast appears to regulate the response to all these diverse environmental changes through a common mechanism, the modification of a factor which co-fractionates with the RNAP I enzyme.

In a similar manner, we identified the chromatographic fraction that restored specific RNAP III activity in extracts prepared from treated cells. Active RNAP III (5S $rRNA^+/tRNA^+$) transcription extracts were loaded onto a Q column which was developed with a KCl gradient. The fraction eluting in 250 mM KCl ("Q-250" fraction) was sufficient to restore 5S rDNA transcription in extracts from nitrogen starved cells and slowly growing

cells (Fig. 6D and E). The synthesis of 5S rRNA in a transition phase cell extract was also restored with this fraction to levels comparable the tDNA transcriptional activity (Fig. 6F). All of the inactive 5S rDNA transcription extracts examined were restored with the Q-250 fraction. This same fraction also restored tRNA synthesis to extracts prepared from slowly growing cultures (Fig. 6G). Thus it appears that a factor(s) in the Q-250 fraction is the target of regulatory mechanisms which are responsible for the coordinate, as well as discoordinate, regulation of 5S rRNA and tRNA synthesis which have been observed both in our extracts and *in vivo*.

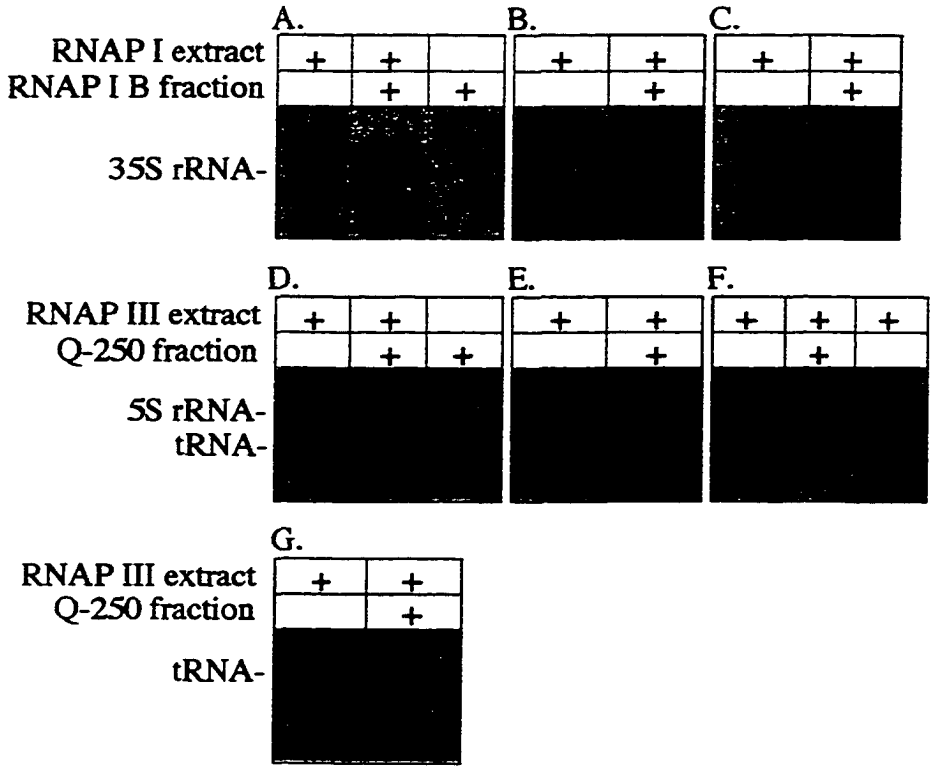


Figure 6: RNAP I and III transcription in extracts were restored by chromatographic fractions prepared from exponential phase cell extracts. RNAP I activity in extracts prepared from cultures which were either starved for nitrogen (*A*), starved for histidine (*B*), or grown in YEP with glycerol as the carbon and energy source (*C*), were all restored by the addition of the RNAP I B fraction (described in (36)). 5S rRNA synthesis in extracts from cultures which had been either starved for nitrogen (*D*), grown with valine as the sole nitrogen source, *E*), or were in transition phase (Fig. 2, *F*) was restored with the same Q-250 fraction. For comparison, tRNA synthesis in this extract was also assayed (*lane 3*). tRNA synthesis in an extract prepared from slowly growing cells (valine as the nitrogen source) was also restored by the same Q-250 fraction (*G*).

Identification of a 5S rRNA specific inhibitor—Either of two simple models could explain the selective inactivation of 5S rDNA transcription (such as in transition phase extracts). Either a 5S rDNA-specific factor in the Q-250 fraction becomes inactivated, or alternatively, an inhibitor interferes with the activity of a factor in the Q-250 fraction on 5S rDNA templates. To distinguish between these possibilities, we performed extract mixing experiments. The addition of a 5S $rRNA^-/tRNA^+$ extract to a 5S $rRNA^+/tRNA^+$ extract resulted in decreased 5S rRNA synthesis (Fig. 7*A*, lanes 2 and 3), suggesting the existence of a 5S rRNA specific inhibitor. This effect did not appear to be due to saturation of the transcription assay, as doubling the amount of the 5S $rRNA^+/tRNA^+$ extract increased the level of 5S rDNA transcription (lane 4).

If this specific inhibitor is solely responsible for the lack of 5S rRNA synthesis in these cell extracts, when the 5S $rRNA^-/tRNA^+$ extract is chromatographed, we should be able to 1) isolate the inhibitor in a chromatographic fraction, 2) show that the inhibitor abolishes the ability of a Q-250 fraction prepared from exponential cells to restore 5S rRNA synthesis, while not affecting the ability of this Q-250 fraction to rescue tRNA synthesis, 3) restore 5S rRNA deficient extracts with the Q-250 fraction derived from the 5S $rRNA^-/tRNA^+$ extract, and 4) restore 5S rRNA synthesis from the 5S $rRNA^-/tRNA^+$ extract by removing the inhibitor. To address these points we chromatographed a 5S $rRNA^-/tRNA^+$ extract on a Q column developed with a KCl gradient. Individual fractions were then assayed for the inhibitory properties of the extract from which they were derived. A fraction eluting in 550 mM KCl was found to have such an activity (Fig. 7B). When this Q-550 fraction was preincubated with a Q-250 fraction from a 5S $rRNA^+$ extract, the Q-250 fraction was no longer able to restore 5S rRNA transcription, although it could restore tRNA synthesis (7C). The most potent inhibition of 5S rRNA transcription required preincubation of the 5S $rRNA^-/tRNA^+$ and 5S $rRNA^+/tRNA^+$extract before addition to the transcription assay, suggesting that this inhibitory property is the result of direct

interactions between factors in these two extracts, interfering with 5S rRNA synthesis and not the stability of the transcript (not shown).

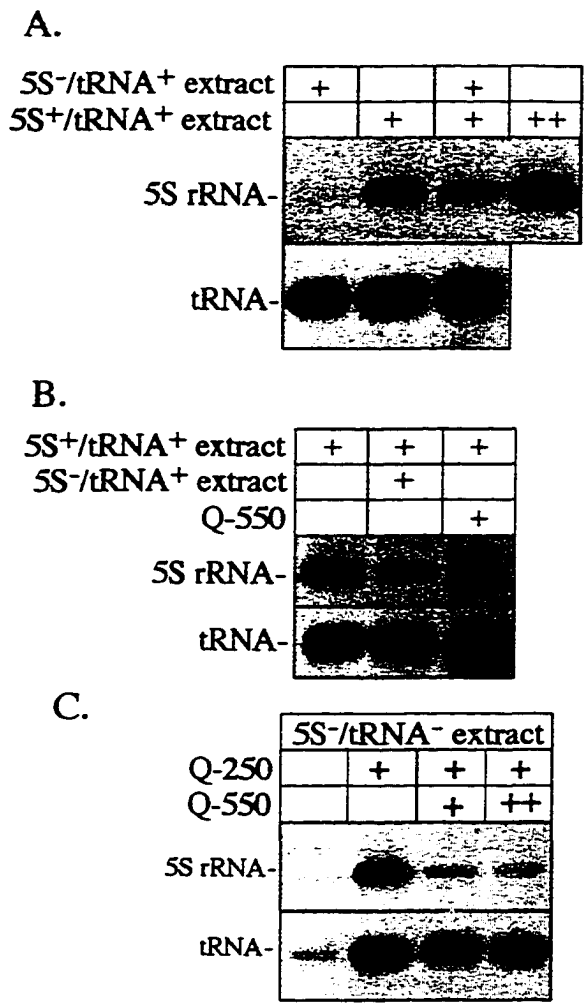


Figure 7: Identification of an activity from 5S rRNA-/tRNA+ extracts which specifically inhibited 5S rRNA synthesis. *A*, RNAP III transcription with a mixture of a 5S rRNA-/tRNA+ and 5S rRNA+/tRNA+ extracts. *B*, Identification of a chromatographic fraction containing a 5S rRNA specific inhibitor. A 5S rRNA-/tRNA+ extract was chromatographed on a Q column and the fractions were assayed for the inhibition of 5S rRNA and tRNA synthesis. A fraction eluting in 550 mM KCl (Q-550) had the same inhibitory effect on 5S rRNA synthesis as the extract from which it was derived (compare *lanes 2* and *3*). The Q-550 fraction from active extracts lack this inhibitory activity (*lane 6*). *C*, The Q-550 fraction interfered with the ability of the Q-250 fraction to rescue 5S rRNA- extracts. The fractions were pre-incubated before addition to a 5S rRNA-/tRNA- extract which was programmed with either a tDNA or 5S rDNA template.

To determine if the presence of the inhibitor in the Q-550 fraction alone might account for the lack of 5S rRNA synthesis in these extracts, we examined the integrity of the 5S rDNA transcription apparatus separated from the inhibitor. The Q-250 fraction from a 5S rRNA deficient extract was tested for activity on a 5S rDNA template. The Q-250 fraction from these extracts was able to rescue 5S rRNA synthesis in deficient extracts (Fig. 8A), suggesting that the 5S rRNA transcription system may still be intact. To directly test this, we chromatographically separated the inhibitor from the RNAP III transcription apparatus. Using reconstitution studies with extracts from exponential cultures, it was determined that none of the components of the RNAP III transcription apparatus bind to a Q matrix in 500 mM KCl. To recover the RNAP III components from a 5S $rRNA^-/tRNA^+$ extract, it was adjusted to 500 mM KCl and then chromatographed through a Q column. A significant amount of 5S rDNA transcriptional activity was recovered in the flow through from these 5S rRNA deficient extracts (Fig. 8B). These experiments are all consistent with the proposal that the selective inactivation of 5S rRNA synthesis, which has been observed *in vivo* and in our extracts, is due to the accumulation of an inhibitor, rather than the inactivation of a RNAP III transcription factor.

We have characterized the Q-250 fraction, as well as other fractions from the Q column, to identify the target of this inhibitor. Using DNase footprinting on a tRNA gene, we detected TFIIIC in the Q-250 fraction. This fraction did not contain a significant RNAP III activity, as measured by non-specific transcription assays. When the Q-250 fraction was supplemented with proteins eluted from a Q column between 300 and 500 mM KCl (the Q300-500 step fraction) tRNA synthesis, but not 5S rRNA synthesis, was reconstituted (Fig. 9A, lanes 1 and 2). 5S rRNA synthesis required the addition of a step fraction eluting from the Q column between 100 and 300 mM KCl. This factor(s) required only for 5S rRNA synthesis eluted in 140 mM KCl from a Q column developed with a salt gradient. We have identified TFIIIA in this fraction based on its distinctive footprint on 5S rDNA

(Fig. 9B). which is identical to previously published footprints (Braun *et al.*, 1989). Additionally we used highly purified RNAP III transcription factors obtained from Drs. George Kassavetis and E. Peter Geiduschek to help characterize our fractions. Using these fractions, we have determined that our Q-250 fraction contains three of the known TFIIIB polypeptides, the TATA-binding factor, BRF and B''. Recently, Dieci *et al.* have identified two components of the RNAP III transcription factor IIIB, BRF and B'', as the target of the regulatory response to the cessation of cell growth in response to cycloheximide treatment (Dieci *et al.*, 1995). Consistent with these observations, the addition of both BRF and B'' restores tRNA synthesis in inactive extracts, although they (as well as TFIIIC) did not restore 5S rDNA transcription in the same extracts (not shown). These restoration experiments and the isolation of 5S $rRNA^{-}/tRNA^{+}$ extracts are consistent with a 5S rRNA specific regulatory mechanism that is apparently independent of the previously observed inactivation of BRF and B'', which mediates tRNA regulation.

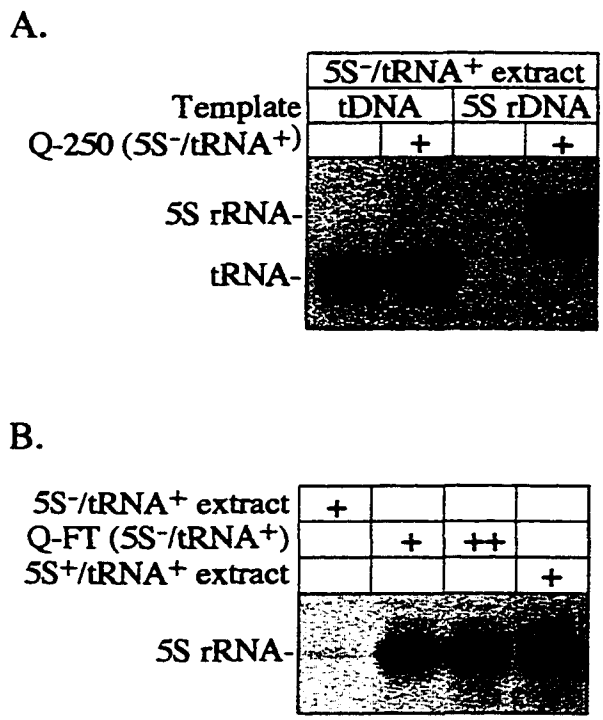


Figure 8: 5S rRNA-/tRNA+ extracts had an active 5S rDNA transcription apparatus. *A*, The Q-250 fraction derived from these extracts restores both 5S rRNA and tRNA synthesis in inactive extracts prepared from nitrogen starved cultures (Fig. 6*D*). *B*, 5S rRNA transcription can be restored in 5S rRNA- tRNA+ extracts by chromatography. A 5S rRNA-/tRNA+ extract (*lane 1*) was chromatographed through a Q column at 500 mM KCl. The flow through (Q-FT) was collected and assayed on a 5S rDNA template (*lanes 2* and *3*). The transcriptional activity of an extract from exponential cells is shown in *lane 4*.

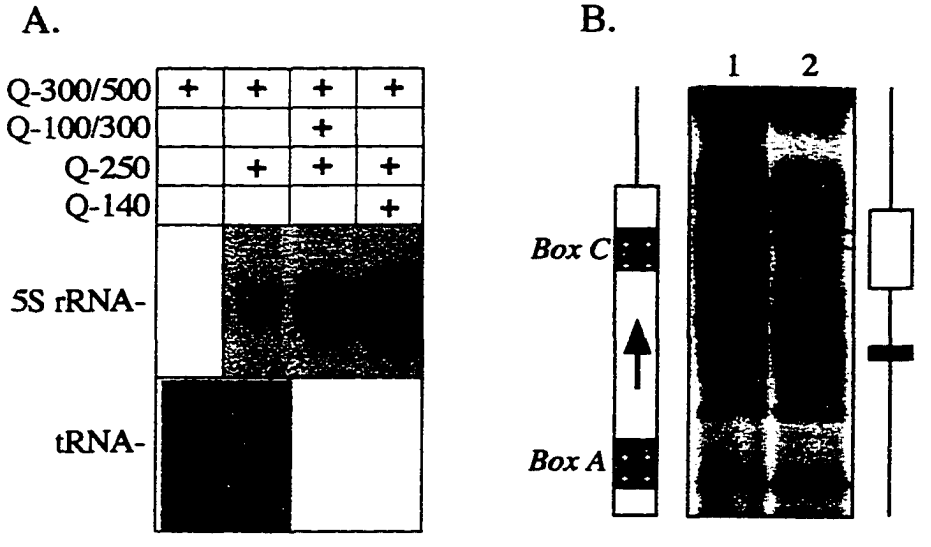


Figure 9: TFIIIA activity eluted from a Q column in 140 mM KCl. *A*, Reconstitution of 5S rDNA and tDNA transcription from Q column step fractions eluting between 100 mM to 300 mM KCl (Q100-300) and 300 to 500 mM KCl (Q300-500), and Q column gradient fractions eluting at 140 and 250 mM KCl. *B*, DNase footprinting the 5S rDNA gene with (*lane 2*) and without (*lane 1*) the Q-140 fraction. The gene (open box) is diagrammed on the left with the important internal control regions (shaded). The positions of the previously observed protections (open box) and enhancements (closed box) of DNase digestion (37) are on the right.

Discussion

Our results indicate that the regulation of yeast stable RNA synthesis observed *in vivo* under a variety of balanced as well as unbalanced growth conditions is mediated by the accumulation of a specific repressor which coordinates synthesis of 5S rRNA by RNAP III with RNAP I, as well as the previously observed inactivation of essential transcription factors associated with RNAP I (Riggs *et al.*, 1995) and RNAP III (Dieci *et al.*, 1995). It seems reasonable that the differential regulation of 5 S rDNA and tDNA transcription, which has been observed *in vivo* (Oliver & McLaughlin, 1977; Shulman *et al.*, 1977) and here *in vitro*, might involve the 5 S rDNA-specific factor, TFIIIA. In addition to binding the 5 S rDNA gene, TFIIIA binds to the gene product, the 5 S rRNA (Honda & Roeder, 1980; Pelham & Brown, 1980), resulting in inhibition of transcription. *In vitro* experiments suggest that free ribosomal protein YL3 might prevent this sequestration of TFIIIA by forming a YL3-5 S rRNA complex (Brow & Geiduschek, 1987), providing a link between a free ribosome component assembly (free YL3) and 5 S rRNA synthesis. Our experiments do not support a role for TFIIIA in this regulation. The TFIIIA-containing Q-column fractions do not rescue 5S rRNA synthesis in 5S $rRNA^{-}/tRNA^{+}$ extracts. Instead this regulation appears to be due to the accumulation of a specific inhibitor which interferes with the function of a factor in the Q-250 fraction on 5S rDNA. This inhibitor does not appear to be the yeast homolog of the transcriptional inhibitor DR1 as DR1 is a potent inhibitor of tRNA synthesis (White *et al.*, 1995). We speculate that the target of the inhibitor may be a 5S rDNA specific factor or activity associated with TFIIIB.

The temporal relationship of the responses to downshift experiments may provide important insight into the mechanisms of the regulation of stable RNA synthesis. RNAP I activity is most responsive to changes in growth conditions, followed by 5S rRNA synthesis, and eventually tRNA synthesis. Although both 35S rRNA and tRNA synthesis

appear to be regulated in a similar manner, that is the inactivation of an essential transcription factor, the differences in responses suggests that they may be mediated by fundamentally different mechanisms. The persistence of tRNA activity in downshifted cells, such as in transition phase or in response to amino acid starvation, is consistent with the loss of BRF and B'' activity under these conditions occurring at the level of factor synthesis or stability and its subsequent dilution during further cell growth. Dieci *et al.* have noted a lack of BRF in down regulated extracts (Dieci *et al.*, 1995) and BRF is limiting *in vivo* (Lopez-De-Leon *et al.*, 1992). Our results suggest that there may be an alternative mechanism, not involving simple regulation at the level of synthesis, to turn off tRNA synthesis. In the absence of cell proliferation, tRNA synthesis was shut off within 5 h in high density cultures (Fig. 3*B*). In contrast, the down regulation of RNAP I was very rapid under all conditions analyzed, consistent with the regulation of the RNAP I associated factor not at the level of synthesis, but rather modification of preexisting protein.

These experiments indicate that stable RNA synthesis is not directly regulated by the growth rate of a cell at the level of cell division. Extracts prepared from cultures subjected to energy starvation or cycloheximide treatment retain the ability to synthesize stable RNA. Since these cells remain viable and rapidly resume growth when conditions allow, it is reasonable to assume that alternative mechanism(s), not involving modification of the transcription apparatus, shut off stable RNA synthesis. Possible targets may be the conformation of the DNA template or nucleoside triphosphate pools. RNAP I transcription has been demonstrated to be very sensitive to the size of the intracellular nucleoside triphosphate pools (Grummt, 1976). The nutritional upshift experiments lead to similar conclusions. The addition of glucose to dense cultures in transition phase restores RNAP I and III activity to cell extracts, although no cell division occurs. These observations enforce the notion that the "trigger" which precipitates the regulation of the transcription complex is not simply cell proliferation, but is rather perhaps more narrowly defined.

This work provides a basis for the further biochemical analysis of the regulation of both RNAP I and III complexes. We have demonstrated that all three of the transcription systems responsible for stable RNA synthesis are directly modified in a manner that tolerates biochemical manipulation. Identification of the conditions which provoke these regulatory responses and the initial biochemical analysis of factors involved in the regulation will facilitate a detailed analysis of the molecular mechanism of stable RNA synthesis in eukaryotes.

Acknowledgments–We thank Kathy Dodd and Daniel Davidson for technical assistance, and Drs. George Kassavetis and E. Peter Geiduschek for the RNAP III transcription factors and advise. This work was supported by grant GM47881 from the National Institutes of Health.

Chapter 3

Transcriptional Regulation of RNAP I

in *Saccharomyces cerevisiae*

Abstract

It has been appreciated for a number of years that the rate of ribosomal RNA (rRNA) gene transcription is altered in response to the need for ribosome production. A key step in this process is the regulation of synthesis of ribosomal RNA. However, the regulatory mechanisms involved in transcription of rRNA remain largely uncharacterized. RNAP I specific transcription has been down-regulated in yeast which had been subjected to various environmental conditions such as starvation for an essential amino acid or nutrient, or upon entry into stationary phase. Upon reversal of these conditions, the level of rRNA synthesis returned to high levels. The RNAP I activity was restored to these inactive extracts by the addition of a chromatographic fraction, from an active extract, which was enriched in the polymerase. This fraction was able to restore this RNAP I specific activity under all conditions tested. These results indicate that the polymerase or something loosely associated with the polymerase is the target of the regulatory mechanism.

The focus of this work is to determine the molecular mechanisms involved in rRNA synthesis. More specifically, this research determined which component of the RNAP I transcription complex is involved in the down-regulation of rDNA transcription as yeast cells enter stationary phase. A tagged chromosomal copy of one of the subunits of the RNAP I enzyme complex will enable the RNAP I complex to be affinity purified and identified from both regulated and unregulated cultures with the hopes of elucidating the specific target of the regulatory mechanism.

Introduction

As described in Chapter 1, a great deal of effort has been put into identifying the components of the RNAP I transcription machinery of higher eukaryotes. However, there is still debate concerning which of these factors participates in regulating rRNA synthesis according to the physiological state of the cell. It is questioned whether the down regulation of transcription of the rDNA involves modification of the polymerase itself, associated growth factors, or if there is presence of a RNAP I specific inhibitor. Below is a brief summary of the most recent models for RNAP I transcription regulation in higher eukaryotes.

Polymerase Modification—In *Acanthamoeba* the *in vitro* transcription of the rDNA requires two factors; TIF-IB and RNA polymerase I. An additional factor, EBF (enhancer binding protein), stimulates transcription but is not required (similar to the function of UBF in mouse) (Yang *et al.*, 1995). When *Acanthamoeba* are starved for essential nutrients they undergo cellular differentiation and become dormant cysts, during which time transcription of the rDNA ceases. Specific rRNA synthesis has been restored to extracts from these cysts by supplementation with highly purified polymerase. This result indicates that during encystment the RNAP I undergoes a modification which prevents specific 35S rDNA transcription without affecting nonspecific transcription activity. As shown by DNase footprinting experiments, this modification prevents the binding of the polymerase to the stable preinitiation complex. 2-D electrophoresis comparison of *Acanthamoeba* RNAP I from growing cells and dormant cysts shows a change in the electrophoretic mobility of the 39 kDa subunit from the down regulated polymerase (Bateman & Paule, 1986). This subunit is the eucaryotic homolog of the *E. coli* α subunit and is common to both RNAP I

and III. It has not been possible to determine if this alteration is the cause of RNAP I inactivation nor is it established what modification causes the mobility change.

The modification of a subunit of the RNA polymerase has also been documented in other eukaryotes. In yeast, the RNAP I subunits A190, A43.5, ABC 23, and AC19 are modified by phosphorylation, but the role of this modification in regulation of rRNA synthesis has not been established (Sentenac *et al.*, 1992). In addition, a decrease in rDNA transcription in extracts from mouse cells which have either entered into stationary phase or have been treated with the protein synthesis inhibitor cycloheximide has been attributed to an exhaustion of factor C* which is the component of the RNA polymerase I holoenzyme necessary for specific transcriptional initiation at rRNA gene promoters (Brun *et al.*, 1994). Although a specific modification of the polymerase is not discussed, these authors suggest that the inactivation of C* involves a posttranslational modification.

RNAP I associated transcription factors involved in growth regulation of rRNA synthesis— In contrast to these findings, others suggest that factors responsible for down regulation of rDNA transcription are separable from the polymerase I enzyme. The reversible inhibition of rRNA synthesis in mouse lymphosarcoma cells after glucocorticoid treatment has been attributed to a decrease in amount or activity of the RNAP I transcription factor TFIC (Mahajan *et al.*, 1990). In these experiments, extracts from hormone treated cells failed to form initiated complexes on the rDNA preventing transcription. However, rRNA synthesis was restored by the addition of highly purified TFIC. TFIC is a multimeric protein which associates very tightly with the polymerase but is clearly not a modified form of the polymerase. It is hypothesized that RNAP I and TFIC associate to form the transcriptionally active enzyme capable of initiating transcription from the rRNA promoter. This factor is suggested to be analogous to the σ subunit of bacterial RNA polymerase (Mahajan & Thompson, 1990). However in a separate study, serum starvation

of the same cell line did not alter the level of TFIC activity even though there was a decrease in rDNA transcription which suggest that regulation of rRNA synthesis could be stimulus dependent (Schnapp *et al.*, 1990).

A different group working on the regulation of rDNA transcription in mouse also attributes the down regulation to a RNAP I specific transcription factor. In mouse ascites cells the growth dependent regulation of rDNA transcription is due to the reduction of activity of the essential transcription factor TIF-IA (Schnapp *et al.*, 1993). This factor's activity has been shown to decrease in cells which have entered stationary phase corresponding to a decrease in RNAP I specific activity. In these down regulated cells, the RNA polymerase is still able to bind to the preinitiation complex at the rDNA promoter however, the formation of the first phosphodiester bond is precluded in the absence of the TIF-IA activity (Schnapp *et al.*, 1990). TIF-IA activity is similar if not identical to the activity of TFIC which is responsible for the regulation of rDNA transcription in hormone treated mouse lymphoma cells. Extracts from both sources can form stable initiation complexes which can not initiate without supplementation of the TFIC/TIF-IA factor. Additionally, both factors associate with the polymerase to convert it into an initiation competent enzyme. However, the major difference between these factors is the identity of the factors' polypeptides. TIF-IA activity corresponds to a single polypeptide with a molecular mass estimated at 75 kDa. In contrast, TFIC activity copurifies with three polypeptides of molecular masses of approximately 55, 50, and 42 kDa.

Psoralen cross-linking experiments on mammalian cells in culture provide additional insights to the regulation of rRNA synthesis in growing and stationary cells. In exponentially growing cultures, approximately half of the rDNA genes are active and are accessible to the psoralen. Nuclear run-on experiments on these same cells show a high level of RNAP I loaded on the DNA. However, in stationary phase cells the level of loading of the RNAP I on the rDNA drops even though the number of genes accessible to psoralen

remains unchanged. These results indicate that there are the same number of preinitiation complexes available but the ability of the RNAP I to initiate decreases due to the inactivation of TIF-IA.

Polymerase I inhibitor— Despite the contradiction of these reports they do share the common theme of a positive growth dependent manner of regulating rRNA synthesis. However, evidence has also been presented by the laboratory of Masami Muramatsu that there is the presence of an inhibitor of RNAP I mediated transcription in extracts from growth arrested mouse cells (Kermekchiev & Muramatsu, 1993). Whole cell extract mixing experiments identified the presence of a negative regulator of rRNA transcription. In these experiments extracts made from cells which were serum starved for 3 hours were deficient in rRNA synthesis. Mixing these inactive extracts with transcriptionally active extracts resulted in an inhibition of rDNA transcription. This was a RNAP I specific inhibition as RNAP II specific transcription was not affected. The inhibitory activity was tested in extracts obtained from different stages of starvation and the results demonstrated that the RNAP I inhibition activity *in vitro* fluctuates according to the physiological state of the cell, showing a maximal level during growth arrest and rapidly disappearing when cells are recovered by dilution into fresh media. Gel retardation assay results indicated that the inactivation of the extracts was not due to the formation of the preinitiation complex but rather inhibits the subsequent steps in transcription. This result is in agreement with the results found in other laboratories. An intriguing question is whether this inhibitor is responsible for the inactivation of the transcription factors implicated in growth rate regulation by other laboratories.

Regulation of transcription in Yeast— Several lines of evidence indicate that yeast also down-regulate rDNA transcription independent of preinitiation complex formation. Psoralen cross-linking studies on exponentially growing yeast have shown that ~46% of the

rDNA genes are accessible to psoralen. These extracts have a high level of RNAP I specific activity. Extracts made from yeast in stationary phase have shut-down rDNA transcription and are inactive in *in vitro* transcription assays. However, these down-regulated cultures only show a modest decrease in the number of open genes which indicates the presence of preinitiation complexes. In addition, studies from our laboratory showed that the RNAP I specific activity in these down regulated extracts could be rescued by the addition of a chromatographic fraction, from active extracts, which was enriched with RNAP I. Taken together these results suggest a model in which the polymerase or a transcription factor closely associated with the polymerase, functionally similar to TIF-IA, is affected during down regulation of rRNA synthesis.

A RNAP I associated factor, encoded for by the RRN3 gene, was recently identified by Nomura and co-workers. The Rrn3p was shown to be required for *in vitro* transcription and was suggested to interact directly with the polymerase since preincubation of the Rrn3p with RNAP I led to stimulation of transcription (Yamamoto *et al.*, 1996). They suggested Rrn3p had a functionally similar role to TIF-IC and was important for elongation without a role in growth regulation. However, Milkereit and co-workers described the resolution and characterization of a distinct RNAP I population from yeast cell extracts using a reconstituted system (Milkereit & Tschochner, 1998). They found only a minor amount of the RNAP I to be active in a promoter driven transcription assay while the bulk of polymerase existed in an inactive form. They provided evidence that <2% of the total RNAP population is in the initiation competent form and that this form exists as a complex of RNAP I and Rrn3p. It is predicted that dissociation of this complex serves as a switch for transcription initiation-growth rate dependent regulation of rRNA synthesis.

Here we provide evidence to support this model of regulating rRNA synthesis in yeast.

Materials and Methods

Buffers and solutions— *TE buffer* (10 mM Tris-chloride, pH 7.5; 1 mM EDTA, pH 8.0). *Incubating solution* (1.2 M sorbitol; 100 mM sodium phosphate, pH 7.4; 2.5 mg/ml zymolyase). *1X TAE electrophoresis buffer* (20 mM Tris acetate, pH 7.5; 0.5 mM EDTA, pH 8.0). *1X Laemmli resolving buffer* (375 mM Tris chloride, pH 8.8; 0.2% (w/v) SDS). *1X Laemmli stacking buffer* (125 mM Tris chloride, pH 6.8; 0.1% (w/v) SDS). *1X Laemmli upper reservoir buffer* (25 mM Tris chloride, 192 mM glycine; 0.1% (w/v) SDS, pH 8.3). *1X Laemmli lower reservoir buffer* (25 mM Tris; 192 mM glycine; pH 8.3). *6X agarose loading buffer* (0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF; 30% (v/v) glycerol). *1X SDS gel loading buffer* (50 mM Tris chloride, pH 6.8; 100 mM dithiothreitol; 2% (w/v) SDS; 0.1% (w/v) bromophenol blue; 10% (v/v) glycerol). *Formamide loading buffer* (80% (v/v) formamide; 10 mM EDTA, pH 6.8; 1% (w/v) xylene cyanol FF; 1% (w/v) bromophenol blue). *Solubilization buffer* (200 mM Tris acetate; 10% (v/v) glycerol; 10 mM magnesium acetate). *TA-0 buffer* (10 mM Tris acetate, 10% (v/v) glycerol; 10 mM magnesium acetate, pH 7.5). *TA-80 mM KCl* (TA-0 buffer containing 80 mM KCl). *TA-250 mM KCl buffer* (TA-0 buffer containing 250 mM KCl). *TA-700 mM KCl* (TA-0 buffer containing 700 mM KCl). *TA-200 KGlu* (TA-0 buffer containing 200 mM potassium glutamate). *Transfer buffer* (1X Tris glycine; 125 mM Tris base, 0.96 mM glycine, 20% (v/v) methanol). *TBST* (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% (v/v) Tween 20).

Culture media— *LB broth* (1% (w/v) tryptone; 0.5% (w/v) yeast extract; 15% (w/v) NaCl). *LB plates* (1% (w/v) tryptone; 0.5% (w/v) yeast extract; 15% (w/v) NaCl; 15 g/l agar) *TB* (Part A: 1.2% (w/v) tryptone; 2.4% (w/v) yeast extract; 0.16% (v/v) glycerol. Part B: 2.3% (w/v) KH_2PO_4; 12.5% (w/v) K_2HPO_4. One part of B in 9 parts of A). *YEPD*

(1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose). *Minimal media lacking tryptophan plates* (1X yeast nitrogen base without amino acids and ammonium sulfate; 2 mg/ml uracil; 10 mg/ml lysine; 2% (w/v) glucose; 8 mM ammonium sulfate; 30 g/l agar).

Plasmids—*pRS304: Saccharomyces/E.coli* phagemid vector. TRP1- f1 ori (*Nae*I) - T7 promoter- lacZ'/MCS- T3 promoter- pMB1ori-bla (Sikorski, 1989). *pDR300:* pRS304 derivative. Hexa histidine tag inserted into the *Xho*I/*Kpn*I sites. *pDR310:* 5'ΔRPA135 inserted into *Pst*I/*Cla*I site of pDR300. *pDR320:* HA1 epitope (derived from the human influenza virus hemmaglutinin protein [Wilson et. al]) inserted into the *Cla*I/*Xho*I site of pDR310.

Basic DNA Manipulation Techniques

Restriction digestion—The restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (NEB. Beverly, MA. USA). Taq DNA polymerase was purchased from Promega (Madison, WI. USA). All manipulations were performed with the accompanying buffer at 1X concentration and incubated in accordance to manufacturer's instructions. When necessary the DNA was extracted with 0.5 volumes of phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 2.5 volumes of 100% ethanol containing 1 M ammonium acetate, and then rinsed with 1 volume of 80% (v/v) ethanol to remove residual salts. The air dried DNA was resuspended in the appropriate volume of TE buffer.

Plasmid DNA isolation— For both large scale and "miniprep" isolation of the plasmid DNA the alkaline/SDS plasmid preparation method described in Sambrook *et al.*, (Sambrook *et al.*, 1989) was used.

Yeast genomic DNA isolation— A 250 ml flask containing 30 ml of YEPD was inoculated with a single yeast colony and grown 18-22 hours at 30°C with aeration. Cells

were harvested by centrifugation (5,000 x g for 5 minutes) and resuspended in 4 ml of 1 M sorbitol. 75µl of zymolyase (2 mg/ml in 1 M sorbitol) was added to the solution and incubated at 37°C for 1 hour. The cells were centrifuged as above and the pellet resuspended in 3.5 ml of H_2O. After resuspension, 250 µl of 1M Tris acetate and 250 µl of 0.5M EDTA were added and mixed gently followed by the addition of 0.5 µl of 20% SDS. The solution was incubated at 65°C for 20 minutes to inactivate the zymolyase. After incubation, 2.5 ml of 3 M potassium acetate was added and the mixture was incubated on ice for 30 minutes. The cellular debris was pelleted at 12,000 x g for 10 minutes and the supernatant fraction was transferred to a clean tube which contained two volumes of 100% ethanol. The DNA was pelleted at 5,000 x g for 5 minutes and the supernatant discarded. The pellet was air dried and resuspended in 500 µl of TE buffer. Twenty five µl of a 10 mg/ml RNAse solution was added and incubated at 37°C for 1 hour. The DNA was precipitated with one volume of 100% isopropanol and the precipitate was removed with a sterile pipette tip and then resuspended in 0.5 ml of TE buffer. The DNA was reprecipitated by the addition of two volumes of 100% ethanol containing 1 M ammonium acetate. The solution was centrifuged at 13,000 x g for 10 minutes, the supernatant decanted, and the DNA washed with 1 volume of 80% (v/v) ethanol. The DNA was resuspended in 0.4 ml of TE buffer. The concentration of DNA was typically 300 ng/µl.

PCR amplification— General conditions: The reactions were performed using 2.5 units of Taq DNA polymerase in a total volume of 100 µl. The reaction contained 1X of the manufacturer's Taq buffer, 2 mM magnesium chloride, 0.2 mM dNTPs, 50 pmols of each primer, and 1 to 10 ng of plasmid DNA or 50 to 200 ng of purified yeast genomic DNA. The PCR reactions were performed using an Ericomp thermocycler (Power Block System. Ericomp. San Diego, CA. USA). Generally, the following cycling information was used:

(1X) 94°C/3 minutes
(29X) 94°C/1 minute; 55°C/1 minute; 72°C/1 minute

(1X) 72°C/3 minutes

The parameters subjected to variation were the annealing temperature and duration of elongation. The annealing temperature was calculated as 5 degrees less than the lowest melting temperature of the two primers. The elongation time depends on the length of the DNA being amplified. Typically 1 minute/ kb amplified was used.

Electrophoresis of DNA— The quality of the DNA was analyzed by gel electrophoresis. Briefly, a 0.8% or 1.6% (w/v) agarose gel in 1X TAE was cast in a 7 cm x 8 cm casting unit and run in a horizontal minigel system (OWL Scientific. Woburn, MA. USA). The agarose gel and buffer both contained ethidium bromide (8 μg/100 ml) to allow visualization of the gel when exposed to UV irradiation. Agarose loading buffer (6X) was added to the DNA samples to a final concentration of 1X prior to loading. The gel was run at 80 volts for approximately 30 to 40 minutes.

Bacterial transformation— *E. coli* strain DH5α (subcloning efficiency, $>1x10^6$ transformants/ μg Gibco BRL Life Technologies. Grand Island, NY. USA) was used for all transformations. The manufacturer's instructions were followed. Briefly, approximately 0.5 μg of plasmid DNA was incubated with 25 μl of competent cells at 4°C for 30 min. The mixture was subjected to a 42°C heat shock for 20 seconds and then returned to the ice for an additional 2 minutes. One ml of LB was added to the transformation mix and the mixture was incubated at 37°C for one hour with shaking to allow for phenotypic expression. The cells were plated on LB Amp (100 μg/ml) plates and incubated overnight at 37°C. A positive control (pUC19) and a negative control (no DNA) were always included.

Yeast transformation— The lithium acetate/SS-DNA/PEG transformation protocol (Gietz *et al.* 1995) was followed. A 250 ml erlenmeyer flask containing 50 ml of YEPD was inoculated with the yeast strain BJ3505 (*pap4::HIS3 prb-Δ1.6R lys2-208 trp1-Δ101 ura3-52 gal2 can1*) and incubated at 30°C with shaking overnight. The overnight culture

was used to inoculate a 100 ml YEPD culture to a starting cell density of $5x10^6$ cells/ml (an OD_{600} of 0.5). Once the culture had reached a density of $2x10^7$ cells/ml the culture was harvested by centrifugation at 5,000 x g for 5 minutes. The cell pellet was resuspended in 25 ml of sterile H_2O and centrifuged again at 5,000 x g for 5 min. The pelleted cells were resuspended in 1 ml of 100 mM lithium acetate and centrifuged as before. The supernatant fraction was decanted and the cell pellet was resuspended in 400 µl of 100 mM lithium acetate. The cell suspension was divided into 50 µl aliquots in 1.5 ml microfuge tubes and pelleted again. After removal of the supernatant, a transformation mix containing the following was added to the microfuge tubes: 240 µl PEG 50% (w/v), 36 µl 1.0 M lithium acetate, 25 µl single stranded DNA (2.0 mg/ml), 50 µl sterile H_2O and 0.1 -10.0 µg of linearized plasmid DNA. The suspension was vigorously vortexed and incubated at 30°C for 30 minutes and subjected to a 5 minute heat shock at 42°C. The cells were pelleted (5,000 x g for 5 minutes) and then resuspended in 1.0 ml of sterile H_2O. Finally, 200 µl of the transformation was plated onto minimal media plates lacking tryptophan and incubated for three days at 30°C.

Plasmid and Yeast Strain Construction

pDR300 construction—Two oligonucleotides 5'-TCG AGC ACC ACC ACC ACC ACC ACT GAT AGG GTA C -3' and 5'-CCT ATC AGT GGT GGT GGT GGT GGT GC-3' were annealed to create a hexa histidine cassette. The oligonucleotides were rehydrated with TE buffer at a concentration of 50 pmol/µl. Fifty pmols of each primer was added to 165 µl of TE for a final concentration of 300 fmols/µl. The solution was heated to 100°C for 5 minutes and allowed to cool to 70°C for 1 hour. The ends of the annealed cassette were designed to create a to *Xho*I and *Kpn*I overhang at the 5' and 3' ends of the cassette respectively. In order to prepare the vector for ligation with the hexa histidine cassette the yeast integrating plasmid pRS304 (ATCC #77139) was digested with *Xho*I and

*Kpn*I. A 5:1 molar ratio of cassette to vector was ligated with T4 DNA ligase at room temperature overnight. Prior to transformation into DH5α, the ligation mix was digested with *Apa*I in order to linearize any of the original vector. The recombinants were analyzed by restriction digestion and sequenced.

pDR310 construction—The last 699 bp of the RPA135 gene was PCR amplified using the primers 5' TAT CTG CAG GTA AAG GGG GGG CAT TG3' and 5' ATA ATC GAT TTT GGG CTC TAC ATT ATA ACG C 3'. The vector pDR300 and the PCR fragment were both prepared for ligation by digestion with *Pst*I and *Cla*I. A 5:1 molar ratio of vector to insert was ligated with T4 DNA ligase at room temperature overnight. The ligation mix was transformed into DH5α competent cells. The transformants were analyzed for recombinants by restriction analysis.

pDR320 construction— A pair of synthetic double stranded complementary oligonucleotides of 27 bp that encoded for the "HA1 epitope" YPYDVPDYA, was cloned into the *Cla*I and *Xho*I sites of pDR310 just downstream and in frame with the hexa histidine tag.

Construction of the mutant yeast strain DLRY-320— In order to construct the strain DLRY-320, 10 μg of pDR320 was linearized with *Nco*I. This enzyme cuts within the truncated RPA135 gene which would direct the integration to the chromosomal copy of the RPA135 gene. The yeast strain BJ3505 was transformed as previously described.

Yeast Cell Growth and Protein Extract Preparation

Large scale yeast growth— Twenty liters of yeast culture was prepared using the following protocol. Sterile techniques were employed to decrease the level of bacterial contamination. A 100 ml overnight culture of DLRY-320 grown at 30°C was used to inoculate a 4 L flask containing 1 L of YEPD to a starting OD_{600} of 0.1. When the culture reached an OD_{600} of 1.0 to 1.5 it was added to 20 L of YEPD (containing 50 μg/ ml of

ampicillin to prevent bacterial contamination). The culture was aerated and the growth was monitored. The strain grows with a doubling time of 1.5 hours. At an OD_{600} of about 1.0, ice was added to the culture to stop growth and it was harvested through glass microfiber filter paper (Glass Fiber; 1.0 μm thick and 15 cm in diameter. Whatman. Clifton, NJ. USA). The cells were then scraped from the filter paper with a rubber spatula and transferred to a beaker which was chilled on ice. The yeast slurry was then pelleted at 14,000 x g for 5 minutes. Finally, the pellet was weighed and the cell pellet frozen at –80°C. A typical yield was 80 to 100 g of cells (wet weight).

Large scale lysate— The protocols for cell breakage, extract preparation, Q chromatography, and transcription assays have been previously described (Riggs & Nomura, 1990).

Ni-NTA agarose purification of Rpa135p— Affinity purification of the his-tagged Rpa135p on Ni-NTA agarose resin (Qiagen. Santa Clarita, CA. USA) was performed following the protocol for batchwise native conditions. Briefly, 200 μl of Ni-NTA agarose was added to a siliconized 1.5 ml microfuge tube and washed with 3 volumes of TA-325 mM KCl. The Q column fraction containing the tagged protein was added directly to the resin (typically the volume of the fraction was twice the volume of the resin bed). The slurry was incubated at 4°C with rocking for 3 hours to allow the protein to bind to the resin. The resin was pelleted by centrifugation at 1,000 x g for 30 seconds and the supernatant was saved for analysis. Unbound protein were removed by washing the resin three times with 2 volumes of TA-325 mM KCl. An additional wash with TA-325 mM KCl buffer containing 5 mM imidazole was used to remove any nonspecifically bound proteins. Finally, the bound proteins were eluted from the resin with TA-325 mM KCl buffer containing 500 mM Imidazole. The fractions were dialyzed against TA-200 KGlu and analyzed for activity.

Western Blot — The proteins of interest were added to 1 X SDS gel loading buffer, heated to 100°C for 3 minutes and resolved in a discontinuous 10% SDS PAGE (38:2 acrylamide:bisacrylamide). The gel was run in a vertical electrophoresis system (OWL Scientific. Woburn, MA. USA) at 300 volts until the bromophenol blue reached the bottom of the resolving gel. The SDS gel and a piece of Immobilon P filter paper (Millipore. Bedford, MA. USA) were soaked in distilled water and equilibrated for 10 minutes in transfer buffer. The transfer was performed using a semidry blotting apparatus (Fisher Scientific. Pittsburgh, FL. USA), at 9 volts for 8 hours. After transfer, the membrane was then rinsed with TBST buffer pH 7.5 for 30 minutes and then blocked in blocking solution (4% (w/v) dried milk in TBST) for one hour. The membrane was washed three times in TBST, each rinse for 10 minutes. The membrane was soaked in 10 ml of primary antibody solution (mouse monoclonal antibody anti-HA1 (clone 12CA5, Boehringer Manhheim. Indianapolis, IN. USA) diluted to a concentration of 5 μg antibody/ml in TBST containing 0.02% (w/v) sodium azide for 2 hours at room temperature with gentle rocking. The membrane was again rinsed as above and then incubated with the secondary antibody (sheep anti-mouse IgG horseraddish peroxidase conjugate, (Amersham Life Science. Arlington Heights, IL. USA) at 1/10,0000 dilution in TBST) for 1 hour. The membrane was rinsed as above and then incubated for 2 minutes with 1 ml of ECL western blotting detection reagents as instructed by the manufacturers (Amersham Life Science. Arlington Heights, IL. USA). Finally, the membrane was wrapped in Saran Wrap and exposed to X-ray film for 15 to 45 seconds.

Results

With the goal of better understanding the molecular mechanisms involved in the regulation of rRNA synthesis, we combined both the well developed genetic and biochemical techniques to further purify the rescuing activity and characterize the components of this fraction. A strain was made which bears two epitope tags on the 3' end of the RPA135 gene which encodes for the second largest subunit of the RNAP I enzyme. There are two major advantages to tagging the chromosomal copy of the gene using this method rather than using a centrameric plasmid. First, it ensures that the expression of the RPA135 gene will be under the control of its native promoter preventing overexpression of the subunit which could have an adverse affect on cell growth. Second, the product of the chimeric gene is the only active copy of the gene present in the cell, ensuring that the entire population of polymerase will be tagged. Below is the description of the construction of the yeast strain and a report on the further characterization of the rescuing activity found in the Q-B fraction.

Creation of a yeast strain bearing an epitope tagged RNA polymerase I

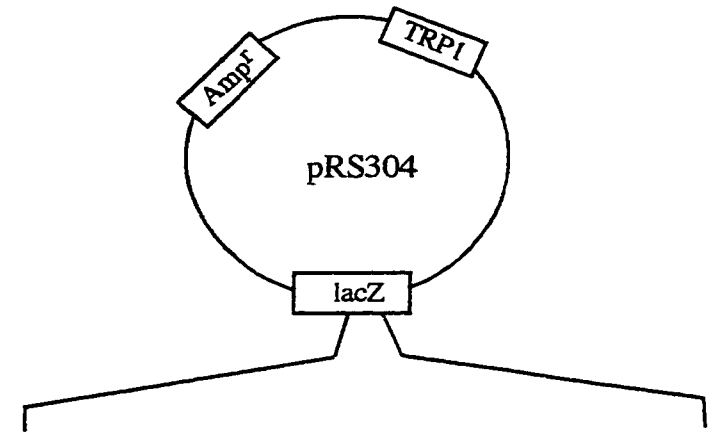
Construction of the strain—The hemi-zapper method (illustrated in Figure 6) tags genes on the chromosome via an integrating plasmid. This technique uses a yeast integrating plasmid which contains two epitope tags. Located upstream and in frame of these tags is a gene fragment encompassing only the 3' end of the gene to be targeted. Once constructed the plasmid will be linearized by a restriction enzyme, which cuts uniquely within the truncated gene, in order to direct the integration into the chromosome. The linearized plasmid will be transformed into a yeast strain which is trp- as the plasmid has the TRP 1 gene as the selectable marker. The integration of the plasmid creates a yeast strain

which has a C-terminally tagged chimeric gene as well as a truncated inactive gene. Figure 6 illustrates this integration scheme.

We modified the yeast integrating plasmid pRS304 by adding two affinity tags; the 6X His tag and the HA epitope tag. The 6X His tag, which adds six histidines to the C-terminus of the subunit, allows for affinity purification of the polymerase complex based on its affinity for a commercially available Ni-NTA resin. There are commercially available monoclonal antibodies against the HA epitope, a nine amino acid epitope from the human influenza virus hemagglutinin, which will allow for identification of the polymerase throughout the purification process. An advantage to using these small peptides is that they are unlikely to perturb the overall structure of the subunit allowing it to integrate into the multi-subunit polymerase complex.

The yeast integrating plasmid pRS304 (Figure 1A) was first modified by the addition of the hexa histidine tag. Two complementary oligonucleotides which comprise the 6X His cassette (Figure 1B) were annealed and ligated into the *Xho*I and *Kpn*I sites of pRS304 to create the plasmid pDR300. The construction was designed so that the recombinants could be screened based on the retention of the *Xho*I and *Kpn*I sites and the elimination of the *Apa*I site. Agarose gel analysis of the restriction digestion of several recombinants (Figure 2) shows the correct recombinant does not digest with *Apa*I (2A; Row 1, lane 3), but linearizes with *Xho*I (2A; Row 1, lane 4) and *Kpn*I (2B; lane 2).

A.



B.

Primer #1

5' - T/CGA/GCA/CCA/CCA/CCA/CCA/CCA/CTG/ATA/GGG/TAC-3'

Primer #2

5' - CC/TAT/CAG/TGG/TGG/TGG/TGG/TGG/TGC-3'

6X His tag

5' - **T/CGA/G**CA CCA CCA CCA CCA CCA **CTG/ATA/GGG/TAC**-3'
3'- **C**GT GGT GGT GGT GGT GGT **GAC/TAT/CC - 5'**

Figure 1: Construction of a yeast integrating plasmid (pDR300) which contains a 6X His tag in the multiple cloning site. (A) The yIP pRS304 (ATCC# 77139) was digested with *Xho*I and *Kpn*I which are contained in the MCS. The sites within the poly-linker which are not unique are underlined. (B) A pair of complementary oligonucle-otides were annealled to form the 6X His cassette which was ligated into pRS304. The 6X His tag is indicated in red while the bold type shows the overhangs complimentary to the vector. The ligation was designed so that the insertion did not interupt the reading frame of the lacZ gene. The *Apa*I site was destroyed upon ligation of the 6X His cassette.

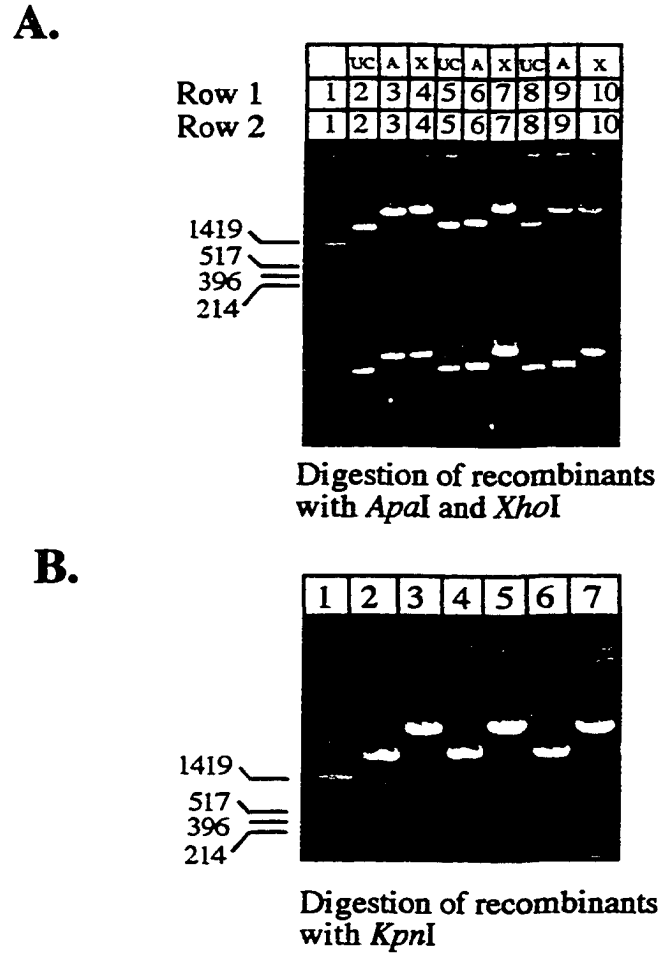


Figure 2: Restriction analysis of putative pDR300 recombinants. The ligation reaction of the 6X His cassette with pRS304 would result in recombinants which retained both the *Xho*I and the *Kpn*I sites and eliminated the *Apa*I site. The agarose gel in panel A shows the results of a miniscreen of five transformants digested with *Xho*I and *Apa*I (lanes 2, 5, and 8 uncut; lanes 3, 6, & 9 *Apa*I; lanes 4, 7, & 10 *Xho*I). Row 1 shows pRS304 uncut (lane 2), digested with *Apa*I (lane 3), and *Xho*I (lane 4). Row 1, lane 5 and Row 2, lanes 5 and 8 are possible recombinants and were further digested with *Kpn*I (shown in panel B). (B) Lane 1 contains pUC19 *Hinf*I markers, lanes 2, 4, and 6 are uncut, lanes 3, 5, and 7 are plasmid digested with *Kpn*I.

As described earlier, the yeast RNAP I enzyme is composed of 2 large subunits and 12 smaller polypeptides each of which have been cloned and sequenced. The second largest subunit of the polymerase was chosen to tag for several reasons. First, due to the large size of this subunit, 135 kDa, it was reasoned that the tags would have a greater chance of being exposed allowing for more efficient purification of the polymerase complex. Subunits which had been implicated in the assembly process of the enzyme, such as the A12.2 and AC40, were not chosen for fear they would be buried within the enzyme complex (Mann *et al.*, 1987; Nogi *et al.*, 1993) In addition, the A49 subunit was eliminated as a possibility because it had been shown to be loosely associated from the polymerase (Liljelund *et al.*, 1992). Finally, those subunits which were common with the other two polymerases were avoided to ensure the exclusive isolation of RNAP I.

Primers with specific restriction sites were created to PCR amplify the last 699 bp of the RPA135 gene from yeast genomic DNA (Figure 4, lane 2). The primers included the *Pst*I and *Cla*I restriction sites (Figure 3, shown in bold type) on the 5' and 3' ends of the RPA135 gene respectively. The fragment was inserted between the *Pst*I and *Cla*I sites of pDR300, which is upstream of the two tags, creating the plasmid pDR310. The recombinant retained both the *Pst*I and *Cla*I sites (Figure 5, lane 6) and acquired a *Nco*I site (Figure 5, lane 7), unique to the RPA135 gene.

In order to create pDR320, which contained an HA tag inserted between the RPA135 gene and the 6X His cassette, two oligonucleotides which encoded for the 9 amino acid epitope "YPYDVPDYA" were annealed and ligated into the *Cla*I and *Xho*I sites of pDR310 (Figure 6). Recombinants were screened based upon the loss of the *Cla*I site which was eliminated upon ligation.

Restriction digestion with *Nco*I, which cuts uniquely within the truncated gene, linearized the plasmid prior to transformation in order to direct integration into the

chromosome. The plasmid was transformed into the trp- yeast strain BJ3505 and the integrants were selected on minimal media plates minus tryptophan.

Verification of strain— PCR was used to verify the correct integration of the plasmid. Primers, designed to anneal to the 5' end of the RPA135 gene and the 3' end of the 6X his tag, were used to amplify the chimeric gene (Figure 6). Amplification would only occur in the case of the correct recombination event. The growth of the mutant strain (DLRY320-1) was also monitored to ensure that the tagged subunit could assemble into the polymerase without interfering with the cell growth (data not shown). DLRY320 had the same growth rate as the wild type strain BJ5305 in both complex and minimal media indicating that the tags have no effect on the RNAP I enzyme. Extracts were made with DLRY320 (as previously described) and assayed for RNAP I specific transcription activity. These extracts had identical activity as the wild type (data not shown) which also indicated that the tags did not effect the specific transcription activity of the RNAP I transcription complex.

A.

Primer #1 (5' RPA135 *Pst*I)
5' - TAT/**CTG/CAG**/GTA/AAG/GGG/GGG/CAT/TG

Primer #2 (3' RPA135 *Cla*I)
5' - ATA/**ATC/GAT**/TTT/GGG/CTC/TAC/ATT/ATA/ACG/C

B.

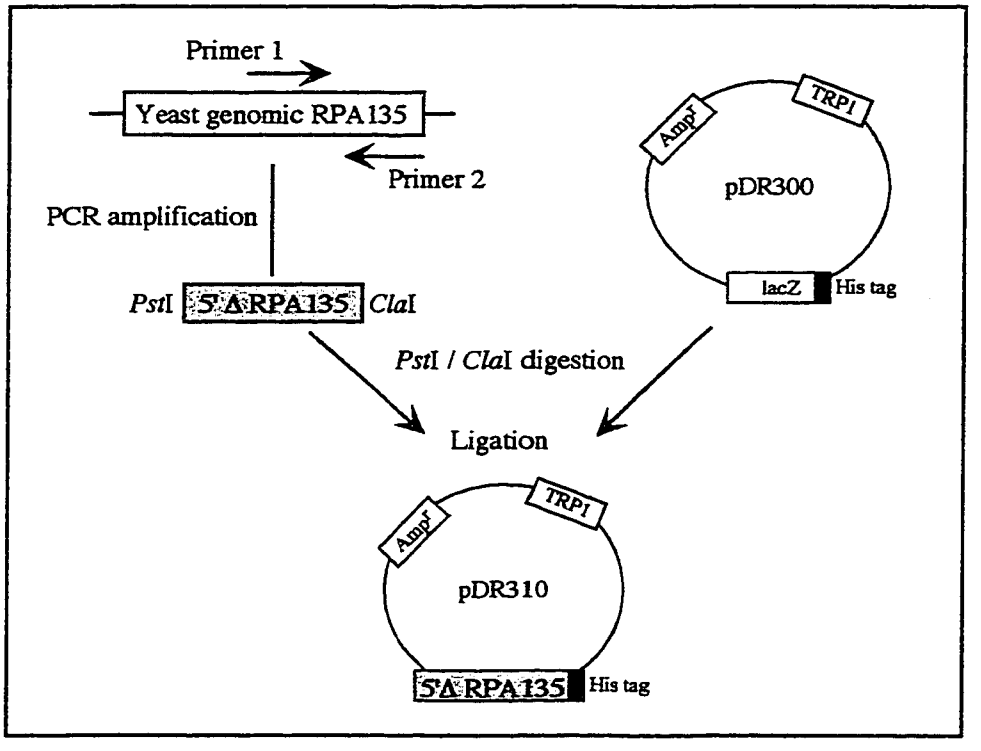
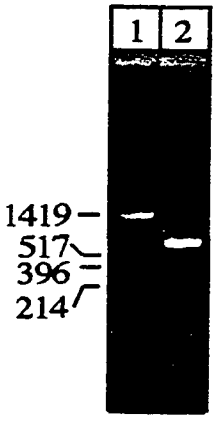
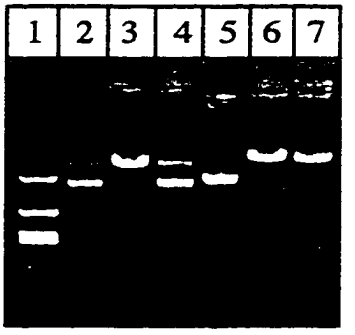


Figure 3: Construction of Yeast integrating plasmid pDR310. The primers in panel A were used to PCR amplify the last 699 bp of the RPA135 from yeast genomic DNA. *Pst*I and *Cla*I sites were encoded for by the primers (in bold) and subsequently contained in the PCR product. The underline indicates the nucleotides which annealed during the first several rounds of amplification. The truncated gene and vector were both digested with *Pst*I and *Cla*I, ligated together, and transformed into *E. coli* DH5α.



1.6% Agarose Gel

Figure 4: Analysis of PCR amplification of 5'ΔRPA135 gene from Yeast genomic DNA. The last 699 bp of the RPA135 gene was PCR amplified from Yeast genomic DNA. The PCR product was digested with *Cla*I and *Pst*I to prepare it for ligation with pDR300, which was also cut with these enzymes. Lane 1 is pUC19 *Hinf*I markers. Lane 2 is 10 μl of a 100 μl PCR amplification of the RPA135 gene.



0.8% Agarose gel of minipreps
digested with *Cla*I or *Nco*I

Figure 5: Restriction analysis of putative pDR310 recombinants. The correct ligation would result in recombinants which retained the *Cla*I site and gained a *Nco*I site as a result of the insertion of the RPA135 gene. Lanes 2 (uncut), 3 (*Cla*I), and 4 (*Nco*I) show the digestion patterns of pDR300. Lanes 5 (uncut), 6 (*Cla*I), and 7 (*Nco*I) are the restriction pattern of a pDR310 recombinant.

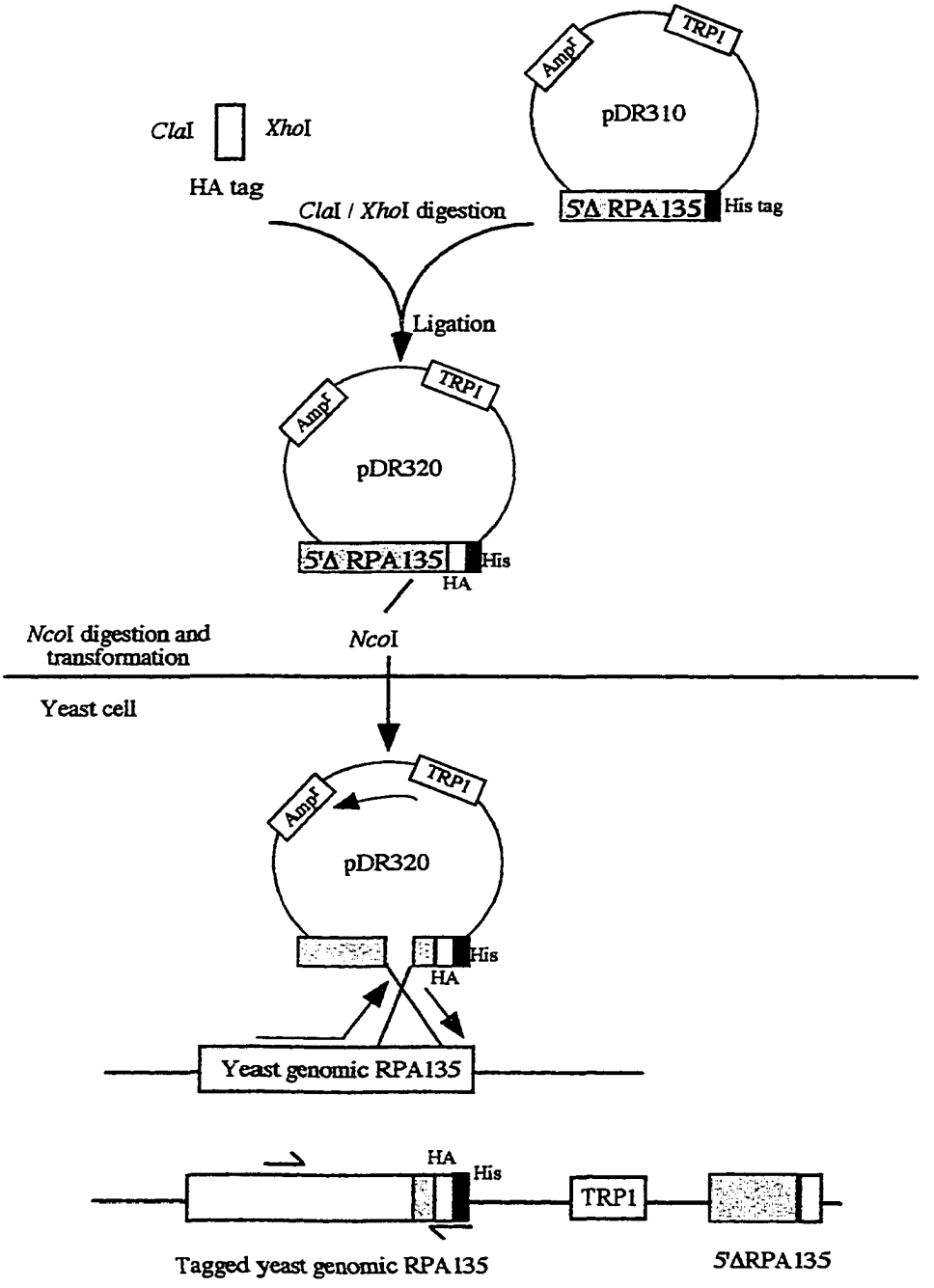


Figure 6: Construction of the yeast mutant strain DLRY-320. The plasmid pDR320 was created by inserting a cassette encoding for the HA epitope into the *ClaI/XhoI* site of pDR310. The plasmid was linearized with *NcoI* and transformed into a *trp-* yeast strain. Integrants were selected on minimal media lacking tryptophan. Through homologous recombination the yeast integrated the plasmid and the chromosomal RPA135 gene was tagged with both the 6X His and the HA tags.

Characterization of rescuing activity

The Q-B activity co-elutes with the rescuing activity on a high Q column— We had previously characterized the components of the RNAP I transcription machinery by fractionating an active extract over an anion exchange column (Riggs & Nomura, 1990). We reconstituted specific RNA polymerase I transcription from three partially purified chromatographic fractions (termed Q-A, Q-B, and Q-C). (Figure 11 represents this purification scheme). As shown by gel retardation assays, the Q-A fraction contained proteins which bound specifically to the rDNA promoter. Nonspecific transcription assays identified the presence of RNAP I in the Q-B fraction. Although the contents of the Q-C fraction remain to be characterized, it is necessary to reconstitute RNAP I specific activity. In order to further characterize the components of the Q-B fraction, we cultured the strain DLRY320, the strain containing the tagged polymerase, in rich medium with aeration to allow optimal growth. The exponentially growing culture was harvested at an OD_{600} ~1.0 and a whole cell extract was prepared as previously described. The extract was tested for RNAP I specific transcription and then fractionated over an anion exchange column to isolate the Q-B fraction.

The Q column fractions were first assayed for the ability to reconstitute specific RNAP I transcription *in vitro*. Fractions which eluted from the Q-column were added to an RNAP I specific transcription assay containing Q-A and Q-C fractions from a previous column and a 35 S rDNA template. The Q-B activity was found in fractions 65-70, which eluted from the Q-column at 250-350 mM KCl, with the peak of activity in fraction 67-69 (Figure 7A).

We had previously identified that the rescuing activity coeluted with the Q-B fraction from an anion exchange column. We next wanted to examine if the Q-B fractions from the tagged strain had the ability to rescue RNAP I specific activity in an inactive extract. Fractions 64-70 were added to an inactive extract, which was prepared from a

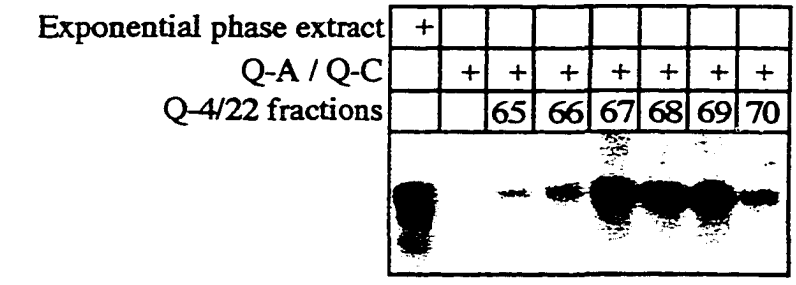
stationary phase culture, and assayed for recovery of RNAP I specific transcription activity. The rescuing activity was found in fractions 66-70 with the peak of activity in fraction 68 (Figure 7B) which corresponded to the Q-B activity.

Next, we wanted to determine if the presence of the RNAP I corresponded with the rescuing activity. Western blot analysis was performed using antibodies against the HA tagged RPA135 subunit to confirm the presence of RNAP I in the Q-B fraction. A cell extract from an exponentially growing culture of DLRY320 was fractionated over a high Q anion exchange column (Figure 8). The fractions which contained the rescuing activity were identified by assaying the fractions for the ability to restore RNAP I transcription to an extract from stationary phase cells (Figure 10A). The rescuing activity was identified in fraction 70 to 73 with the peak of activity in fraction 72. These fractions were subjected to SDS-PAGE and the proteins were transferred to a nitrocellulose membrane. Monoclonal antibodies against the HA epitope were used for western analysis. RNAP I was identified in fractions 68 to 76 with only a relatively small fraction of the polymerase in fraction 72 (Figure 10B). These results indicate that of the total population of RNAP I present in the cell, only a small portion is present in an initiation competent form, and that the polymerase or a factor which associates with the polymerase is down-regulated as yeast enter stationary phase.

In order to determine the relationship between the regulated factor and the RNAP I enzyme we further purified the his-tagged RNAP I enzyme from this fraction using a Ni-NTA resin. The Q-fractions which contained the rescuing activity, fractions 70 through 73, were combined and applied to Ni-NTA resin in a batchwise manner. The proteins were incubated with the resin for several hours at 4°C to allow binding of the polymerase complex. In order to remove unbound proteins the resin was washed three times with buffer. Nonspecifically bound proteins were eluted in a second wash with buffer containing 5 mM imidazole. Finally, the bound proteins were eluted with buffer containing 500 mM

imidazole. The flow through, washes, and eluting fractions were dialyzed against TA200 KGlu and assayed for the ability to rescue RNAP I specific transcription to inactive extracts. The first fraction which eluted from the resin with 500 mM Imidazole was able to only slightly restore activity to the inactive extract (Figure 9A). This result indicated that perhaps the rescuing activity had "split" on the resin and a second component was present in one of the other fractions. To test this, we used a combinations of the Ni-NTA fractions to assay for the rescuing activity. We found that specific transcription could be more fully restored to an inactive extract with the combination of the flowthrough and the first eluting fraction (Figure 9B). In order to determine which fraction contained the polymerase, western blot analysis was performed on all of the Ni-NTA fractions (Figure 9C). The polymerase was only identified in the eluting fraction and was not present in the flow through fraction. These results suggest the presence of an additional regulatory factor(s).

A.



B.

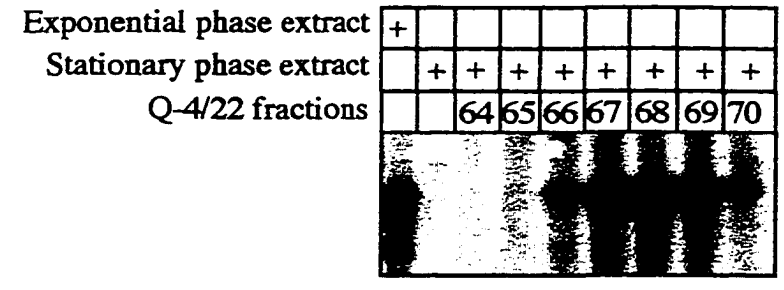


Figure 7: Q-B activity co-elutes with rescuing activity on high Q column. (A) In order to assay for Q-B activity, 10 µl of fractions from the Q4/22 column were added to specific transcription assay containing 10 µl of Q-A and Q-C activity from a previous columns. The first lane in both panels is a positive control assay of a TA-0 pellet from an exponential phase extract. The specific fractions added to the assay are noted. (B) To determine which fractions contain the rescuing activity 10 µl of the Q4/22 column were added to 0.5 µl of a crude inactive extract from a stationary phase culture. The Q-B activity and the ability to restore specific transcription were found in fractions 66-70 with the peak of activity in fraction #67.

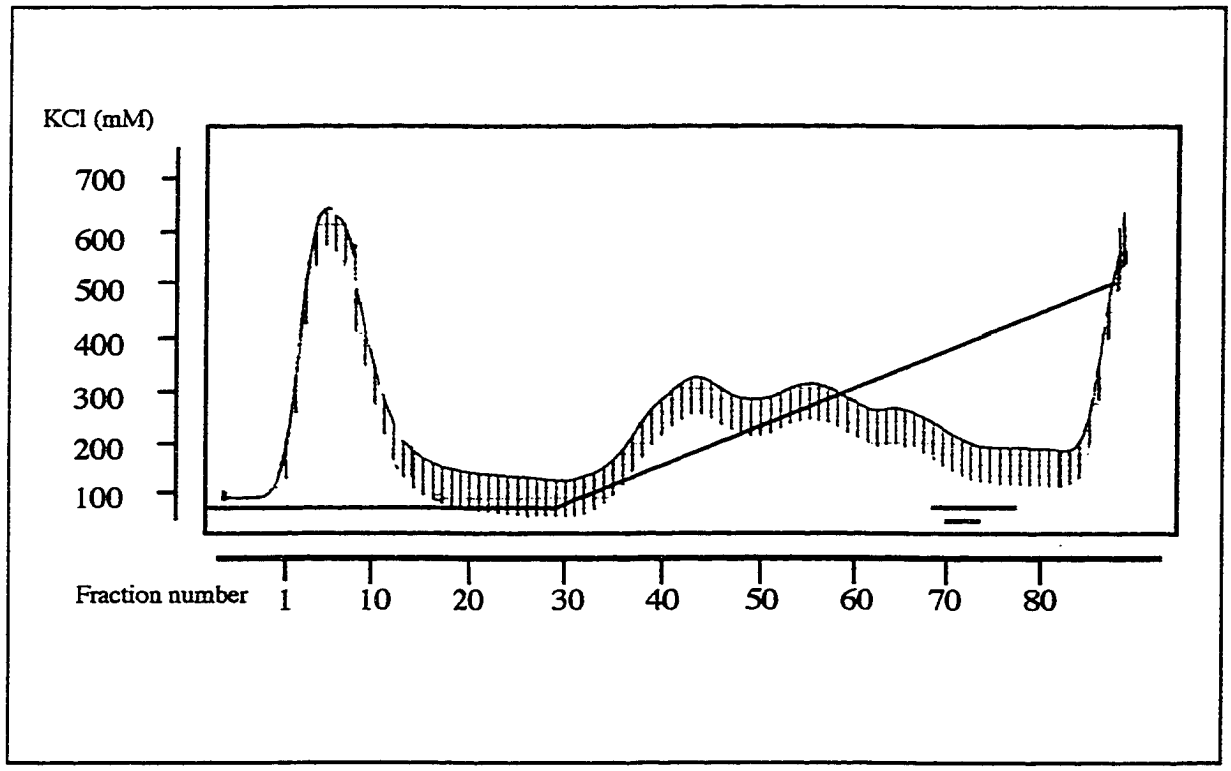


Figure 8: Protein profile of DLRY320 exponential phase extract chromatographed over a high Q column. The blue band indicates the fractions which contain the RNA polymerase I specific A135 subunit as detected by western blot analysis. The red line indicates fractions which were able to restore RNAP I specific transcription to inactive stationary phase extracts.

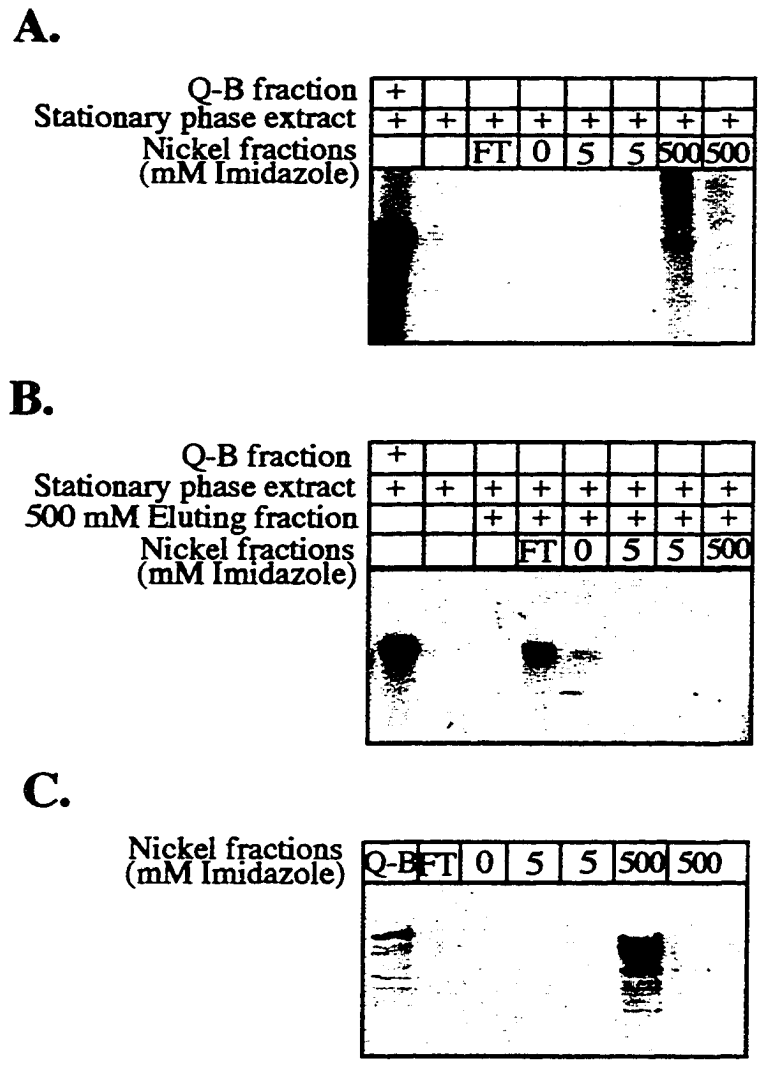
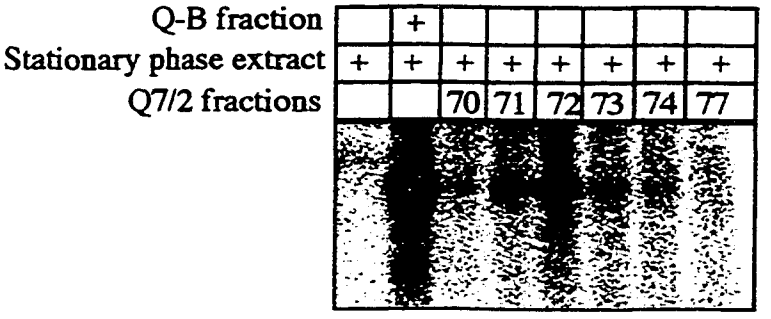


Figure 9: Restoration of inactive extract requires a combination of Ni-NTA fractions. To further purify the rescuing activity, a Q-B fraction containing the his-tagged RPA135 subunit was bound to Ni-NTA resin. The unbound proteins were removed by washing the resin three times with buffer containing no imidazole and twice with buffer containing 5 mM Imidazole. Bound proteins were eluted with buffer containing 500 mM Imidazole. (A) The Ni-NTA fractions were assayed for the ability to restore specific transcription to a stationary phase cell extract. The first eluting fraction (lane 7) was able to slightly restore activity to the inactive extract. (B) The combinations of Ni-NTA flowthrough and 500 mM elution fraction (lane 4) were able to restore activity to an inactive extract. (C) Western blot analysis of the Ni-NTA fractions indicate that only the 500 mM elution contained the polymerase.

A.



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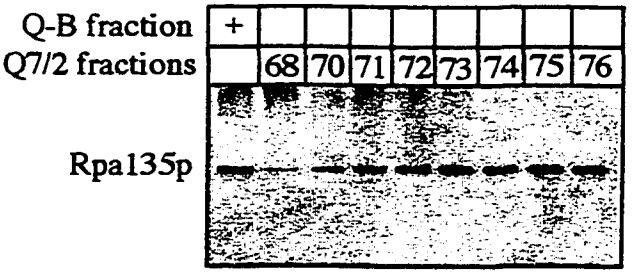

Figure 10: A small portion of the RNA polymerase I is associated with the rescuing activity. (A) Q column fractions from an exponentially growing culture were assayed for the ability to restore specific RNAP I transcription to an inactive extract. Lane 2 is a positive control from a previous column. In lanes 3-8, 10 µl of the specified fractions were added to the inactive extract. (B) Western blotting analysis, with antibodies against the pol I specific subunit A135, of the Q7/2 fractions showed the polymerase in fractions #76 with the peak in fraction #75.

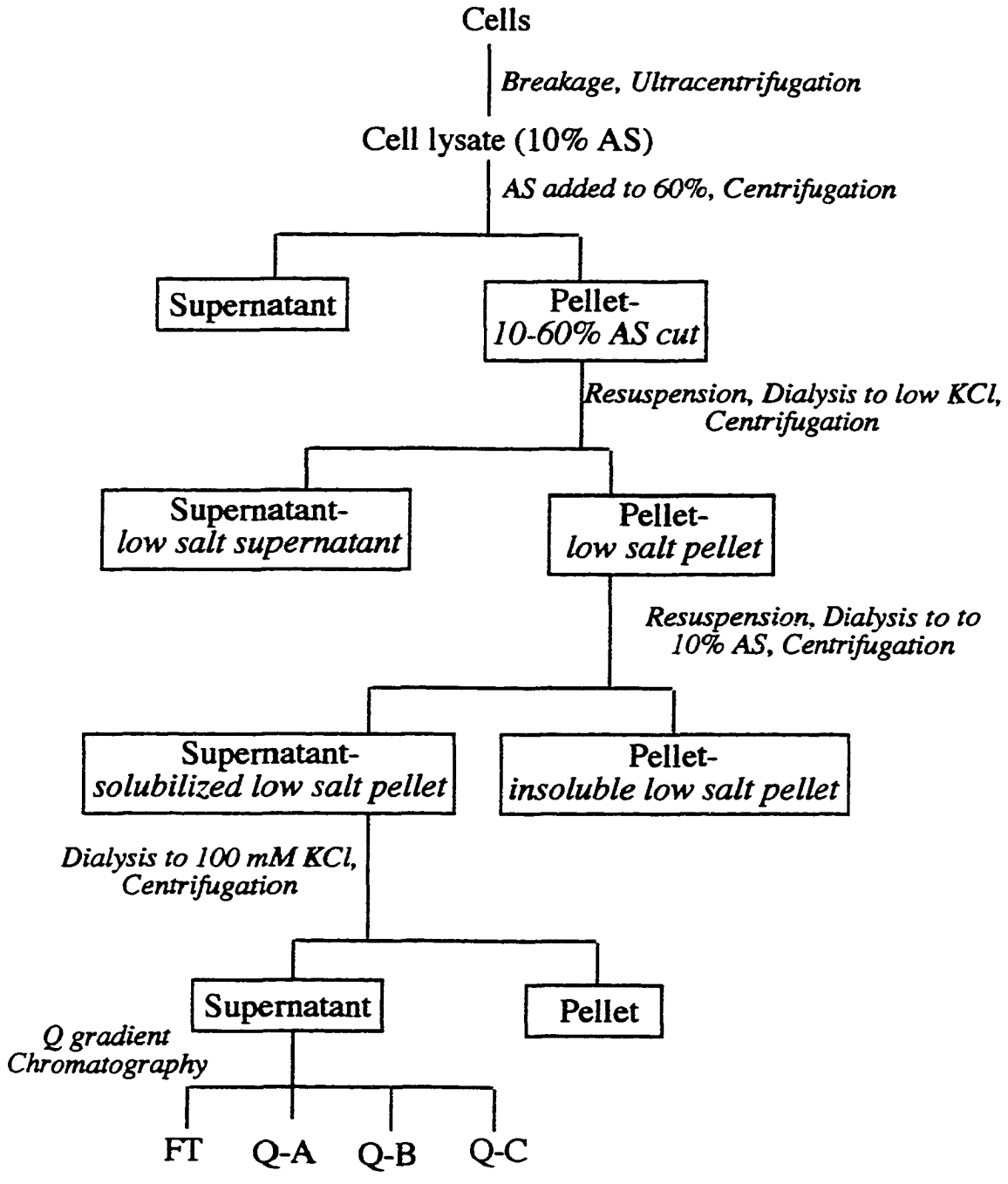

Figure 11: The fractionation scheme of the RNAP I transcription complex. (AS, ammonium sulfate).

Discussion

Here we present further characterization of the components of the yeast RNAP I transcription apparatus which are involved in the regulation of rDNA transcription. We have shown, by western blot analysis and *in vitro* transcription assays, that only a small percentage of the total population of RNAP I found in an exponentially growing culture is competent for specific RNAP I transcription. Although the reason for this is unclear, it is possible that either the remainder of the polymerase was inactivated during the purification process or that a specific transcription factor(s) must associate with the polymerase to enable it to specifically recognize the rDNA promoter and initiate transcription. This second possibility seems more likely if it is considered that most of the RNA polymerase in the cell would be in a form which would allow for elongation of transcription while only a small portion would be involved in recognition of the promoter. A plausible hypothesis is that perhaps after initiation, the factor, which conveys the initiation competence, would then dissociate from the polymerase allowing it to transform into the elongation form. This is seen in *E. coli* RNA polymerase, where the holoenzyme, upon release of the σ factor from the core enzyme, is transformed into a stable elongating complex. Our observation is further supported with the recent results of Milkeriet and Tschochner, who found that less then 2% of RNAP I from whole-cell extracts appeared to be competent for specific initiation at the ribosomal gene promoter in a yeast reconstituted transcription system (Milkereit & Tschochner, 1998). In this study, initiation-competent RNAP I from a sizing column was applied to a MonoQ column and eluted with a salt gradient. As determined by western blot analysis, the peak fractions of RNAP I did not coincide with the peak of promoter-dependent transcription activity.

Upon further purification of the initiation competent RNAP I complex using a Ni-NTA affinity resin, we found that the polymerase could be partially separated from the

factor(s) which conveys this initiation competent form. Restoration of specific RNAP I transcription to extracts from stationary phase cells required the combination of two Ni-NTA fractions, one of which contained RNAP I. Rrn3p, as previously described, is a protein essential for the efficient transcription initiation by yeast RNAP I (Yamamoto *et al.*, 1996). It seemed likely that the Rrn3p protein could be this associated factor which transformed the RNAP I enzyme into an initiation competent form. In order to determine if this factor was present in either of our two Ni-NTA fractions which restored RNAP I transcription to stationary phase extracts, we created a yeast strain which had the HA and His tags fused to the RRN3 gene.

However, shortly after the yeast strain was made, Milkeriet published results which indicated that this indeed was the case. Western analysis showed that the fractions which contained the initiation-competent form of RNAP I coincided with the presence of Rrn3p. Although the majority of the Rrn3p present in the cell was not associated with the RNAP I, immunoprecipitations of Rrn3p from initiation competent fractions also coprecipitated RNAP I (Milkereit & Tschochner, 1998). In addition, Milkeriet and co-workers could not use initiation inactive RNAP I (from exponentially growing cell) or recombinant Rrn3p to restore specific transcription. However, they could exchange other components of the RNAP I transcription complex from stationary and exponential extracts. We have previously shown this to be true as the Q-A and Q-C fractions from exponentially growing cells did not rescue specific activity. Taken together, these results suggest that either the RNAP I of Rrn3p has to be modified to enable an interaction between these two partners and that this modifying factor is yet to be identified. It is possible to imagine a scenario in which the RNAP I-Rrn3p initiation complex is present in our Ni-NTA eluting fraction and that the factor(s) which modifies either the polymerase or the Rrn3p in a way which allows for their interaction is found in our Ni-NTA fraction. Further experiments should be done

to test whether and how the RNAP I-Rrn3p complex is modified to restore the capability to assemble and identify the factor(s) involved in this modification.

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PART 2:

Interactions Between the *Escherichia coli* RNA Polymerase Alpha Subunit and the Transcription Elongation Factor NusA

Chapter 4

Literature Review

Introduction

Transcription in Escherichia coli— Unlike eucaryotes which have three DNA dependent RNA polymerases, prokaryotes rely on only one RNA polymerase to transcribe all of their genes. In *Escherichia coli* (*E. coli*), the core RNA polymerase is composed of three types of protein subunits: beta (β), beta prime (β'), and two alpha subunits (α). The core enzyme is capable of elongating preinitiated transcripts but is unable to recognize the specific start site of transcription on the DNA (Burgess, 1969). This specificity is conferred by the sigma (σ) subunit which joins the core enzyme to form the holoenzyme (Burgess & Travers, 1969). The two largest subunits of the polymerase, β and β', contain the catalytic site of the enzyme. The alpha subunit can be divided into two functionally separable domains: the N-terminal domain (NTD) and the C-terminal domain (CTD). It has been shown, by reconstituting polymerase lacking the αCTD, that the NTD is necessary and sufficient for the formation of the functional enzyme (Igarashi *et al.*, 1991). The CTD responds to regulatory signals encountered throughout each step in the transcription cycle (Igarashi *et al.*, 1991; Ishihama, 1992; Ishihama, 1993; Russo & Silhavy, 1992). The transcription cycle (illustrated in Figure 1) can be divided into several sequential steps: i) initiation, ii) promoter escape, iii) elongation, iv) termination, and in certain situations v) antitermination (reviewed in (Yager & von Hippel, 1987)).

Transcription initiation— Initiation of transcription requires the location and binding of the holoenzyme to the start site of transcription. The RNAP recognizes the promoter by interacting with several *cis* elements found near the start site of transcription: a hexamer located -10 basepairs upstream of the start site of transcription (termed the -10 region or pribnow box), a second hexamer located at -35 (-35 region), the spacer region located between these two hexamers, and, at certain promoters an UP element (typically located between -40 and -60) (Brunner & Bujard, 1987). Once the promoter is located, the RNAP reversibly binds to one face of the double helix and forms a closed complex which

contacts a region covering approximately 80 bp from -55 to +20 (Record *et al.*, 1996). The polymerase then melts approximately 12 base pairs (bp) of the DNA (deHaseth & Helmann, 1995), including the initiation site, to form an open complex. This transformation involves a major conformational change in the polymerase (deHaseth *et al.*, 1998; Record *et al.*, 1996; Roe *et al.*, 1985). After the formation of the stable open complex, the initiated complex can follow one of three pathways: i) formation of abortive transcripts, (short transcripts of 2 to 8 nucleotides may be released) ii) reiterative synthesis or stuttering, resulting in homopolymer extensions of initial RNA transcripts (Chamberlin & Hsu, 1996; Liu *et al.*, 1994), or iii) release of the σ factor (Burgess & Travers, 1969). Upon release of σ, RNAP translocates away from the promoter and transforms into the stably bound transcription elongation complex.

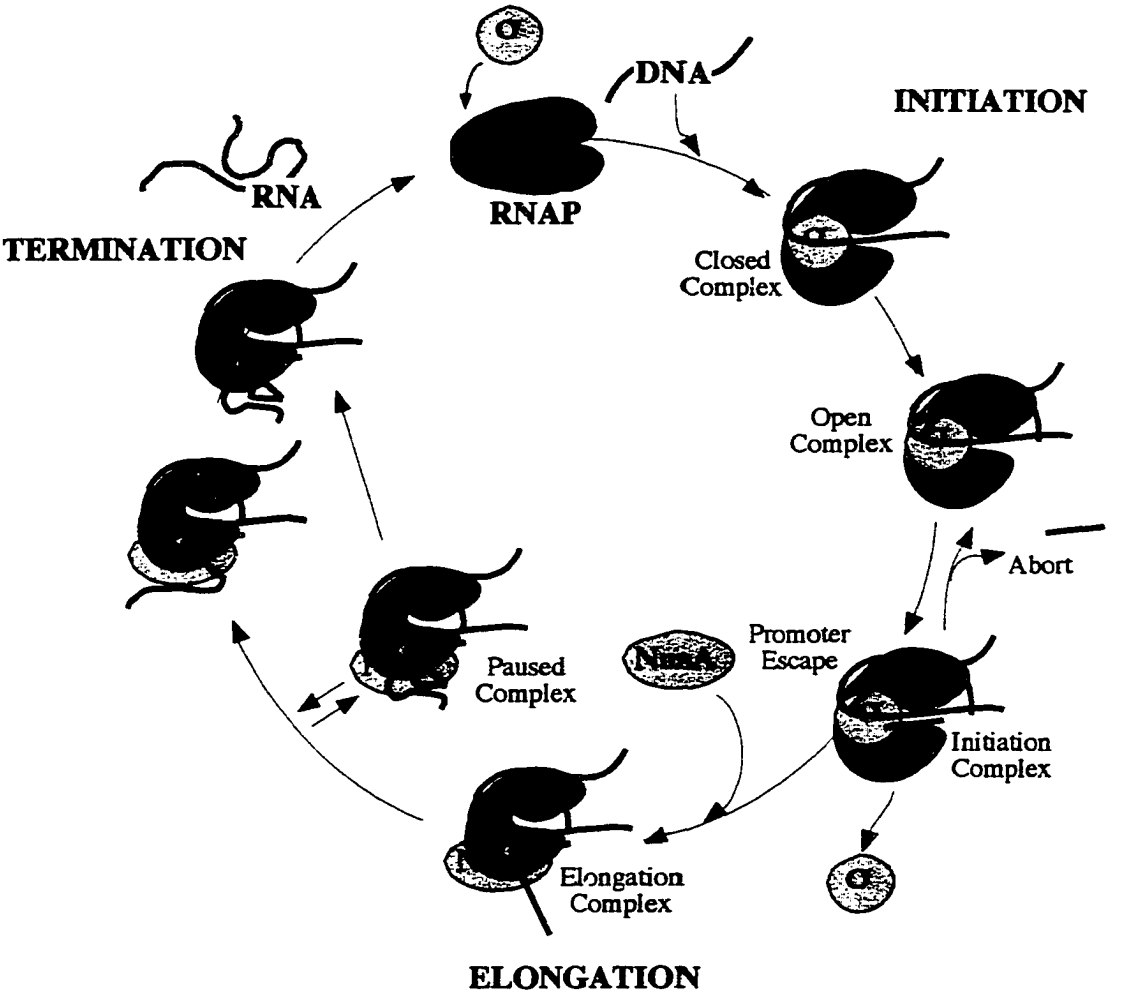


Figure 1: The transcription cycle. Intermediates of the phases are discussed in the text. (The figure is a modification of Landick *et al.*, 1998, as NusA is indicated in the transcription cycle).

Elongation— The elongation phase of the transcription cycle involves an enzymatically catalyzed polymerization reaction during which the RNA is formed by the assembly of nucleotide triphosphate (NTP) monomers into a growing polymer. The NTPs are selected by the polymerase in an order dictated by the base sequence of the template DNA. During this process the RNAP elongation complex is stably bound (i.e. able to resist dissociation in 1 M KCl or at 65°C (Naito & Ishihama, 1975; Rhodes & Chamberlin, 1974)) and traverses thousands of basepairs of DNA. However, the details of how this reaction occurs and the regulatory mechanisms involved are not fully understood.

The features of the transcription complex can be presented in a form of a model (illustrated in Figure 2). The structure of the transcription complex, which is derived from studies of halted transcription complexes, indicates that the RNA polymerase protects a region of ~25 to ~40 bp of DNA from digestion by various endo- and exonucleases (Krummel & Chamberlin, 1992). Within the elongation complex, the DNA is unwound in the transcription bubble (~17 bp). Two independent and precise measurements, one by Kumar and Krakow (Kumar & Krakow, 1975), the other by Hanna and Meares (Hanna & Meares, 1983a; Hanna & Meares, 1983b), have determined the average length of the RNA-DNA hybrid to be 12 ± 2 base pairs. Kumar and Krakow showed that short fragments of RNA, of length 12 ± 2 base pairs, are protected when RNAases were added to an *in vitro* transcription system. Photocrosslinking experiments by Hanna and Meares showed strong cross-linking to the DNA template strand at positions 1 through 12 (counting back from the growing point of the RNA), but no crosslinking to the DNA template strand at positions 13 or beyond. Other groups argued for an ~8 bp hybrid based on the protection of template DNA bases from single-stranded specific reagents (Kainz & Roberts, 1992; Lee & Landick, 1992) and RNA-DNA crosslinking (Nudler *et al.*, 1997).

The boundaries of the transcription bubble are maintained by a tight interaction of the RNA polymerase with the DNA, downstream and upstream from the melted region.

Kornberg, Ddarst and others used electron crystallographic methods to deduce low-resolution (16 to 25 Å) structures of *E. coli* holo- and core RNAPs (Darst *et al.*, 1989; Polyakov *et al.*, 1995). The structures have revealed a large channel (~25 Å) surrounded by "jaws". It has been suggested that these "jaws" close around the downstream duplex DNA in the transition from holoenzyme to the elongation complex (Asturias *et al.*, 1997; Polyakov *et al.*, 1995). Two tunnels have been identified that run through the enzyme from near the channel to the opposite side, one of which may function as the RNA exit tunnel (Darst *et al.*, 1991; Kim *et al.*, 1997). The overall structure resembles the hand-like motif of DNA polymerases whose X-ray crystal structures have been determined.

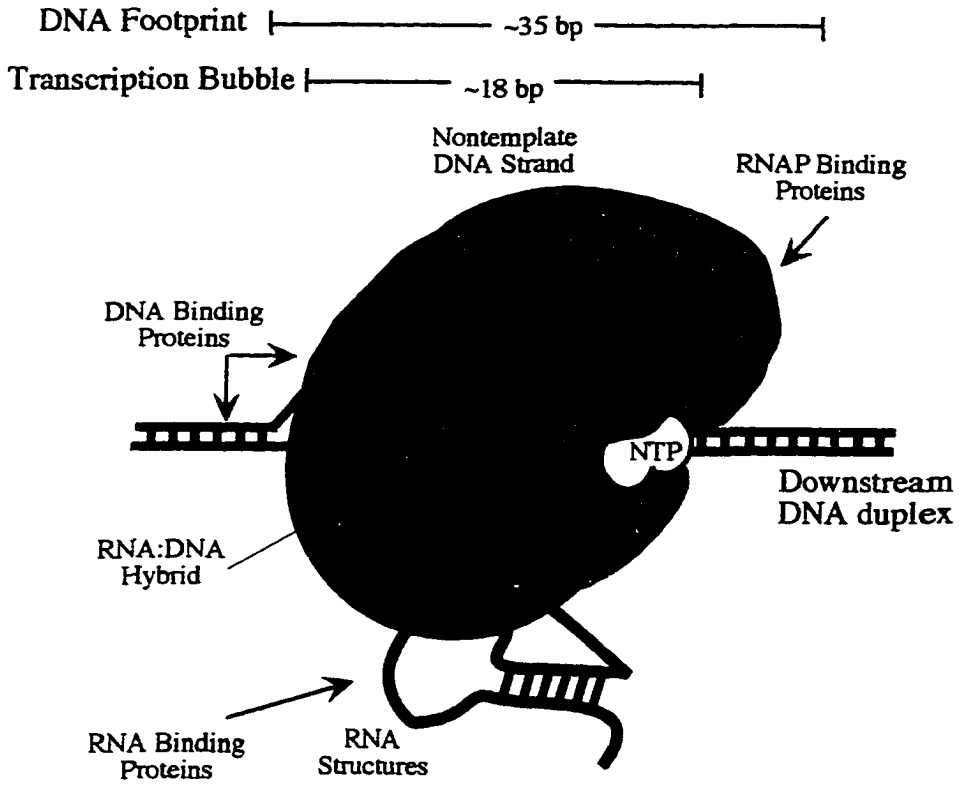


Figure 2: Structure and regulatory inputs of a transcription complex (Landick et al. 1998).

In the traditional view of transcriptional elongation, the RNAP was believed to translocate along the DNA template in a monotonic manner, advancing 1 bp with each nucleotide addition (Yager & von Hippel, 1987). The stability of the binding of the RNA to the complex is determined only by the stability of the DNA-RNA hybrid, and the hybrid was solely responsible for maintaining the integrity of the transcription elongation complex. In this model, it is implied that the structure of a normal elongating RNA polymerase complex would remain invariant as the enzyme moved through a particular transcription unit.

An alternative model incorporated the modification that the addition of 2 or more nucleotides could occur without the complete translocation of DNA and RNA through RNAP followed by chain translocation of 2 or more base pairs (discontinuous movement, sometimes called inchworming) (Chamberlin, 1995). This model was created from evidence of variable sized footprints of halted complexes (Krummel & Chamberlin, 1992; Nudler *et al.*, 1994). In this inchworming model, the RNAP is considered a flexible structure which contains two different RNA/DNA binding sites. One site is located at the front edge of the enzyme (site 1) and the other at the back edge (site 2). These sites can exist in two states, either locked or sliding, and the DNA/RNA hybrid is proposed to be only 2 base pairs in length. In such a model, elongation involves two separable phases of synthesis: nucleotide addition and translocation. In the nucleotide addition phase, the DNA/RNA site at the front of the enzyme is locked, while the DNA/RNA site located at the rear of the polymerase is unlocked and can slide. The catalytic site of the polymerase moves along the DNA, adding ribonucleotides. The addition of nucleotides leads the back edge of the polymerase to move progressively closer to the front. This phase is completed when the RNA binding site 1 is filled. During translocation, DNA/RNA site 2 is locked and site 1 is unlocked. This allows the front edge of the polymerase to move forward, leading to a translocation of

approximately 10 base pairs. As the site 1 moves forward, RNA site 1 is emptied, and the cycle begins again (Chamberlin, 1995).

New evidence, which contradicts the two basepair DNA-RNA hybrid theory, promotes a reinterpretation of Chamberlin results. Footprinting evidence demonstrates that a potentially rigid RNAP can sometimes slide freely along the RNA and DNA chains and displace the 3' end from the active site, resulting in larger or smaller footprints (Komissarova & Kashlev, 1997a; Nudler *et al.*, 1997; Reeder & Hawley, 1996). The most recent model for translocation suggests that the RNAP is distributed among all accessible positions by rapid sliding, with the occupancy of each template position dependent on its relative free energy (Guajardo & Sousa, 1997; Komissarova & Kashlev, 1997b; Landick, 1997). This thermal ratchet mechanism suggests that the energy for directional translocation along DNA is derived when nucleotide addition shifts the positional equilibrium of the RNAP sliding back and forth along the RNA and DNA chains towards the forwardly translocated conformation (Gelles & Landick, 1998).

Regulation sites throughout elongation— RNA chain elongation is punctuated by certain control sites which are recognized by the RNAP. When the elongation complex interacts with a pause site, the polymerase hesitates but is able to resume elongation. These interactions are distinct from transcriptional arrests which have been shown to occur *in vitro*. At arrest sites, the polymerase halts and the ternary complex remains intact with no release of the RNA. At a third type of site, transcriptional terminators, the elongation complex dissociates and releases the RNA transcript. There are many *cis* and *trans* acting factors which can regulate the RNAP complex at each of these sites.

Transcriptional pausing can be a result of natural variations, such as local NTP concentrations, or can have a specific regulatory function. Temporary halting of the polymerase within a transcriptional unit allows for *trans*-acting factors, such as ribosomes, termination factors or antitermination factors, to interact with the elongation complex and

alter RNA synthesis. It is also thought that pausing allows the surveillance of defective mRNA by allowing for rho-dependent release of untranslatable messages (Mooney *et al.*, 1998).

Pause sites are found in the leader regions of some amino acid biosynthetic operons and are thought to synchronize attenuation by delaying transcription of the attenuator until a ribosome begins synthesis of the leader peptide. Upon release of the polymerase from the paused site by the translating ribosome, the polymerase synthesizes the RNA transcripts that compete to form the terminator or antiterminator structures just as the ribosome arrives at the leader peptide control codons. *E. coli* RNA polymerase has also been shown to pause at the sites +16 and +17 when transcribing bacteriophage λ late gene operon (Grayhack *et al.*, 1985; Kainz & Roberts, 1992). It is thought that the stalled complex is a substrate for the late gene regulatory antiterminator Q protein (Grayhack *et al.*, 1985)

Although there is no single origin of transcriptional pauses, there are certain sequences and structures which contribute to pausing by *E. coli* RNA polymerase. RNA hairpins have been shown to induce pausing by either disrupting the DNA:RNA hybrid or by directly interacting with the RNA polymerase (Chan & Landick, 1993; Farnham & Platt, 1981; Reynolds & Chamberlin, 1992). GC-rich regions in the DNA can induce pausing about 10 base pairs downstream, perhaps by impairing elongation through a region requiring higher energy to unwind the DNA:RNA hybrid in the transcription complex (Gilbert *et al.*, 1974). Other pause sites have neither of these characteristics and are thought to involve interactions of regions of the DNA or nascent RNA with RNA polymerase that impede elongation in some unspecified way (Levin & Chamberlin, 1987). The transcription elongation factor NusA has been shown to enhance pausing (Farnham *et al.*, 1982; Greenblatt *et al.*, 1981b; Kassavetis & Chamberlin, 1981; Kingston & Chamberlin, 1981; Yager & von Hippel, 1987) either through direct competition for NTPs (Schmidt & Chamberlin, 1984) or through a NusA-directed change in the interactions between the

RNAP and the RNA (Zhang & Hanna, 1994). A more detailed description of NusA function will be given in Chapter 4.

Transcript release—Transcription termination in *E. coli* occurs via two mechanisms: factor dependent or factor independent termination (also referred to as intrinsic termination). Both mechanisms involve the dissociation of the ternary complex concomitant with collapse of the transcription bubble and release of the RNA transcript. Factor dependent termination requires the termination protein rho, a hexameric protein identified based on its ability to terminate transcription which had initiated from the early phage promoters P_L and P_R (Roberts, 1969). The rho protein binds to the nascent RNA of the transcription complex and releases it at defined rho-dependent terminators along the template.

Intrinsic terminators are characterized by a stable RNA hairpin followed by a 7 to 9 nucleotide 3' proximal region that usually contains 6 to 8 U's. Although the actual function of the RNA hairpin in transcription termination remains unclear, several models have been proposed to explain its importance. It is thought that the hairpin may partially disrupt the RNA-DNA hybrid within the ternary transcription complex and facilitate the release of the RNA by partially destabilizing the complex (Yager & von Hippel, 1991). The hairpin may also cause elongation complexes to pause at positions of termination and increase the probability of termination (Farnham & Platt, 1981). Finally, others propose that the hairpin acts to destabilize specific binding interactions between the polymerase and the nascent RNA (Chamberlin, 1995).

Several *trans*-acting factors can influence the efficiency of termination at intrinsic terminators. The NusA protein has been shown to increase the termination efficiency of several intrinsic terminators. The t_{R2} terminator of phage λ, which is 40% efficient in a minimal system, terminates with 90% efficiency in the presence of the NusA protein (Schmidt & Chamberlin, 1987a). It is not known how the NusA protein enhances the

efficiencies of intrinsic terminators. Other extrinsic factors decrease the termination (called antitermination) by modifying the elongating complex in such a way that it is unable to recognize the terminator signals. The best characterized examples of antitermination are found in the lambdoid bacteriophages. The phage λ N protein, in concert with several *E. coli* host proteins, prevents termination at multiple sites in both early operons which allows the polymerase to transcribe genes necessary for replication of the phage DNA (Friedman, 1988; Greenblatt *et al.*, 1993). Similarly, the Q protein prevents termination during transcription of the late p_R operon which allows for transcription of the late genes involved in lysis of the infected cell (Grayhack *et al.*, 1985).

The focus of Part 2 of this dissertation is to examine the role of the RNA polymerase α subunit in pausing and in intrinsic termination. Emphasis will be placed on the α subunits specific interaction with the transcription elongation factor NusA. A model of how this interaction affects RNA polymerase function in pausing and intrinsic termination is proposed.

Chapter 5

Interaction of the Transcriptional Elongation Factor NusA with *E. coli* RNA Polymerase: Identification of the Critical Residues on the C-terminal Domain of the Alpha Subunit

Abstract

The *E. coli* RNA Polymerase (RNAP) core enzyme is composed of four subunits, $\beta\beta'\alpha_2$. The α subunit is critical for assembly of the polymerase and also mediates the activation of transcription initiation by a number of factors. We have recently shown that α plays an important role in transcription elongation, which is mediated by a direct interaction of α with the elongation factor NusA. When the C-terminal domain of α (αCTD) is deleted, NusA no longer enhances pausing or termination at intrinsic terminators, or enhances antitermination by the bacteriophage λ Q protein.

Here we present evidence for a specific NusA binding domain on αCTD. To determine the specific amino acids on αCTD that contact NusA, we have utilized alanine scanning mutants of αCTD covering residues 258-329. *In vitro* binding experiments of α to immobilized NusA (glutathione-S-transferase (GST)-NusA on Glutathione Sepharose) were performed using *E. coli* extracts which overexpress these mutant proteins, and the amino acid residues on αCTD which are critical for NusA binding have been identified.

Loss of any of the five leucines in helix 4 and one leucine in helix 3 virtually abolished binding, suggesting a predominantly hydrophobic interaction between NusA and αCTD. Changes in a second group of amino acids, involving primarily acidic and basic amino acids in helix 3 and helix 4, also caused significant decreases in NusA binding. Interestingly, R265, known to be required for most other interactions with αCTD, was not required for NusA binding. The two groups of defective amino acids form a contiguous patch on one side of αCTD. The stoichiometry of binding was one NusA for each αCTD, suggesting that two NusA molecules can bind to RNAP, either directly, or through NusA-NusA interactions.

Introduction

Gene expression involves numerous of protein-protein, protein-DNA, and protein-RNA interactions. To fully understand the regulation of gene expression, the molecular interactions that occur at each step in the transcription cycle must be characterized. The RNA polymerase in *E. coli* consists of a core enzyme composed of two large subunits, beta (β) and beta prime (β'), and two copies of a smaller subunit alpha (α). The core enzyme is assembled *in vitro* and *in vivo* in the following order: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (Ishihama, 1981). The holo enzyme, responsible for specific transcription, contains an additional subunit, sigma (σ), which confers promoter specificity upon the core enzyme (Gross *et al.*, 1992), The sigma factor is released from the core shortly after promoter recognition and initiation of transcription.

Once σ is released, the elongation factor NusA can bind to the transcription complex (Burgess & Travers, 1969; Gill *et al.*, 1991; Greenblatt & Li, 1981). NusA, as part of the transcription complex, slows the elongation rate of RNA polymerase, enhances pausing at some sites (Chan & Landick, 1993) and increases termination efficiency at intrinsic terminators and at some Rho-dependent terminators (Kainz and Gourse 1998). RNA polymerase terminates transcription via two different mechanisms, factor dependent or factor independent termination. RNA polymerase receives regulatory signals at each of the steps in the transcription cycle. Examining how the polymerase is modulated by these signals is central to understanding the regulation of gene expression.

The alpha subunit is composed of 329 amino acids that form two structurally independent domains separated by a flexible linker of 13-36 amino acids (Blatter *et al.*, 1994). The N-terminal domain (αNTD), amino acids 20-235, is necessary and sufficient for the proper assembly of RNA polymerase, both *in* vitro and *in vivo* (Murakami *et al.*, 1997). The αNTD also participates in the activation of transcription at Class II CRP-

dependent promoters by directly contacting CRP (cAMP receptor protein) to facilitate open-complex formation (Savery *et al.*, 1998). The C-terminal domain (αCTD), amino acids 249-329, contains the binding site(s) for several regulatory proteins, including OxyR (Igarashi *et al*, 1991), OmpR (Igarashi *et al.*, 1991; Jair *et al.*, 1996; Savery *et al.*, 1998; Tao *et al.*, 1995; Yang *et al.*, 1997), CRP (Savery *et al.*, 1998), SoxS (Tao *et al.*, 1995), & TyrR (Yang *et al.*, 1997). In addition, αCTD interacts directly with DNA promoter regions upstream of the −35 consensus hexamer (UP elements) of some promoters, including the ribosomal RNA P1 promoters (Gaal *et al.*, 1996).

Recently, we have shown that αCTD also contacts the nascent RNA in actively transcribing elongation complexes (Liu & Hanna, 1995b). We have shown by RNA-protein crosslinking that NusA can interact with RNA in transcription elongation complexes even when αCTD is absent. However, this requires high concentrations of NusA, and the interaction made by NusA with polymerase lacking αCTD is nonfunctional for enhanced pausing, intrinsic termination, or Q-mediated antitermination. Although NusA can bind to core enzyme lacking αCTD through interactions with β and β', this binding is lost when the ionic strength of the solution is decreased and, although NusA can bind to intact α even in the absence of β and β', NusA cannot bind to α that lacks the CTD (Liu *et al.*, 1996). Interaction of NusA with the elongation complex eliminates the α-RNA contact, with concomitant interaction of the RNA with NusA (Liu & Hanna, 1995b). The removal of the RNA from α could be due to an allosteric interaction of NusA elsewhere on the RNA polymerase, causing a conformational change affecting α. Alternatively, the loss of α-RNA interaction could be caused by direct contact of NusA with αCTD, either masking the RNA binding site on αCTD, or lifting the RNA off of α. Here we describe experiments which distinguish between these two possibilities and define a NusA-binding domain on αCTD.

Materials and Methods

Bacterial Strains and Plasmids

The *E. coli* strain BL21 (DE3) (Novagen), which bears the inducible T7 RNA polymerase gene on the chromosome, was used to express all of the *E. coli* α subunit genes on plasmids. The strain BL21 (Novagen), which lacks the T7 polymerase, was used to express the GST-NusA fusion protein (Zhang & Hanna, 1995). The *E. coli* strain M15 from Qiagen was used to produce a hexahistidine tagged portion of α containing only the linker and the CTD (amino acids 235-329).

The plasmid pHTT7f1α (Tang *et al.*, 1995), which encodes α that contains a N-terminal hexahistidine tag expressed from the bacteriophage T7 gene 10 promoter, was used to construct the complete set of His-tagged alanine scanning mutants in α from amino acid 258 to 329. The *Hind*III-*BamH*I fragment from pHTf1α, containing single alanine substitutions at amino acids 258-273 (a gift from Dr. Richard Ebright), and pREIIα derivatives, encoding single alanine substitutions at amino acids 278, 284-301, 303-304, and 307 (given by Dr. Gail Christie and Dr. Rick Gourse) were used to replace the analogous segment in pHTT7f1α (Tang *et al.*, 1995). The remaining alanine scanning mutations were created using the mega primer method of site-directed mutagenesis (Picard *et al.*, 1994). The DNA sequence of each mutated α gene was verified.

The plasmid encoding only the His-tagged αCTD was created by PCR amplification of amino acids 235 through 329 from plasmid pHTT7f1-NHα. The PCR primers contained *BamHI* and *HindIII* sites, so these sites were on the 5' and 3' ends of the PCR product, respectively. The DNA fragment was digested with *BamHI* and *HindIII* and subcloned into those sites of the vector pQE30 (Qiagen). The recombinant plasmid was transformed into the *E. coli* strain M15.

Binding of αCTD to immobilized GST-NusA

αCTD was expressed in *E. coli* M15 cells and purified with Ni^{2+} agarose (Tang *et al.*, 1995). Purified αCTD (20 μg) was mixed with 100 μg purified GST-NusA fusion protein immobilized to Glutathione Sepharose 4B (Zhang & Hanna, 1995), and incubated at 4°C for 10 minutes with gentle shaking. The beads were pelleted and washed 3 times with PBST (20 mM K_3PO_4; 150 mM KCl; 1% v/v Triton X-100; 1 mM β-mercaptoethanol). The washed beads were resuspended in LLB (100 mM Tris-HCl, pH 8.0; 200 mM DTT; 4% w/v SDS; 0.02% w/v bromophenol blue; 20% v/v glycerol), heated at 94°C for 3 minutes and analyzed by electrophoresis on a 12.5% SDS Tris-glycine gel.

Binding of the mutant alpha proteins to immobilized GST-NusA

GST pull-down assays using a GST-NusA fusion protein were performed to determine which of the amino acids in α are critical for binding to NusA. Crude cell lysates containing the overexpressed wild type α or α mutant protein were prepared by growing the appropriate strains at 37°C with shaking in 5 ml of LB broth plus 100 μg of ampicillin. At an OD_{600} = 0.5 - 0.7, expression of α was induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM. After induction, the cultures were shaken 3 hr at 37°C and then harvested by centrifugation (13,000 x g; 30 sec at 4°C). The cell pellets were resuspended in 0.3 ml of PBST, the cells were lysed by sonication (3 × 15 sec bursts at 5 watts, Fisher Model 60 Sonic Dismembrator, Pittsburg, PA.), and the lysate was cleared by centrifugation (13,000 x g for 5 min at 4°C). The GST-NusA fusion protein was prepared as previously described (Zhang & Hanna, 1995) and immobilized to Glutathione Sepharose 4B (Pharmacia) equilibrated with PBST, at a concentration of 2.5 μg of protein per μl of resin. For the pull-down assays, 40 μl of a 50% slurry of GST-NusA (50 μg) bound to resin was incubated at 4°C for 30 min with 300 μl of the crude cell extract containing the overexpressed αWT, α235 and each of the alanine scanning α proteins. The

resin was pelleted by centrifugation (1,000 x g; 15 sec at RT) and the supernatant fraction was saved for SDS PAGE analysis. The resin was washed 3 times with 600 µl of PBST. The washes were carried out by adding the buffer, mixing the beads briefly and then centrifuging (1,000 x g; 15 sec at RT). The resin was then resuspended in 20 µl of LLB, heated for 3 min at 100°C and analyzed by 12% SDS Tris-glycine electrophoresis.

Results

The C-terminal domain of α binds to NusA

We have previously shown that NusA makes direct contact with the *E. coli* RNAP alpha subunit but does not bind to α containing only the NTD (lacking amino acids 235 to 329 ; Liu *et al.*, 1996). To determine if the αCTD alone binds to NusA, binding studies using a GST-NusA fusion protein immobilized to glutathione sepharose were performed. Fusion of GST to the N-terminus of NusA does not interfere with its function in enhancement of pausing, termination, or Q-mediated antitermination (Zhang & Hanna, 1995) and allows normal binding of NusA to RNA polymerase. The His-tagged WTα, αNTD (amino acids 1-235) and αCTD (amino acids 235-329) were overexpressed and purified using Ni^{2+}-NTA agarose (Qiagen) and passed over the GST-NusA columns after washing the resin to remove unbound proteins. WTα (Figure 1, lane 1) and αCTD (lane 3) remained bound to the GST-NusA column, while αNTD (lane 2) did not. None of the proteins were retained by a column containing resin alone or immobilized GST (data not shown). The interaction of NusA with the alpha subunit therefore requires only the CTD, from amino acids 235 to 329.

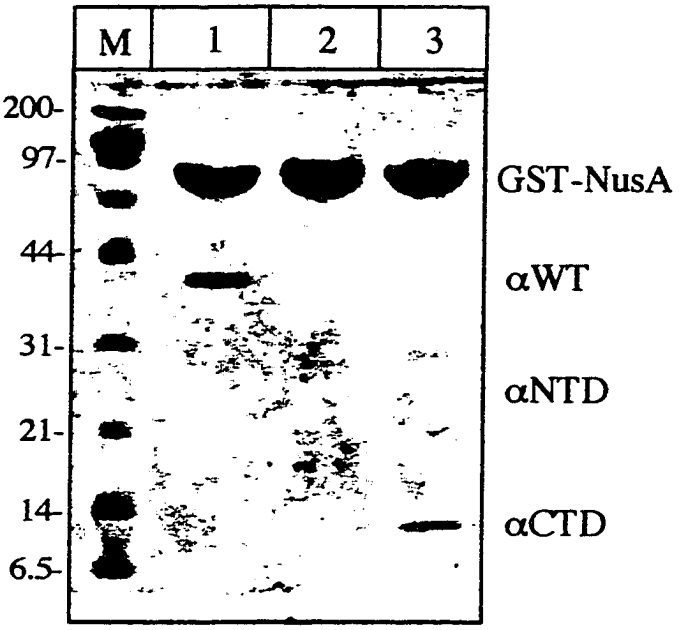


Figure 1. Interactions between immobilized GST-NusA and the *E. coli* α subunit. GST-NusA was immobilized on glutathione sepharose beads and mixed with either αWT (lane 1), αNTD (amino acids 1-235, lane 2), or αCTD (amino acids 235-329, lane 3). After washing, bound proteins were eluted from the resin and the released proteins were analyzed by SDS-PAGE and staining of the gel with Coomassie Blue. Protein molecular weight standards are shown in the lane "M". The molecular weights for α, αCTD and αNTD are 36.5 kDa, 10.4 kDa, and 25.9 kDa, respectively and their positions are indicated on the right. Only the wild type alpha subunit (αWT, lane 1) and the C-terminal domain (αCTD, lane 3) were bound to NusA.

Overexpression of alpha subunits containing single alanine substitutions in the CTD.

To determine which amino acids on αCTD are required for binding to NusA, plasmids encoding the alpha subunit, under the control of the T7 RNAP promoter and containing a hexahistidine tag at the N-terminus of the protein were prepared. Constructs containing alanine substitutions at each of the amino acids from 258 to 329 on αCTD were prepared, and the mutant proteins were expressed. Figure 2 shows the SDS-PAGE analysis of the protein profiles of cell lysates after induction with IPTG (one of three independent experiments is shown). Although the three independent experiments varied somewhat in the level of overexpression of the different α subunits, the level of overexpression did not affect the ability of α to bind the GST-NusA affinity column, as there was an excess of alpha protein added to the column. For example, although the K289A subunit was greatly overexpressed, it failed to bind to the GST-NusA column (Figure 3). In contrast, the G279A subunit was only slightly overexpressed yet bound to the GST-NusA column with the same affinity as the WT subunit.

The cell lysates were then loaded onto the GST-NusA affinity column and then incubated at 4° for 30 minutes. The column was washed to remove proteins not interacting with NusA, and the associated proteins were eluted and subjected to SDS PAGE analysis. Figure 3 represents one of three independent binding experiments performed using the cleared lysates shown in figure 2. The alpha mutants which were unable to bind to NusA as well as WTα can be classified into two groups: those which had severely decreased binding and those which bound to GST-NusA with decreased strength relative to wild type. The αΔ235, L262A, L281, L290A, K297A, L300A, I303A, L307A, L312A, and L314A alpha subunit mutants displayed the most severe defects in binding to NusA, (dark blue in figure 4). Mutants L260A, L270A, E273A, I278A, E286A, V287A, L289A, K291A, K298A, E302A, K304A, D305A, G315A, and L318A, which were able to bind GST-NusA, but less

efficiently then wild type, comprise the second group. (light blue in figure 4). Two of the alanine substitutions, N294A and S309A, increased the binding of αCTD to NusA by 1.5 fold.

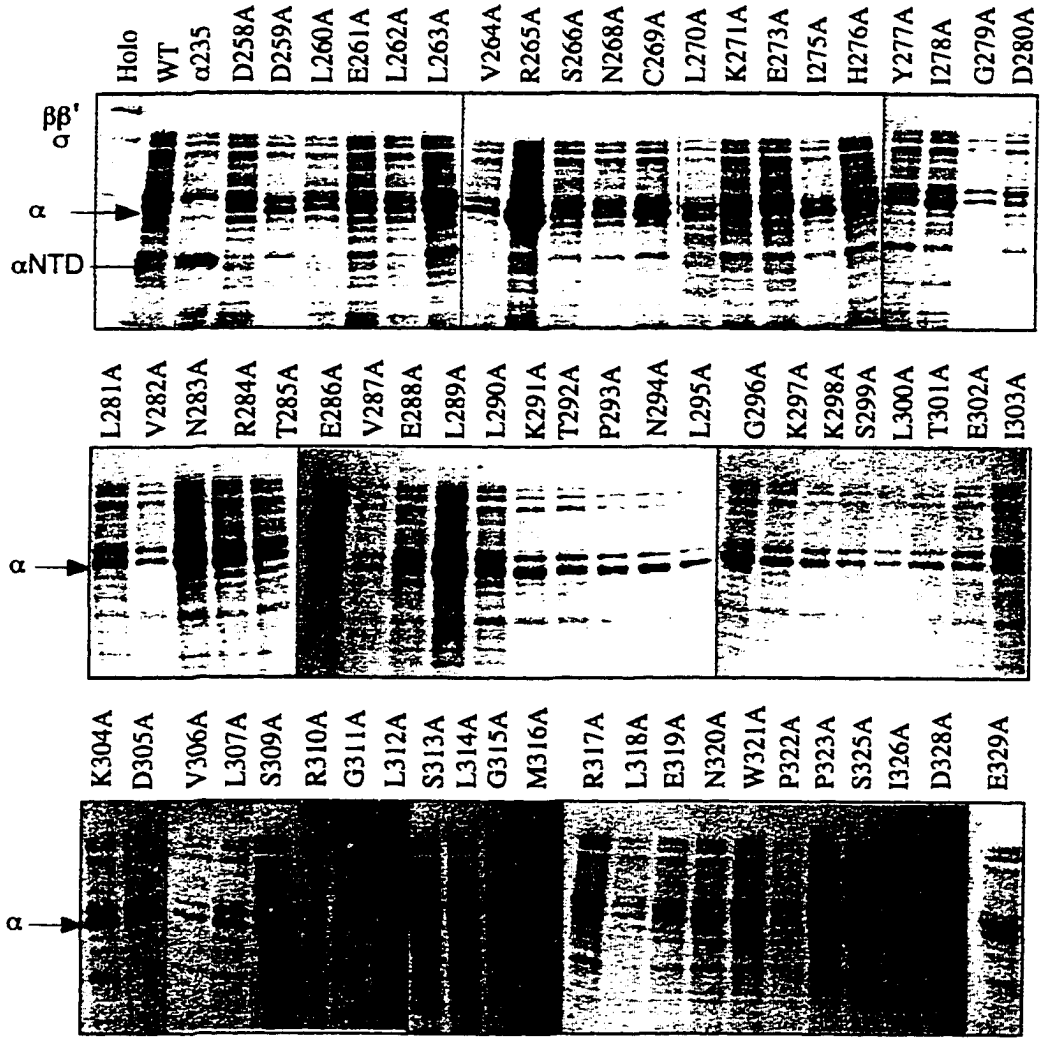


Figure 2: SDS PAGE analysis of clear lysates of cultures overexpressing mutant α subunits. Cultures were prepared as described in Methods. Samples of the clear lysates were analyzed by 12% SDS PAGE. The positions of the RNAP subunits and α containing only the NTD (αNTD) are shown at the left of the top panel.

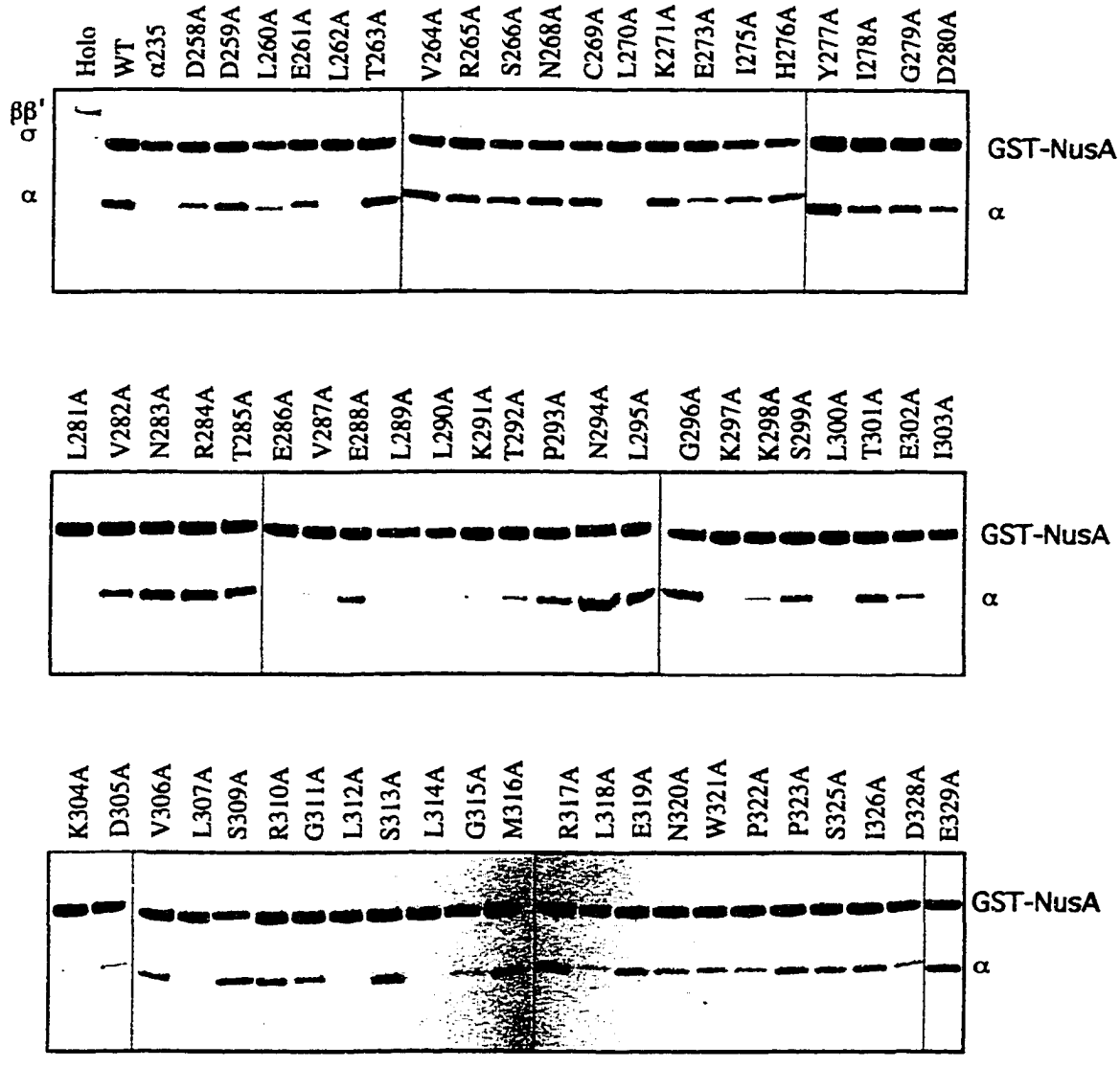


Figure 3: Binding of α subunits mutants to immobilized NusA. GST-NusA was immobilized on Glutathione Sepharose beads and then mixed with crude cell lysates containing overexpressed alpha subunits at 4° C for 30 min. The resin was washed three times with buffer and then bound proteins were eluted from the resin with SDS loading buffer and heated at 100° C for 3 min. Released proteins were analyzed by SDS-PAGE and stained with Coomassie Blue. The positions of the α subunit and GST-NusA are indicated.

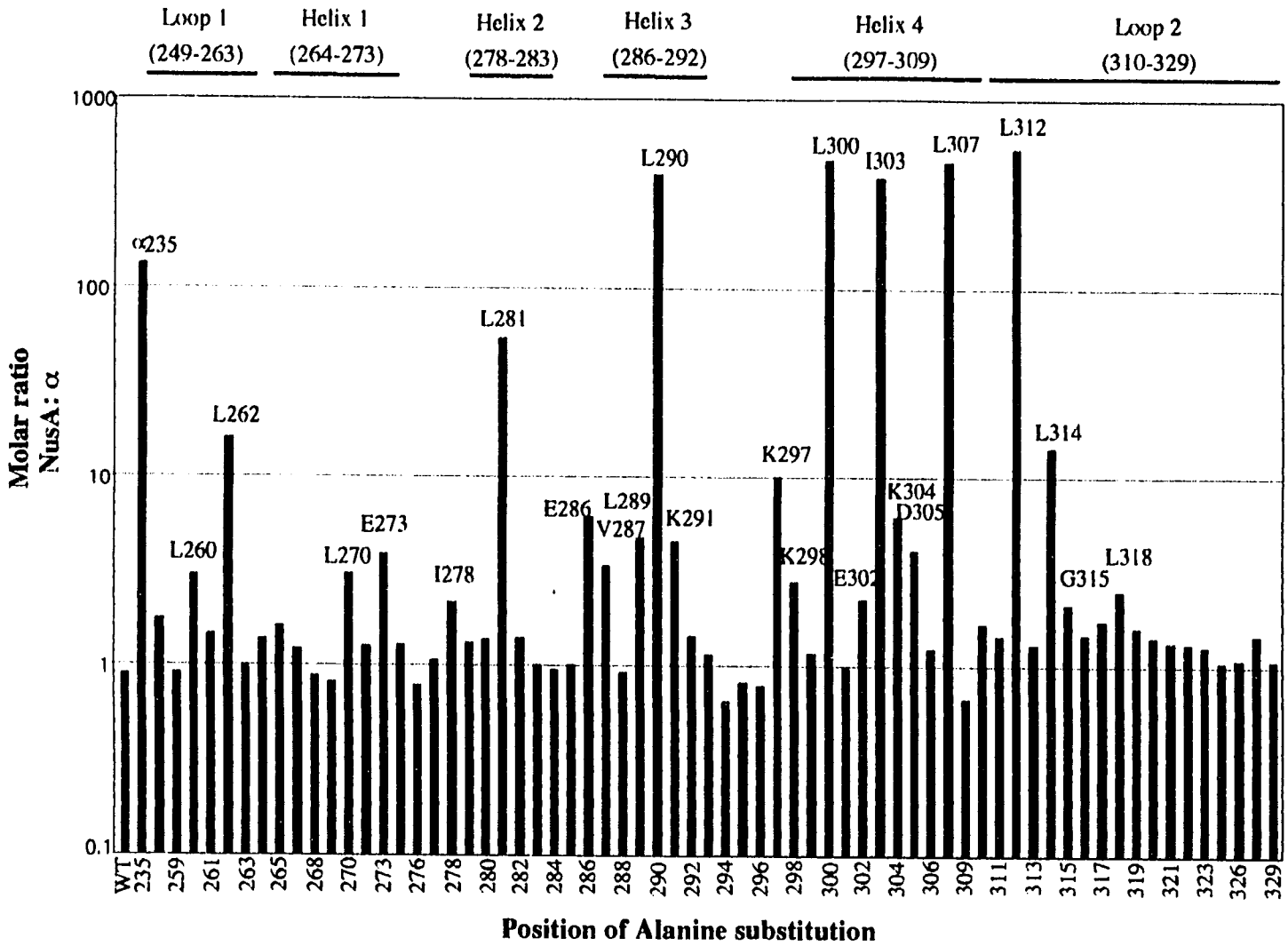


Figure 4: Molar ratio of NusA to α. The molar ratios of NusA to α was determined for each of the alanine substitutions for three independent experiments. Note that the Y axis is in exponential scale. Kodak ID software was used to analyze the amount of NusA and α in each lane.

Discussion

Several studies have identified the specific amino acid residues on the C-terminal domain of the α subunit involved in the direct molecular communication with several transcription activator proteins (both class I and class II activator proteins, Figure 6). In addition, the amino acids involved in protein activator-independent transcription activation at several strong promoters containing an UP element are also identified. Recently, Kainz *et al.* identified residues in αCTD which are important for efficient rho-dependent transcription termination. In this study we supplement the map of the αCTD by indicating which amino acids are involved in the binding of α to the elongation factor NusA.

We have classified two groups of residues on αCTD which exhibit a decreased binding to NusA *in vitro* (Figure 4). Using alanine scanning mutagenesis we have identified eleven amino acid residues, L262, L281, L289, L290, K297, L300, I303, K304, L307, L312, and L314, which are critical for binding of αCTD to NusA. Six of these amino acids substitutions, L290A, L300A, I303A, L307A, L312A, and L314A, completely abolish the binding of αCTD to NusA. Examination of the three-dimensional structure of the αCTD shows that these residues, along with residues K297 and L304, form a surface-exposed patch critical for binding α to NusA. Residues L289 and L270 lie just behind this surface. The location of these residues on the three dimensional NMR structure of αCTD form a contiguous patch which suggests that there is one NusA binding domain (Figure 5). We propose that this surface of αCTD makes direct protein-protein interactions with the transcription elongation factor NusA (Figure 5).

Our studies show that residues R265, N268, E273, L281, G296, I303, K304, and L307 are required for NusA to bind α. Kainz and Gourse (1998) recently reported that NusA stimulated rho-dependent termination two-fold with wild-type RNAP and 1.8 to 2.4-fold with RNAP which have alanine substitutions in αCTD at positions. These results

suggest that while NusA stimulates rho-dependent termination, this stimulation is unlikely to be due to a direct contact of NusA with the αCTD.

Additionally, the L300A substitution completely eliminated the stimulatory effect of NusA on rho-dependent termination *in vitro* even at high concentrations of NusA. We have data which suggests that RNAP reconstituted with this mutant α subunit is unable to respond to NusA in enhancing termination at intrinsic terminators or pausing (unpublished data). This residue is also important in the function of several transcription activators (Figure 6). It will be interesting to determine if this severe mutation causes a structural defect in the αCTD.

We have shown previously that αCTD interacts with the nascent RNA in elongation complexes and that the addition of NusA interferes with this interaction (Liu *et al.* 1996). We have implicated R265 and C269 as the contacts for the RNA (unpublished data). Not surprisingly these residues lie adjacent to the NusA binding site. One can hypothesize a possible model in which, upon binding of NusA to α, the RNA is transferred from the αCTD to the NusA allowing for the increase in pausing. It will be interesting to determine if alanine substitutions at these sites affect NusA function in pausing or termination.

Another intriguing result from this study is the calculated molar ratio of 1:1 α to NusA. We can hypothesis two models either of which is consistent with our data: 1) one αCTD is required for one NusA to bind, or 2) αCTD forms a dimer to which two NusA molecules bind. Figure 7 shows the ribbon model and space filling model of the hypothetical dimer. When located on the dimer of αCTD, in which the two alpha subunits dimerize in two fold symmetry, these residues form a contiguous patch to which NusA can bind.

In conclusion, the alpha subunit, and specifically the αCTD, is a key regulatory target in RNAP which functions through direct contacts with other molecules that regulate all stages of transcription. The transcription elongation factor NusA requires a direct

interaction with αCTD in order to function in elongation, termination, and anti-termination. We have identified specific residues in the αCTD which are required for binding to the elongation factor NusA. Current studies are underway to determine which of these critical residues are required for NusA function throughout the transcription cycle.

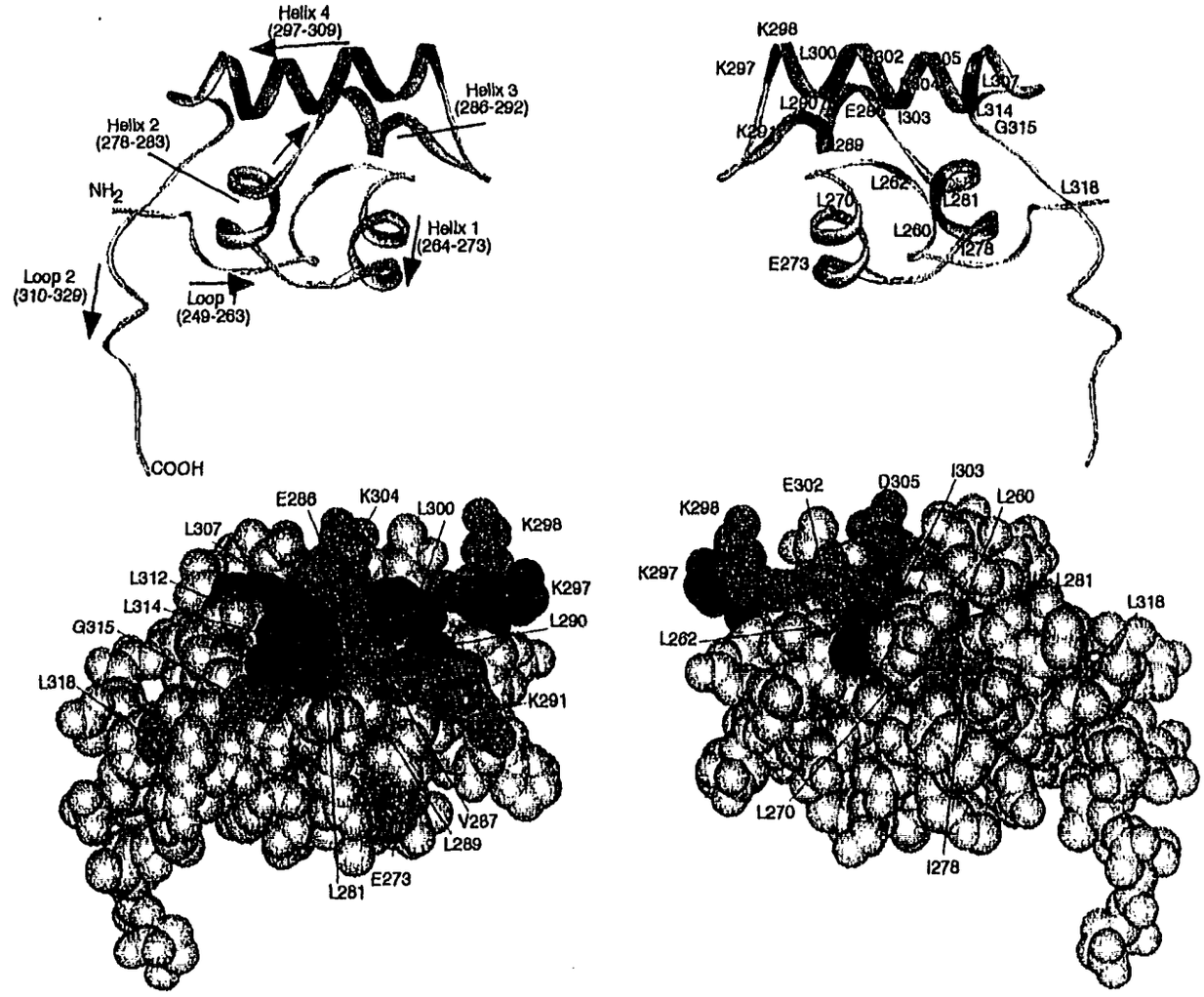


Figure 5: Location of the residues in the αCTD required for binding to NusA (displayed on the three-dimensional NMR solution structure (Jeon *et. al.*,). Residues where alanine substitutions caused a serious defect are colored in dark blue. Alanine substitutions which were less defective in binding to NusA are colored in light blue. The two ribbon models are rotated by 180° around the vertical axis. The spacefill models represent the ribbon model located above.

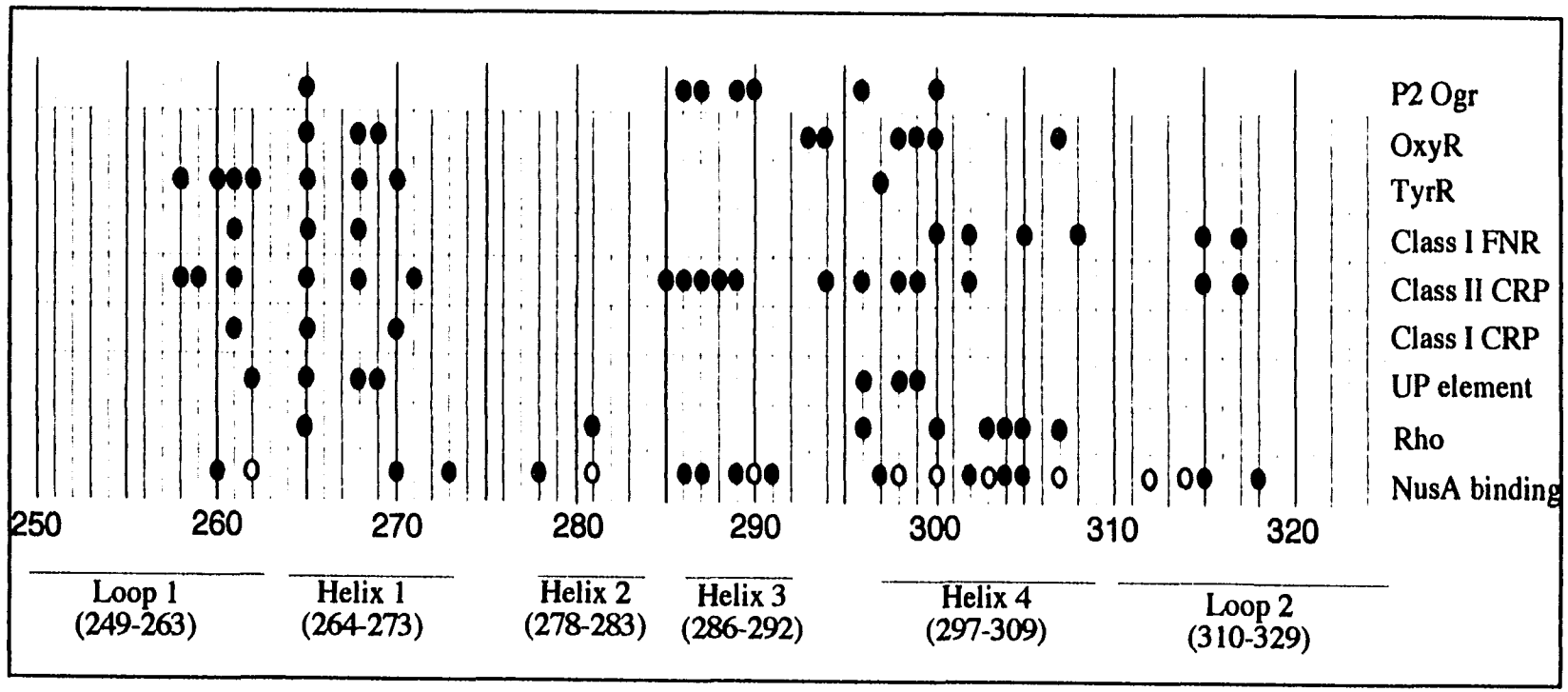


Figure 6: Map of the αCTD showing the amino acids required for binding or function of transcriptional activators and binding of the elongation factor NusA. The circles indicate sites within αCTD (amino acids 250-329) which result in defects in activator dependent transcription, Rho-dependent termination, or NusA binding. The open cirlces represent the alanine substitutions which cause the most serious defects in binding α to NusA (colored dark blue in Figure 5). The gray circles indicate those substitutions which caused the less serious defects (seen in light blue in figure 5).

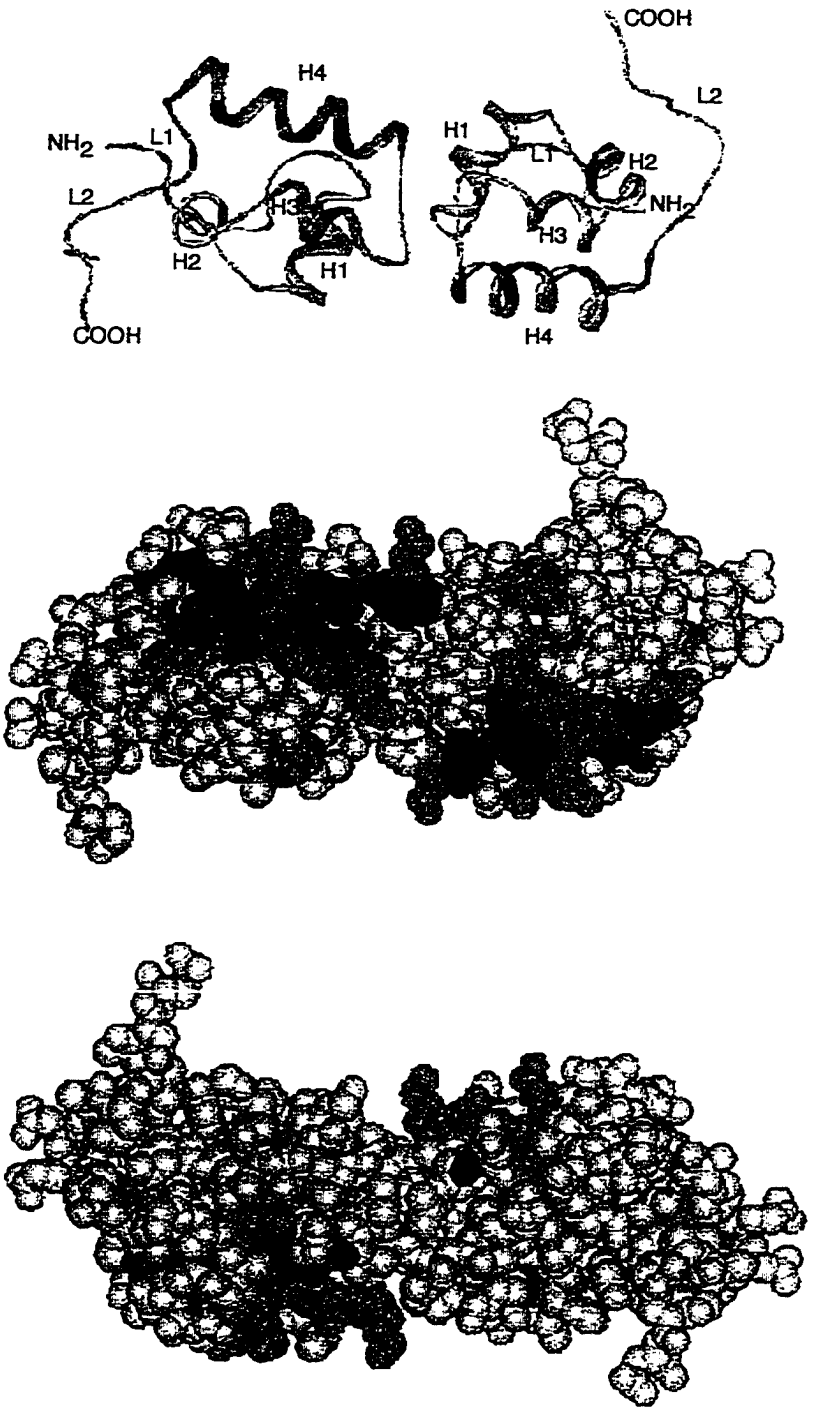


Figure 7: Ribbon and spacefill model of the hypothetical dimer of the αCTD. The alanine substitutions on αCTD which cause the most serious defects (dark blue) and less serious defects (light blue) in binding to NusA are indicated. The ribbon model and top spacefill model both represent the same view of the dimer. The bottom view is a 180° rotation on the horizontal axis showing the opposite face.

Chapter 6

Gene Cloning and Characterization of a NusA-like protein from *Chlamydia trachomatis* which Interacts *in vitro* with *Escherichia coli* RNA Polymerase

Abstract

We cloned and expressed in *Escherichia coli* an open reading frame from *Chlamydia trachomatis* encoding a polypeptide which is homologous to several bacterial NusA factors, a family of proteins regulating transcription elongation and termination. The putative *C. trachomatis* NusA, which differs from *E. coli* NusA in size (434 amino acids, as compared to 495 amino acids for *E. coli*) and sequence (45% homology; 32% identity), was expressed as a Histidine-tagged recombinant protein. Immunoblot analysis of a 2D electrophoretic map of proteins from *C. trachomatis* elementary bodies with antibodies raised against the recombinant protein identified a protein of 48.6 kDa and pI=5.24, in good agreement with the values predictable from the ORF (48.8 kDa, pI=5.19). We show that this protein can bind to *E. coli* RNA polymerase core enzyme (RNAP) *in vitro*. However, the chlamydial NusA is unable to bind to the *E. coli* α subunit, which is a vital interaction for the control of transcript elongation and termination. In agreement with this finding, *C. trachomatis* NusA is unable to increase intrinsic termination in *E. coli* .

Introduction

C. trachomatis can grow only as intracellular parasites of eukaryotic cells (Moulders, 1991) and are pathogenic for humans and several animal species. During their life cycle, the cells alternate between an infectious but metabolically inert, extracellular, spore-like form (the elementary body, EB) and non-infectious, intracellular, replicative forms. The latter form remains contained within a specialized vacuole and undergoes normal bacterial replication by binary fission, but must eventually differentiate back to EBs in order to reinfect the host cells. Due to the features of this unique life cycle, a genetic transformation procedure for Chlamydiae is still essentially unavailable, and therefore there is a general interest in the possibility of studying the molecular genetics of these bacteria by transferring selected molecules into more suitable hosts, or by using reconstituted *in vitro* systems. In particular, the *in vitro* reconstitution of chlamydia-specific RNA transcription processes has been the object of research efforts in recent years (Mathews *et al.*, 1993). A chlamydial initiation factor homologous to *E. coli* σ70 has been described and characterized in an *in vitro* system (Engel & Ganem, 1990; Koehler *et al.*, 1990), and the genes encoding the putative α, β and β' subunits of the chlamydial RNA polymerase core enzyme have been also described (Engel *et al.*, 1990; Gu *et al.*, 1995). It therefore seems reasonable that the chlamydial transcription complex can be reconstituted *in vitro*. We now describe the cloning and expression in *E. coli* of a gene of *C. trachomatis* which encodes a putative component of such a complex, i.e. a protein homologous to *E. coli* NusA, a factor which regulates transcriptional elongation and termination (Farnham *et al.*, 1982; Gill *et al.*, 1991; Greenblatt & Li, 1981; Greenblatt *et al.*, 1981a; Kassavetis & Chamberlin, 1981; Kingston & Chamberlin, 1981). In *E. coli*, NusA interacts both with the core RNA polymerase complex (RNAP) (Greenblatt & Li, 1981) and the nascent RNA (Liu & Hanna, 1995a; Liu & Hanna, 1995b; Yager & von Hippel, 1987). We show here that a recombinant form of this protein from *C. trachomatis* can bind to *E. coli* RNAP, but unlike the *E. coli* factor the

C. trachomatis factor is unable to enhance specific transcription termination at an intrinsic terminator.

Materials and Methods

Construction and Screening of the Expression Plasmids

The oligonucleotides 5'- AAA CTG CAG ATG AAC AAG GAT CTT GTG GCT-3' and 5'- TTT GAA TTC TTA ATC TTC AAC TTG AGG TTT -3', containing *Pst*I and *Eco*RI restriction sites respectively, were used in PCR to amplify the putative *Chlamydia trachomatis* NusA gene from whole genomic DNA. The PCR product was cloned into the corresponding sites of the *E. coli* expression vector pTrcHis/C (Invitrogen Xpress System) to create pTrcHis/C/CTNusA. One positive clone was selected and sequenced (sequence data was deposited with GenBank, accession number U74759).

In order to create a fusion protein of glutathione-S-transferase and the NusA-like protein of *C. trachomatis*, the oligonucleotides 5'-TCC CCC GGG ATG AAC AAG GAT CTT GTG GCT- 3' and 5'-CCT CGA GTT AAT CTT CAA CTT GAG GTT TTT-3', containing *Sma*I and *Xho*I sites respectively, were used to amplify the NusA gene from pTrcHis/C/CTNusA by PCR. The PCR product was ligated into the corresponding sites of pGEX-4T-2 (Amersham Pharmacia Biotech) to generate pMH104. After ligation, plasmids were transformed into DH5α and checked for overexpression of the fusion protein.

Computer analyses

Nucleotide sequences were assembled and analyzed with GCG software (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, Wisc., USA) (Devereux *et al.*, 1984). Data base searches for protein sequence similarity were performed with the BLAST programs (Altschul *et al.*, 1990) available at the NCBI (National Center for Biotechnology Information) Web page (http://www.ncbi.nlm.nih.gov). Multiple alignments of protein sequences were generated with the Pileup algorithm of the GCG software. Theoretical pI and Mw were calculated by the pI/Mw computer program available from the

ExPASy server (http://www.expasy.ch). Analyses of two dimensional protein map and corresponding immunoblots were performed with the Melanie II program (BioRad).

Expression and purification of His-tagged *C. trachomatis* NusA

The pTrcHis/C/CTNusA plasmid was transformed into the *E. coli* strain TOP 10 provided by the Xpress System kit (Invitrogen). Overexpression of the His-tagged *C. trachomatis* NusA protein (CT-His_6-NusA) was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Three hours after induction, cells were harvested and lysed according to manufacturer instructions. The His-tagged CT-NusA protein was purified by loading the cell lysate on a 2 ml Ni-NTA-agarose (Qiagen) column which had been equilibrated with 20 ml of 20 mM phosphate buffer (500 mM NaCl, pH 7.8). The column was then washed with 20 ml of 20 mM phosphate buffer (500 mM NaCl, pH 6.0). The recombinant protein was eluted with 5 ml aliquots of 20 mM phosphate buffer (500 mM NaCl, pH 6.0) containing imidazole at increasing concentrations of 50, 200, 350 and 500 mM. Aliquots of 20 µl from each step were collected and analyzed by SDS-PAGE on 13% polyacrylamide gels (Laemmli, 1970) and stained with Coomassie Blue. The fractions containing a single band of appropriate molecular weight were pooled and the protein was concentrated by precipitation with a 10% TCA, 0.04% deoxycolate solution. The precipitate was collected by centrifugation, the pellet washed with acetone and resuspended in sterile PBS

Preparation of antibodies to recombinant CT-NusA

Polyclonal antibodies were prepared by immunizing a rabbit with the purified CT-His_6-NusA protein. Antigen (50 μg) was injected four times at 15 day intervals, using Freund complete adjuvant in the first injection and incomplete Freund adjuvant in the following ones. Increasing titers of IgGs were monitored by Western blotting analysis with the recombinant antigen.

2D-PAGE protein mapping

Two-dimensional gel electrophoresis of chlamydial proteins was performed using the immobiline/polyacrylamide system, as previously described (Bini *et al.*, 1996). Purified chlamydial cells (mostly EBs) were obtained by growing the *C. trachomatis* strain L2/343/Bu in Vero cell cultures, and purified by density gradient centrifugation, as previously described. Chlamydiae were pelleted by low-speed centrifugation and resuspended in 8 M urea, 4% CHAPS (3-[(3 cholamidopropyl)dimethylammonium]-1-propanesulfonate), 40 mM Tris base, 65 mM dithioerythritol (DTE). Approximately 50 μg of total EB protein was loaded on non-linear immobilized pH gradients (pH 3.5-10) (Pharmacia). The IPG strips were then loaded on 9-16% polyacrylamide linear gradient gels (18 cm x 20 cm x 1.5 mm), and electrophoresis was carried out at 40 mA/gel constant current, 10°C, until the dye front reached the bottom of the gel. Of two gels run in parallel, one was stained with ammoniacal silver nitrate (Hochstrasser *et al.*, 1988; Oakley *et al.*, 1980) and the other was electroblotted onto nitrocellulose membrane (Towbin *et al.*, 1979). Membranes were stained in 0.2% w/v Ponceau S in 3% w/v trichloroacetic acid for 3 min and the position of reference spots was marked to facilitate the matching of the silver stained image with the immunoblot. The immunoreactive spot was detected with the rabbit anti serum raised against the recombinant protein (dil 2500x), followed by incubation with goat

anti-rabbit IgGs conjugated with peroxidase (Cappel, dil. 7000x) and detection by chemiluminescence (Amersham kit).

Expression and purification of GST-tagged CT-NusA

In order to detect the presence of the GST-tagged *C. trachomatis* NusA fusion protein (GST-CT-NusA), DH5α cultures transformed with pMH104 were grown for approximately 2.5 hours at 37°C with shaking and then induced with 1 mM IPTG. After 3 hours of additional growth, the cultures were harvested by centrifugation at 10,000g for 1 min. The pellets were resuspended in 20 µl of LLB (100 mM Tris-HCl, pH 8.0; 200 mM DTT; 4% w/v SDS; 0.02% w/v bromophenol blue; 20% v/v glycerol), and heated at 90°C for 3 min. The expression of the fusion protein was verified by SDS-PAGE.

For large scale purification of the fusion protein, an overnight culture of DH5α harboring the pMH104 overexpression construct was used to inoculate a 3 L culture of LB broth (containing 50 µg/ml ampicillin) to an initial OD_{600} of ~ 0.1. The culture was incubated with aeration until an OD_{600} of 1.0 was reached. IPTG was added to a final concentration of 0.2 mM and 3 hours later the cells were harvested by centrifugation (16,000 x g for 2 min at 4°C) and then resuspended in BC 100 (20 mM Tris-Cl, pH 8.0; 0.2 mM EDTA; 100 mM KCl; 20% v/v glycerol; 0.5 mM PMSF; 0.5 mM DTT; 0.05% Triton X-100) at a concentration of 0.3 grams of cells/ml. Cells were lysed with a French press at 900 lb/in^2 and the lysate was centrifuged at 15,000 x g for 15 min at 4°C. The supernatant was mixed with the appropriate amount of 50% (v/v) slurry Glutathione-Sepharose 4B beads (Pharmacia) equilibrated in buffer BC 300 (20 mM Tris-Cl, pH 8.0; 0.2 mM EDTA; 300 mM KCl; 20% v/v glycerol; 0.5 mM PMSF; 0.5 mM DTT; 1% v/v Triton X-100). The mixture was incubated with rocking for 30 min at room temperature. The resin was sedimented by centrifugation at 500 x g for 5 min and washed three times with 10 volumes of BC 300 and three times with 10 volumes of BC 100 without Triton. In

order to release the GST-NusA, the resin was resuspended in 1 ml of elution buffer (10 mM reduced Glutathione in 50 mM Tris-HCl, pH 8.0) and incubated at room temperature for 10 min. The resin was sedimented as described above and then the supernatant was dialyzed against storage buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 0.1 mM DTT; 100 mM NaCl; 50% v/v glycerol). Protein concentration was determined by the Bradford protein assay (BioRad).

Binding of immobilized *C. trachomatis*NusA to core *E. coli* RNAP

To determine if the *C. trachomatis* NusA is able to directly interact with *E.coli* RNA polymerase, 50 μg of GST-NusA (from either *E. coli* or *C. trachomatis*) was as immobilized to Glutathione Sepharose 4B (Pharmacia) at a concentration of 2.5 μg of protein per μl of resin as previously described (Zhang & Hanna, 1995). For the pull-down assays, 40 μl of a 50% slurry of GST-NusA bound to resin was incubated at 4°C for 30 min with purified *E. coli* core RNAP (10 μg) in a final volume of 100 μl of PBST. The resin was pelleted by centrifugation (1,000 x g; 15 sec at room temperature) and the supernatant fraction was saved for SDS PAGE analysis. The resin was washed 3 times with 600 μl of PBST. The washes were carried out by adding the buffer, mixing the beads briefly and centrifuging (1,000 x g; 15 sec at room temperature). After the supernatant was removed the resin was then resuspended in 20 μl of LLB (100 mM Tris-HCl, pH 8.0; 200 mM DTT; 4% w/v SDS; 0.02% w/v bromophenol blue; 20% v/v glycerol), heated for 3 min at 100°C and analyzed by 12% SDS-PAGE followed by Coomassie staining.

Binding of the alpha subunit of *E. coli* RNAP to immobilized GST-CT-NusA

GST pull-down assays using a GST-NusA fusion protein were performed to determine if the α subunit of *E. coli* RNAP directly interacts with *C. trachomatis* GST-NusA. Crude cell lysates containing the overexpressed wild type α subunit were prepared

by growing the *E. coli* strain BL21(DE3), transformed with the plasmid pHTT7f1-NHα (Tang *et al.*, 1995), at 37°C with shaking in 5 ml of LB broth containing 100 μg of ampicillin. At an OD_{600} of 0.5 - 0.7, expression of the α gene was induced by the addition of IPTG (1 mM). After induction, the cultures were shaken an additional 3 hours at 37°C and harvested by centrifugation (13,000 x g; 30 sec at 4°C). The cell pellet was resuspended in 0.3 ml of PBST (20 mM K_3PO_4, pH 7.4; 150 mM KCl; 1% v/v Triton X-100; and 1 mM β-mercaptoethanol), the cells were lysed by sonication (3 times with 15 sec bursts at 5 watts, Fisher Model 60 Sonic Dismembrator, Pittsburg, PA.), and the lysate was cleared by centrifugation (13,000 x g; 5 min at 4°C). The GST-NusA fusion protein was prepared and immobilized to Glutathione Sepharose 4B as described above. For the pull-down assays, 40 μl of a 50% slurry of GST-NusA (50 μg) bound to resin was incubated at 4°C for 30 min with 300 μl of the crude cell extract containing the overexpressed α protein. The resin was washed as above and the eluted proteins were analyzed by 12% SDS PAGE followed by Coomassie Blue staining.

In Vitro Transcription Assays

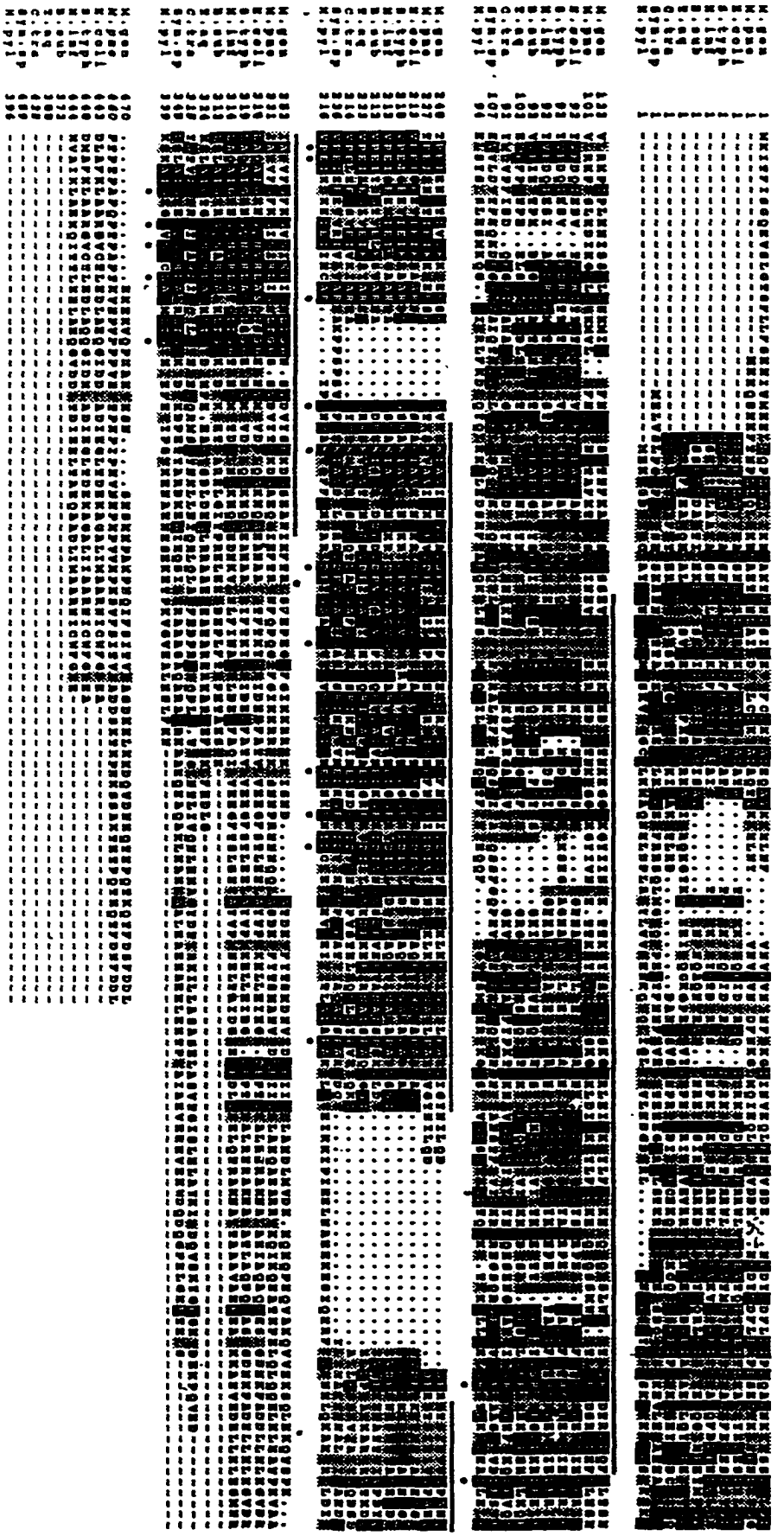
For the analysis of NusA-enhanced Rho-independent termination, transcription complexes were formed by pre-incubation of 20 nM RNAP, 10 nM DNA (the template is a PCR fragment which contains the λ P_R' promoter and the λ t_o terminator from plasmid pHA100-R63 (Hanna & Meares, 1983a) and an initiating trinucleotide (ApApC) (100 nM) for 5 min at 37°C in buffer A (20 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 50 mM KCl; 2% v/v glycerol; 20 μg/ml acetylated BSA) with or without NusA at the indicated concentrations. Transcription was then initiated by the addition of $MgCl_2$ (7 mM), heparin (20 μg/ml), ATP, UTP (200 μM each) and 50 μM [α-^{32}P] GTP ($2x10^5$ cpm/pmol). After incubation at 37°C for 3 min, the transcription was chased by the addition of CTP (200 μM). After another 10 min incubation at 37°C, the reaction was stopped by the addition of

2 volumes of stop solution (1.5 M Ammonium acetate; 37.5 mM EDTA; 50 µg/ml tRNA) and the RNA was precipitated by the addition of ethanol. The RNA was resuspended in 15 µl of load buffer (7 M urea; 1 mM EDTA; 0.02% w/v bromophenol blue; 0.02% w/v xylene cyanol), heated at 100°C for 3 min and separated on a 5% polyacrylamide urea gel.

Results

Cloning of the putative *C. trachomatis* nusA gene

In a program of random genomic walking, which had the scope of connecting the *C. trachomatis* L2 genome to a previously determined protein map (Bini *et al.*, 1996) PCRs were performed on "vectorette" genomic libraries. The DNA segments obtained were cloned and screened by sequencing. Nucleotide sequences, which contained significant open reading frames (ORFs), according to data base homology searches, were extended on either side by further specific PCRs in the appropriate restriction libraries. Several expressed genes were identified by matching the theoretical and experimental data on N-terminal amino acid sequence, Mw and pI values, and/or by immunoblot analysis with immune sera raised against the recombinant product of the cloned ORF. One of the ORFs selected encodes a 434 amino acid long polypeptide which could be significantly aligned with several other bacterial proteins belonging to the NusA family of transcription elongation/termination factors (Bini *et al.*, 1996; Friedman, 1988; Richardson, 1993) An alignment of the *C. trachomatis* peptide with other nine bacterial proteins of the NusA family is shown in Figure 1. This alignment was obtained with the Pileup computer program, which orders the sequences according to calculated evolutionary distances. Interestingly, nucleotide sequence data shows that the next gene downstream of the putative *C. trachomatis nusA* gene encodes for the putative protein-synthesis initiation factor IF2 (see DNA sequence data deposited in GenBank with accession number U74759): an arrangement which is also found in the genomes of other bacteria, including *E. coli* and *B. subtilis* (Shazand *et al.*, 1993) and is consistent with the identification of the ORF as a chlamydial *nusA* gene.



Expression of the putative nusA gene in *E. coli* and identification of the corresponding native gene product

The ORF which encodes for the putative *C. trachomatis* nusA protein was cloned in the expression vector pTrcHis/C and overexpressed as a His-tagged fusion protein by IPTG induction in the *E. coli* strain TOP10. The expressed protein (CT-His_6-NusA) was purified from bacterial extracts by adsorption onto Ni-NTA-agarose resin followed by elution with increasing concentrations of imidazole. Typical results are shown in Figure 2. The purified CT-His_6-NusA protein was used to raise specific rabbit antibodies. In order to confirm that the protein encoded by the *nusA* ORF is actually expressed *in vivo*, proteins from purified EBs of the *C. trachomatis* strain L2 were fractionated by isoelectrofocusing on immobiline strips followed by SDS PAGE to obtain a two dimensional map. A Western blot of the gel was then probed with the antiserum raised against the recombinant protein. The results (Figure 3) show that the rabbit serum identified a single spot corresponding to a protein species of 48.6 kDa and pI=5.24, in good agreement with values predictable from the cloned ORF (48.8 kDa, pI=5.19).

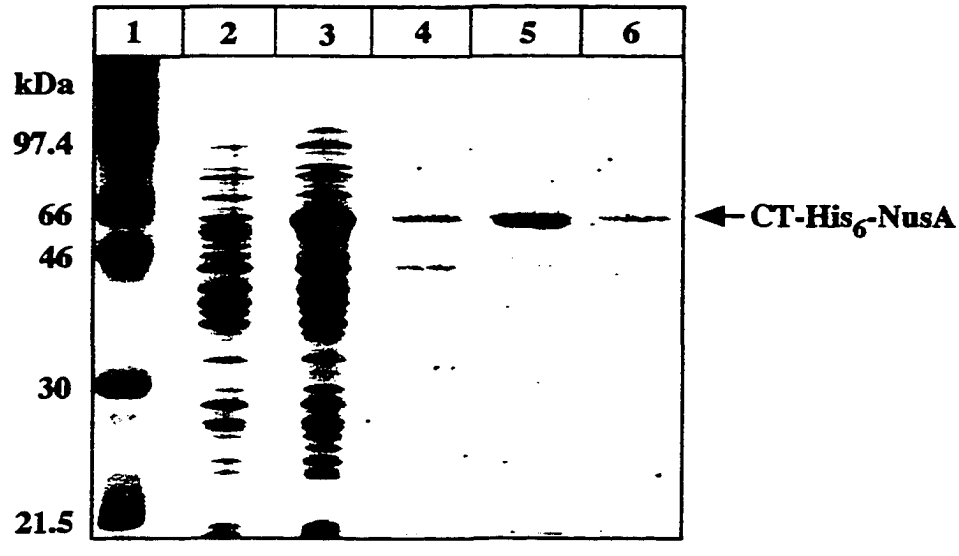


Figure 2: Analysis by SDS-PAGE and Coomassie blue staining of the expression and purification of His-tagged CT-NusA protein in *E. coli*. Aliquots of culture were collected and lysed both before IPTG induction and three hours later (lane 2 and 3). Purification of CT-His_6-NusA was achieved by elution from a Ni-NTA agarose column with 50 mM (lane 4), 200 mM (lane 5), and 350 mM (lane 6) imidazole solution. Molecular weight markers are shown in lane 1 with MW values shown on the left.

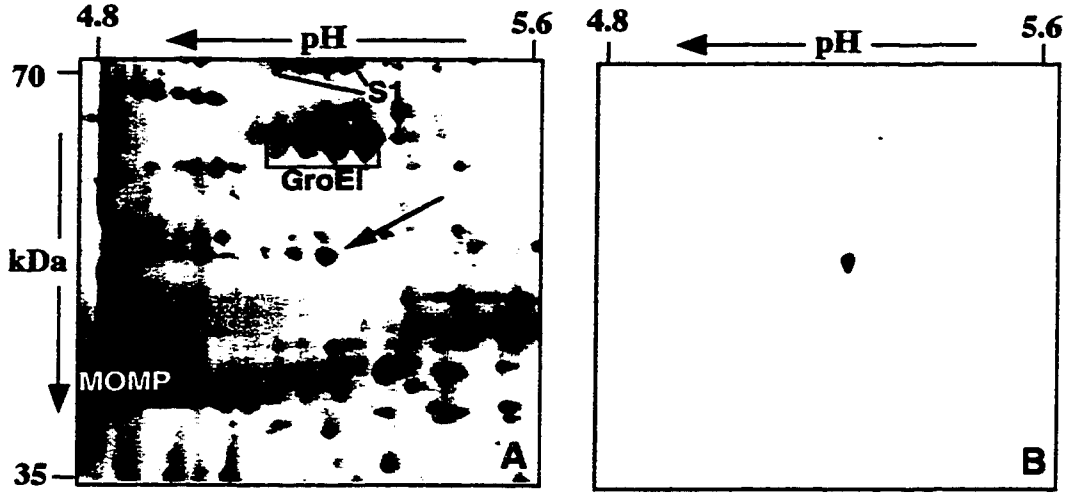


Figure 3: Section of a two dimensional map of *C. trachomatis* proteins (A), and an immunoblot of the corresponding area (B) probed with rabbit antiserum to recombinant *C. trachomatis* NusA. Molecular weight and pI ranges are marked, as well as internal map markers (MOMP, and GroEL-like and S1 proteins according to the reference map previously described). The arrow in panel A shows the spot identified (by computer assisted image matching) as due to the native *C. trachomatis* NusA protein.

C. trachomatis NusA binds to *E. coli* core RNA polymerase

To determine whether the recombinant *C. trachomatis* GST-NusA (CT-GST-NusA) could bind to *E. coli* RNAP core enzyme *in vitro*, as had been previously observed for *E. coli* GST-NusA (E-GST-NusA) (Gill *et al.*, 1991; Greenblatt & Li, 1981), binding studies using a GST-NusA fusion protein immobilized to Glutathione Sepharose were performed. E-GST-NusA (prepared as described by (Zhang & Hanna, 1995)) or CT-GST-NusA protein were immobilized to the Glutathione Sepharose and mixed with *E. coli* core RNAP. The resins were washed several times to remove any unbound proteins (Figure 4, lanes 3 and 5). The *E. coli* RNA polymerase subunits α, β and β' co-eluted with both the immobilized E-GST-NusA (lane 4) and CT-GST-NusA (lane 6). These results indicate that the CT-GST-NusA protein could directly interact with the *E. coli* core RNAP *in vitro*.

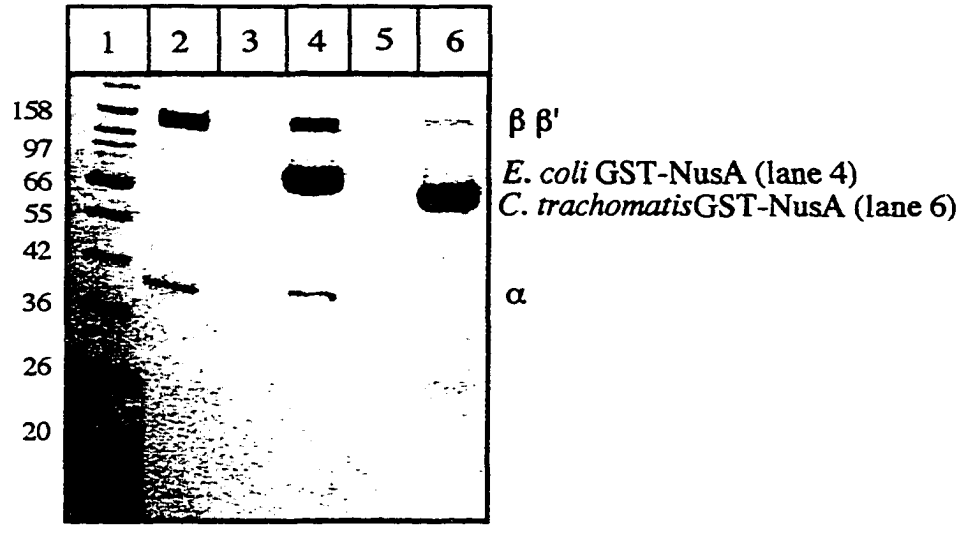


Figure 4: Interaction between immobilized GST-NusA from either *E. coli* or *C. trachomatis* and *E. coli* core RNA polymerase. GST-NusA from *E. coli* (lane 4) or *C. trachomatis* (lane 6) was immobilized to Glutathione agarose. Purified *E. coli* core RNA polymerase was added to the mixture and incubated 37°C for 30 minutes. The resin was spun down and the unbound proteins were removed (lanes 3 and 5). The resin was washed three times with buffer and the specifically bound proteins were eluted by the addition of LLB and heating 3 min. at 100°C. The eluted proteins were analyzed by 12% SDS PAGE (lanes 4 and 6). Molecular weight markers are in lane 1 and core markers are in lane 2.

CT-NusA is unable to enhance intrinsic termination of transcription of *E. coli* RNAP

After demonstrating that *C. trachomatis* NusA binds to the *E. coli* RNAP, we next tested the ability of the *C. trachomatis* NusA to function *in vitro* by enhancing pausing and intrinsic termination. The ability of CT-His_6-NusA and CT-GST-NusA to enhance transcription termination by interacting with the *E. coli* RNA polymerase core enzyme was compared to that of E-GST-NusA and E-His_6-NusA. A 1153 bp long DNA template containing the P_R' promoter and t_o terminator sequences from λ bacteriophage was obtained by PCR from the plasmid pHA100-R63. When RNAP transcribes this template *in vitro* in the absence of the NusA transcription factor, it initiates transcription from the λ P_R' promoter and terminates approximately 55% of transcription 465 nt downstream at the λ t_o terminator (Figure 5B), with the remaining RNAP running off (RO) the end of the template to produce a 619 nt transcript at the top of the gel (Figure 5A). When E-GST-NusA was added to the reaction, an increase in the termination efficiency was observed which was proportional to its concentration, starting from 20 nM and reaching a maximum of 95% termination at 500 nM (Figure 5B). However, CT-GST-NusA was unable to increase termination efficiency even at the higher protein concentrations (Figure 5, lanes 9-12). In order to exclude the possibility that the 23 kD GST tag was interfering with the ability of the CT-NusA from functioning, the CT-His_6-NusA was also assayed (Figure 5, lanes 5-8). The increase in intrinsic termination efficiency was not observed upon the addition of either CT-NusA fusion protein. These results indicate that although CT-NusA can bind to RNAP, it appears to lack a critical interaction necessary for it to function in enhancing termination.

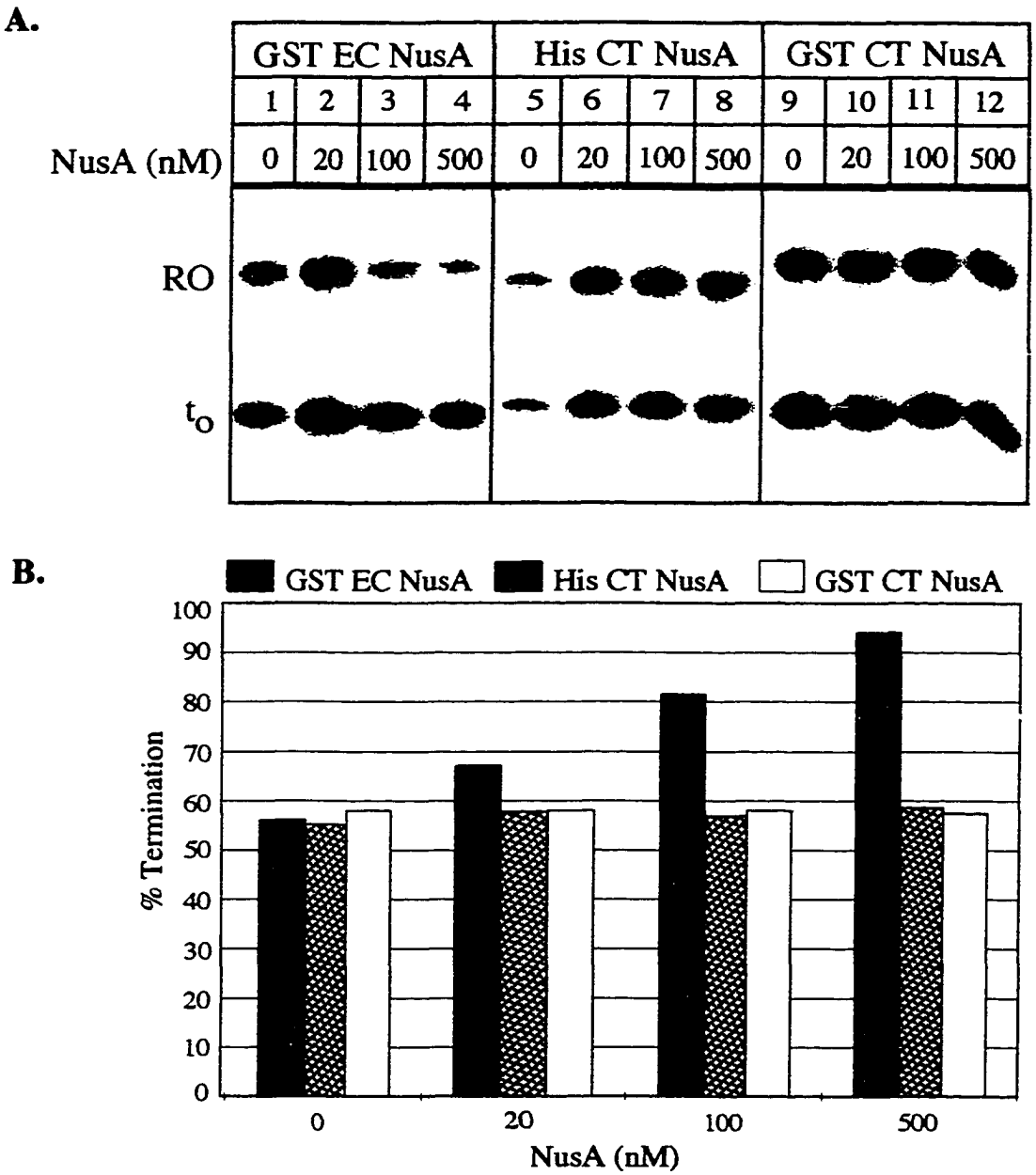


Figure 5: Effect of *C. trachomatis* NusA on termination efficiency. (A) Transcription was initiated from the P_R' promoter of template 1 using *E. coli* RNAP. GST-EC-NusA (lanes 1-4), CT-His_6-NusA (lanes 5-8), or GST-CT-NusA (lanes 9-12) was present at the indicated concentrations. Positions of the transcripts generated by termination at the Rho-independent terminator t_o (465 nt) or from readthrough of t_o to produce the run-off RNA (RO) (619 nt) are indicated. (B) The histagram represents the percent of terminated transcripts.

NusA fusion tags decrease the efficiency of termination compared to Wild Type NusA

In order to determine the effect of the affinity tags, *E. coli* NusA with either the His_6 tag or the GST tag was assayed for the ability to enhance termination efficiency and compared to WT *E. coli* NusA (Figure 6). Transcription was initiated as described above and either untagged wild type *E. coli* NusA (lanes 1-4), E-His_6-NusA (lanes 5-8) or E-GST-NusA (lanes 9-12) was added at the indicated concentrations. As seen in the histogram in Figure 6B, the affinity tags decrease the efficiency of termination by approximately 10% at all concentrations tested. Interestingly, the His tagged proteins were consistently less efficient at enhancing termination then those tagged with the larger 23 kDa GST tag. However, this decrease in termination efficiency does not account for the inability of the CT-NusA proteins to enhance termination at intrinsic terminators.

***C. trachomatis* NusA is unable to bind to the *E. coli* α subunit of RNAP.**

We have previously shown that the specific interaction between the *E. coli* RNAP α subunit and NusA is vital for the control of transcription termination (Liu *et al.*, 1996). Since *C. trachomatis* is able to bind *E. coli* RNAP but is unable to enhance intrinsic termination, we hypothesized that CT-NusA does not make this critical contact with the α subunit. In order to determine if CT-NusA could bind the α subunit, we immobilized GST or GST-NusA from either *C. trachomatis* or *E. coli* to Glutathione Sepharose and passed over the affinity column a crude cell lysate overexpressing the α subunit. After the column was washed to remove proteins not interacting with NusA, the associated proteins were eluted and analyzed by SDS PAGE (Figure 7). The α subunit bound to the *E. coli* GST-NusA (lane 4) but did not bind to either the GST control (lane 2) or *C. trachomatis* NusA (lane 3). We conclude that *C. trachomatis* NusA cannot bind to the α subunit of RNAP.

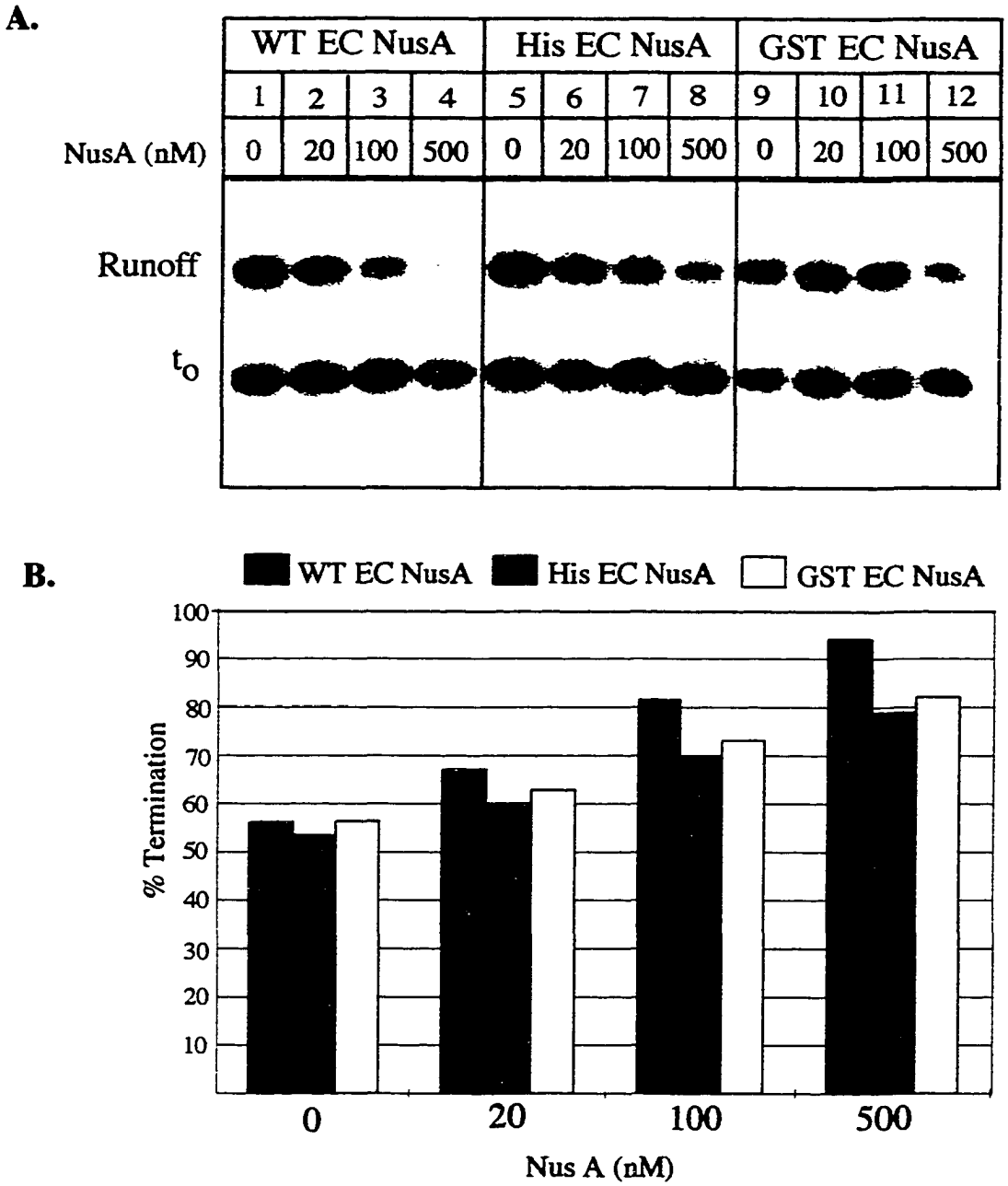


Figure 6: Effect of affinity tag on NusA enhanced termination efficiency. (A) Transcription was initiated from the P_R' promoter from a template derived from pHA100-R63. Either WT untagged *E. coli* NusA, his tagged NusA, or GST tagged NusA was added at the indicated concentrations. The positions of the transcripts resulting from termination at t_o or from readthrough of t_o to generate the run-off RNA are indicated. (B) The amount of terminated transcripts was detemined as described.

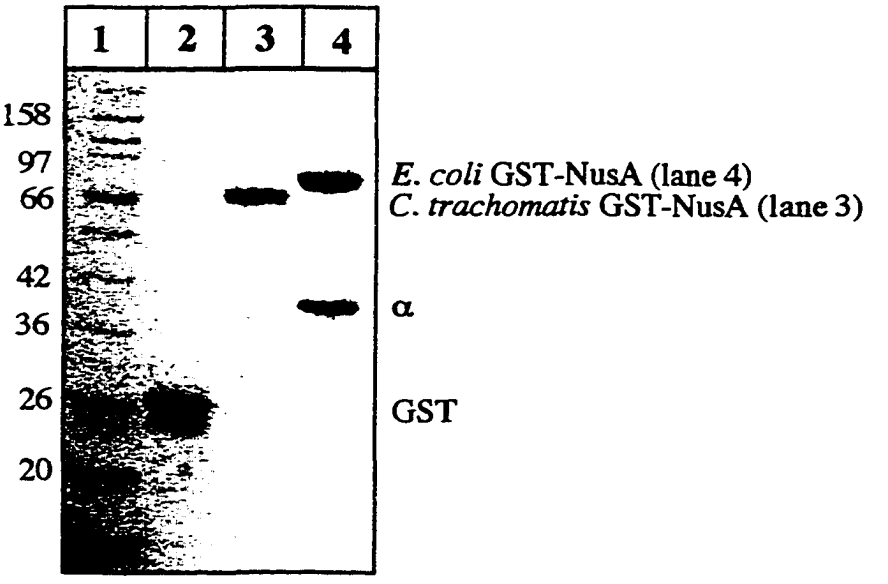


Figure 7: Interactions of the *E. coli* RNA Polymerase alpha subunit with Immobilized NusA Proteins from *C. trachomatis* and *E. coli*. GST (lane 2) or GST-NusA from either *C. trachomatis* (lane 3) or *E. coli* (lane 4) were immobilized on glutatione agarose beads. A crude crude cell lysate which contained the overexpressed α subunit from *E. coli* RNA polymerase was incubated with the immobilized proteins for 30 minutes at 37°C. The resin was washed three times with buffer and then the bound protein were eluted from the resin with SDS loading buffer and heating at 100°C for 3 minutes. The released proteins were analyzed by SDS-PAGE followed by Coomassie staining of the gel.

Discussion

We identified an ORF of *C. trachomatis* which encodes for a protein which amino acid sequence is significantly homologous to other bacterial NusA factors. We showed that this gene is expressed *in vivo* since antibodies raised against the recombinant ORF product specifically recognized, in a two-dimensional electrophoretic map of Chlamydial proteins, a single protein species which had a molecular weight and isoelectric point values expected for the predicted *nusA* gene product.

There are currently available several putative NusA amino acid sequences from different bacterial species. The multiple alignment of nine of these sequences with the putative *C. trachomatis* NusA protein we describe (Figure 1) shows a moderate variability of the structures in their N-terminal third and very high variability in the C-terminal portions, apparently comprising independent domains which can be completely missing in some members of the family. However, the central third of the alignment shows the occurrence of a set of completely invariant amino acid residues, so that the following profile can be proposed to identify this protein family : R-X9-L-X5-PE-X12-R-variable segment-G-X3-K-X10-D-X6-G-X11-E-X3-E-X2-D-X17-P-variable segment-G-X2-G-X-N-X2-L-X5-G The first of these invariant residues, the R-200 in the *C. trachomatis* sequence, is part of a domain (amino acids 143 and 206 in the *E. coli* sequence), which, in the prototype *E. coli* NusA, has been denoted as "arginine-rich" (Ito *et al.*, 1991) and has been described as an RNA binding domain, homologous to the S1 RNA-binding domain of *E. coli* polynucleotide phosphorylase (Bycroft *et al.*, 1997; Gibson *et al.*, 1993). We note that according to the alignment in Figure 1 only the last arginine residue of the domain appears to be essential. At the C-terminal end, after amino acid corresponding to 343 of *E. coli*, there is a significant loss of homology between the *E. coli* and *C. trachomatis* proteins. Interestingly, a truncated *E. coli* NusA protein lacking all amino acids beyond amino acid 343 is fully functional at 32°C for transcription termination, but it is defective primarily in

transcription anti-termination involving the N protein of bacteriophage λ (Tsugawa *et al.*, 1988), a function which would not be required in *C. trachomatis*.

The *in vitro* binding of CT-NusA to *E. coli* core RNAP suggests some structural homology between the two proteins. GST-CT-NusA is clearly able to bind to *E. coli* core RNAP although slightly less efficiently than *E. coli* NusA. However, neither CT-GST-NusA nor CT-His_6-NusA were able to increase transcription termination efficiency at an *E. coli* intrinsic terminator as previously shown for E-GST-NusA (Liu *et al.*, 1996; Rosenthal & Calvo, 1987; Schmidt & Chamberlin, 1987b). In a comparative *in vitro* assay (Figure 5) this defect was not due to the tagging of the NusA protein, as the effect of the fusion tags could only account for a decrease in termination efficiency of approximately 10-15%. It is interesting to note that the addition of only six histidines to the N-terminus of NusA causes a greater defect in NusA function when compared to the larger, 23 kDa, GST tag. It is likely that the N-terminus is involved in an important interaction which is perturbed by the charged histidine residues.

As previously reported, the interaction between the αCTD and NusA is required for optimal termination at intrinsic terminators. Here we report that this interaction is lacking between the *E. coli* α subunit and the *C. trachomatis* NusA, therefore, preventing the CT-NusA from functioning *in vitro*. Analysis of the alignment of the NusA proteins from *E. coli* and *C. trachomatis* shows that the *E. coli* NusA protein is larger than that of *C. trachomatis* . Although an αCTD binding site on *E. coli* NusA has not been identified, it is tempting to postulate that the αCTD binding site is likely to be at the C-terminal end of the *E. coli* NusA which is one of the highly variable domains and is lacking in the *C. trachomatis* NusA.

The presence of a relatively large amount of NusA in the EB's, the infectious but metabolically dormant form of *C. trachomatis* seems noteworthy. A well stained spot could be observed in the silver stained 2D map (Figure 3A). Since on the same map we could

also identify, by N-terminal sequencing (data not shown), the α subunit of the chlamydial RNAP, we compared the amount of protein in the two spots by laser photodensitometry of the gel followed by quantitative analysis with the Melanie II software. Interestingly, the results indicate that the molar ratio of α to NusA was 1.93, i.e. very close to an expected value of 2 for an elongation RNAP/NusA complex. Obviously this calculation is based on the assumption that both NusA and the α RNAP subunit spots stain with silver in a proportional manner; it is nevertheless tempting to speculate that in the spore-like EB cells there may be present already assembled transcription elongation complexes in which initiation has already occurred (possibly at genes selected at the end of the replicative cycle) for a prompt resumption of mRNA synthesis, after entry into the host cell, allowing a rapid differentiation to metabolically active replicative forms. This result is in contrast with our data from the previous chapter which indicated a molar ratio of α to NusA of 1. It must be considered that the α binding experiments were performed with α which was not in complex with the polymerase. However, it is tempting to speculate that the number of NusA proteins interacting with the polymerase plays a role in its function in enhancing pausing and termination. Obviously, more experiments need to be performed to answer these questions.

Chapter 7

Interaction of the Transcription Elongation Factor NusA with *E. coli* RNA Polymerase: Identification of the α Binding Domain on the C-Terminus of NusA

Abstract

The *Escherichia coli* transcription factor NusA binds to the transcription complex shortly after the release of a factor from core RNAP. It interacts with both the core enzyme and the nascent RNA resulting in a slower elongation rate, enhanced pausing and termination. It has been established that direct interaction between the α subunit of RNAP and NusA is required for NusA function. In Chapter 5, the specific amino acids of the α subunit which are critical for this functional interaction are identified. This Chapter presents evidence for the α binding domain on NusA.

In order to determine which region of NusA was important to allow binding to α, two GST-NusA fusion proteins having deletions of the C-terminus of NusA (from amino acids 354 and 432) were compared to the wild type NusA. *In vitro* binding experiments of α to the truncated NusA proteins, immobilized to Glutathione Sepharose, were performed. The C-terminus on NusA was determined to be critical for this interaction. *In vitro* termination and pausing experiments using the truncated NusA proteins emphasized the importance of this interaction for NusA function.

Introduction

NusA, a 54.5 kDa acidic protein of *Escherichia coli*, serves as a transcription elongation and termination factor and as a protein which couples antitermination factors to the elongation complex (Friedman & Gottesman, 1983; Friedman *et al.*, 1984; Kung *et al.*, 1975). It has been shown that the addition of NusA to a coupled transcription-translation system prevents premature transcription termination (Kung *et al.*, 1975; Zarucki-Schulz *et al.*, 1979). In a purified system NusA causes pausing of RNA polymerase at specific sites and enhances termination by RNA polymerase (Farnham *et al.*, 1982; Greenblatt *et al.*, 1981a; Kassavetis & Chamberlin, 1981; Kingston & Chamberlin, 1981). NusA is one of four *E. coli* host factors (The others are NusB, NusE, and NusG) involved in transcriptional anti-termination by the bacteriophage λ N protein (De Vito & Das, 1994). In addition, NusA is the only host factor involved in Q-mediated transcriptional antitermination in phage λ (Barik & Das, 1990; Yarnell & Roberts, 1992). The role of NusA in these processes include interactions with the core polymerase, the nascent RNA, and other transcription factors (Greenblatt *et al.*, 1993; Liu & Hanna, 1995a). However, the direct mechanism of action of NusA remains obscure.

The *E. coli nusA* gene has been the subject of numerous genetic studies which have produced a number of mutants which have provided information on the functional domains of NusA. The *nusA* gene was first identified by isolation of the *nusAI* mutation, which restricts bacteriophage λ growth by preventing the antitermination activity of the λ N protein (Friedman, 1971; Friedman & Baron, 1974). Mapping of the *nusAI* mutation showed a substitution of arginine for leucine at amino acid 183 (Craven & Friedman, 1991; Saito *et al.*, 1986). A second mutant, the temperature sensitive NusA mutant *nusAII*(ts), is defective in the ability to terminate transcription normally at both ρ-dependent and ρ-independent terminators, although at various levels depending on the terminator (Nakamura *et al.*, 1986a; Nakamura *et al.*, 1986b). This mutation, which is a substitution of glycine at amino acid

181 with aspartate, does not restrict λ growth under permissive or nonpermissive conditions (Nakamura *et al.*, 1986a). Both of these mutations map in the same hydrophobic amino acid cluster which apparently constitutes an important domain of the NusA protein.

Sequence and structural alignments have implicated other domains in NusA as important for RNA binding. A region of the NusA protein extending from amino acid 133 to 207 was aligned with the S1 structure derived from polynucleotide phosphorylase (Bycroft *et al.*, 1997). The S1 RNA-binding domains were first identified in the *E. coli* ribosomal protein S1, which has six of these RNA-binding domains, and were located in other proteins thought to interact with RNA (Boni *et al.*, 1991). A second set of alignment data showed two regions of NusA which contain KH domains, one between amino acids 233 and 295 and the other from amino acid 302 to 348. It has been suggested that the KH domain might bind RNA on the basis of its presence exclusively in proteins involved in RNA metabolism (Siomi *et al.*, 1993). Although it has been shown that NusA binds RNA, conclusive proof that either of these regions of NusA are involved in RNA binding has not been presented.

In the previous chapters it has been shown that NusA interacts specifically with the α subunit of RNA polymerase, and the region of NusA involved in this functional interaction, is lacking in the NusA protein from *Chlamydia trachomatis*. Here I provide evidence that the C-terminal 60 amino acids of NusA are essential for this interaction. A model proposing how this interaction affects NusA function in transcription elongation is also presented.

Materials and Methods

Construction of NusA deletion plasmids

The oligonucleotides 5' GGC AAG CTT CAT CGT GAC TGA CTG ACG 3', and either 5' TGC AAG CTT TTA CGC TGC GTG CGC TTC CG 3' or 5' TGC AAG CTT TTA ATC GTC AGC CGG TTT GTT ATC 3' (Figure 1) were used to PCR amplify pHA200A (Zhang & Hanna, 1995), the *E. coli* expression plasmid which encodes for the NusA protein fused to GST. The amplification produces a plasmid which encodes for a NusA protein lacking either the C-terminal 63 or 141 amino acids. The first oligonucleotide anneals to the vector immediately downstream of the NusA gene and was used in both reactions. The second and third oligonucleotides anneal to the NusA gene and encode for a stop codon at amino acid 433 or 355 respectively. All of the primers encode a *Hind*III site at the 5' end to allow for ligation of the PCR product. PCR reactions were performed as previously described in Chapter 3 except 5 Units of Taq Plus Long DNA polymerase and 1X High Salt Buffer (Stratagene) were used. After amplification, the PCR products were digested with *Hind*III and ligated as described in Chapter 3. After ligation, the plasmids were transformed into DH5α and transformants were analyzed for the overexpression of the fusion protein as described in of Chapter 6.

Expression and purification of truncated GST-NusA fusion proteins

The GST Δ432 NusA and GST Δ354 NusA proteins were purified using Glutathione Sepharose as described in Chapter 6.

Binding of immobilized truncated proteins to E. coli core RNAP and the alpha subunit

The truncated NusA proteins were assayed for the ability to bind core RNAP and the α subunit of RNAP as described in Chapter 6.

Transcription assays

For analysis of NusA enhanced pausing, transcription complexes containing radioactively labeled 20-mer RNA (A20) were prepared from the T7 A1 promoter of pAR1707 with RNA polymerase in the presence and absence of NusA, as previously described (Liu & Hanna, 1995b). The 20-mer RNA was then chased by the addition of ATP, GTP, CTP and UTP to 20 μM. Samples were removed at various times, and transcription was stopped by addition of an equal volume of urea loading buffer (7 M urea; 1 mM EDTA; 0.02% w/v bromophenol blue; 0.02% w/v xylene cyanol). The RNA was analyzed by electrophoresis on a 10% polyacrylamide-urea gel. Analysis of NusA-enhanced ρ-independent termination was performed as described in Chapter 6.

Results

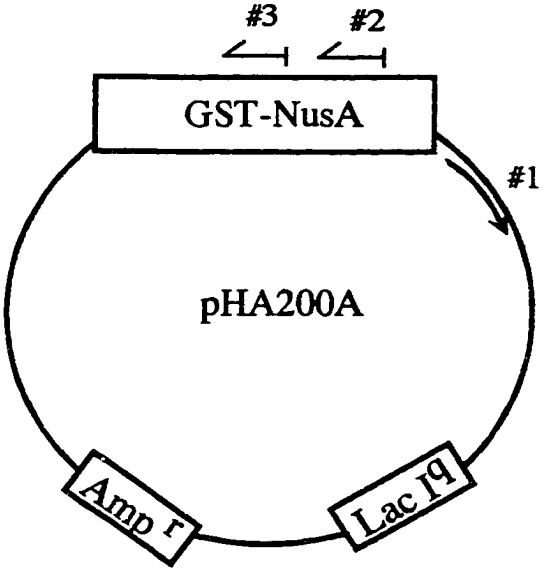
As reported in Chapter 6, the NusA protein from *Chlamydia trachomatis* is capable of binding to the *E. coli* RNA polymerase *in vitro* but is unable to function in enhancing termination at intrinsic terminators. It was demonstrated by binding assays that the NusA protein from *C. trachomatis* is unable to interact with the α subunit of RNAP which is a necessary interaction for NusA function *in vitro* (Liu & Hanna, 1995b). Upon alignment of the amino acid sequences of the *E. coli* and *C. trachomatis* NusA proteins, we recognized that the N-terminus of the two proteins contains the highest regions of homology and that the C-terminus from *C. trachomatis* is lacking approximately 60 amino acids which are present in *E. coli*. In order to determine if the C-terminal region of *E. coli* NusA was the region of NusA necessary for binding α, two plasmids were constructed to express GST-NusA fusion proteins that are truncated at amino acid 432 and 354.

Expression of truncated GST-NusA proteins

In order to construct plasmids which would express a truncated GST-NusA fusion protein, we performed deletion PCR on the plasmid pHA200A (Zhang & Hanna, 1995), which encodes for the full-length fusion protein (illustrated in Figure 1, panel A). Two PCR reactions were performed using either primers #1 and #2, or primers #1 and #3, to create pMH113 or pMH114 respectively. Primer #1 (Figure 1, panel B) anneals to the vector sequence immediately downstream of the NusA stop codon and contains a *Hind*III site at the 5' end. Primers #2 and #3 (Figure 1, panel B), which also contain *Hind*III sites, anneal to the NusA protein and encode for stop codons at amino acid 433 or 355 respectively. After amplification (Figure 2, lanes 2 and 3), the PCR product was ligated and transformed into DH5α. Transformants were screened for expression of the truncated proteins by inducing the cultures with IPTG. SDS PAGE analysis of cell lysates from six

of the transformants showed overexpression of the both Δ432 (Figure 3, lanes 3 and 5), and Δ354 (Figure 3, lanes 6 and 7).

A.



B.

Primer #1
NusAΔ C-terminus 5' - GGCAAG CTT/**CAT/CGT/GAC/TGA/CTG/ACG** - 3'

Primer #2
NusA Δ432 5' - TGC/AAG CTT/**TTA/ATC/GTC/AGC/CGG/TTT/GTT/ATC** - 3'

Primer #3 5' - TGC/AAG CTT/**TTA/CGC/TGC/GTG/CGC/TTC/CG** - 3'
NusA Δ354

Figure 1: Construction of GST-NusA deletion plasmids. (A) The GST-NusA expression plasmid pHA200A and the binding sites of the PCR primers are indicated. (B) Primers were designed for deletion PCR of the NusA gene. Each primer contains a *Hind*III site at the 5' end (shown in red) so that the PCR product could be ligated after amplification. Stop codons are underlined in black and the nucleotides which anneal to the plasmid during the first rounds of amplification are in bold. The plasmids created were used to express GST-NusA proteins which had been truncated at either amino acid 432 or 354.

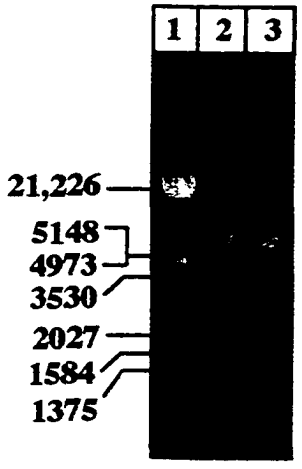


Figure 2: PCR amplification of the NusA deletion plasmids. Lane 1 is λ *Hind*III/*Eco*RI molecular weight standards. Lanes 2 and 3 contain 10 μl of the 100 μl PCR reactions of Δ432 and Δ354, respectively.

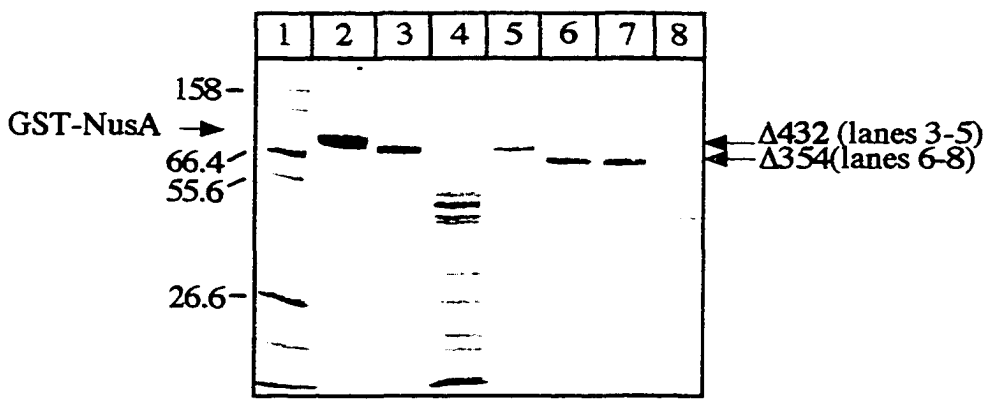


Figure 3: PAGE analysis of transformatns containing the GST-NusA deletion plasmids. Transformants were inoculated into 1 ml of LB broth and allowed to incubate at 37°C for 2.5 hours. Overexpression was induced with the addition of 0.2 mM IPTG and growth continued for 3 hours. Whole cell lysates were analyzed by SDS PAGE followed by Coomassie blue staining. Molecular weight markers are in lane 1 with sizes indicated on the left. Full-length GST-NusA (lane2), Δ432 (lane 3 and 5), and Δ354 (lane 6 and 7) are indicated. Lane 4 and 8 are transformants not containing the plamids.

Δ432 and Δ354 NusA proteins bind to E. coli core RNA polymerase

To determine whether the truncated GST-NusA (Δ432 or Δ354) could bind to *E. coli* RNAP core enzyme *in vitro*, binding studies using a GST-NusA fusion protein immobilized to Glutathione Sepharose were performed. Truncated GST-NusA proteins were immobilized to the Glutathione Sepharose and mixed with *E. coli* core RNAP as described in Chapter 6. The resins were washed several times to remove any unbound proteins. The *E. coli* RNA polymerase subunits α, β and β' co-eluted with the immobilized full-length GST-NusA (Figure 4, lane 2), the Δ432 GST-NusA (lane 3) and the Δ354 GST-NusA (lane 4). These results indicate that the C-terminal region of NusA is not required for binding to core RNAP *in vitro*.

Δ432 and Δ354 are unable to function to enhance intrinsic termination of transcription or pausing in vitro.

The truncated NusA proteins were next tested for the ability to enhance termination at ρ-independent terminators. Termination assays were performed using a 1153 bp long PCR fragment containing the P_R' promoter and t_o terminator sequences from λ bacteriophage. The correctly terminated RNA transcript is a 465 nt long (t_o) and the readthrough RNA (run-off; RO) is 619 nt transcript in length (Figure 5A). In the absence of any additional factors, ~40% of the polymerase terminates at t_o. When full-length GST-NusA was added to the reaction, an increase in the termination efficiency of 45% was observed, with the addition of 20 nM NusA, the termination efficiency was increased to 65% with 100 nM NusA (Figure 5B). However, neither of the truncated NusAs were able to increase termination efficiency to levels as comparable to the full-length NusA, even at the higher protein concentrations (Figure 5, lanes 4-7).

Since pausing and termination are thought to be intimately related and are both affected by the presence of NusA, we next tested the ability of the truncated NusA proteins

to enhance pausing. It has previously been shown that NusA enhances pausing just downstream from an RNA hairpin (P1), 80 nt downstream from the T7 A1 promoter contained on pAR1707 (Dissenger & Hanna, 1991). Synchronously started transcription complexes were formed by initiation of transcription in the absence of one nucleoside triphosphate so that all complexes contained an RNA of 20 nucleotides long. All four nucleoside triphosphates were then added and aliquots were removed at various time points and the resulting transcripts were analyzed by denaturing polyacrylamide gel electrophoresis. Experiments were performed in the absence or presence of 20 nM (Figure 6A) and 150 nM (Figure 6B) of full-length or truncated NusA. In the absence of NusA, over 50% of the transcripts had passed through the P1 pause at the 2 minute timepoint (Figure 7). The addition of full-length NusA enhanced the length of the pause at P1, as 50% of the transcripts had not passed through this site until 3 minutes. The addition of either NusAΔ432 or NusAΔ354 had no affect on pausing at either concentration. These results indicate that the C-terminal 63 amino acids of NusA are critical for function in termination and pausing.

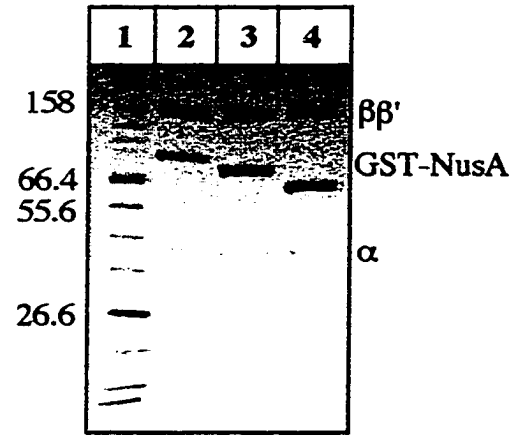


Figure 4: Interaction between immobilized GST-NusA (full-length or truncated) and *E. coli* core RNA polymerase. Full-length GST-NusA (lane 2), Δ432 (lane 3), or Δ354 (lane 4), were immobilized to Glutathione Sepharose. Purified *E. coli* core RNA polymerase was added to the mixture and incubated at 37°C for 30 minutes. The resin was spun down and the unbound proteins were removed. The resin was washed three times with buffer and the specifically bound proteins were eluted by the addition of LLB and heating 3 min. at 100°C. The eluted proteins were analyzed by 12% SDS PAGE. Molecular weight markers are in lane 1 and the sizes of the proteins are indicated on the left.

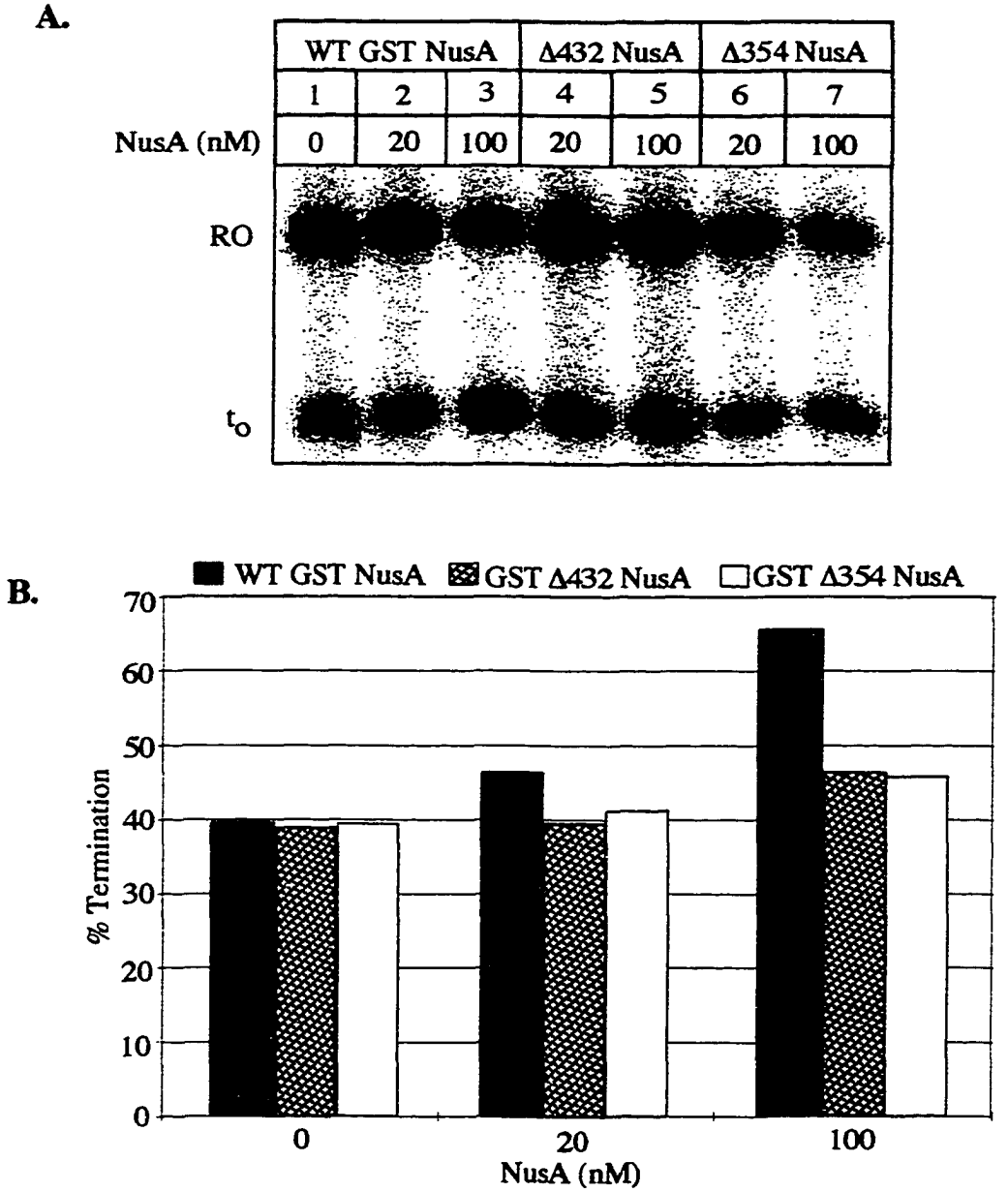
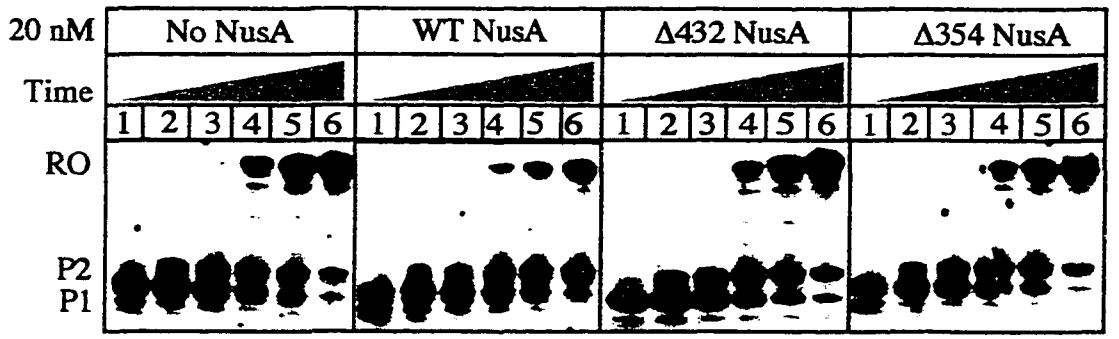


Figure 5: Effect of NusA deletions on termination efficiency. (A) Transcription was initiated from the P_R' promoter of template 1 using *E. coli* RNAP. Full length GST-NusA (lanes 1-3), Δ432 NusA (lanes 4 and 5), or Δ354 NusA (lanes 6 and 7), was present at the indicated concentrations. Positions of the transcripts generated by termination at the ρ-independent terminator t_o (465 nt) or from readthrough of t_o to produce the run-off RNA (RO) (619 nt) are indicated. (B) The histagram represents the percent of terminated transcripts.

A.



B.

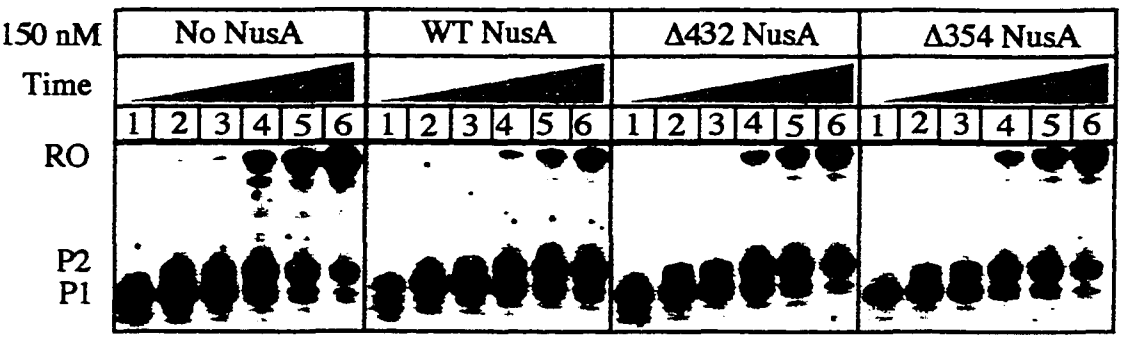


Figure 6: Effect of NusA deletions on pausing efficiency. Panel A and B are autoradiograms of an RNA gel showing the transcripts made during a transcription time course from the T7 A1 promoter using either 20 nM (A) or 150 nM (B) of NusA. Ternary transcription complexes containing 20 nucleotide RNA were formed with *E. coli* RNAP. The 20-mer was chased with or without either full length NusA or the truncated NusAs and aliquots were taken at 20 sec (lane 1), 40 sec (lane 2), 1 min (lane 3), 2 min (lane 4), 3 min (lane 5), or 5 min (lane 6). Pause RNAs are identified as RNA species which persist with time, but eventually chase to full length RNA. P1 and P2 indicate the positions of the major pause site RNAs from this template, and RO indicates the position of the run off transcript.

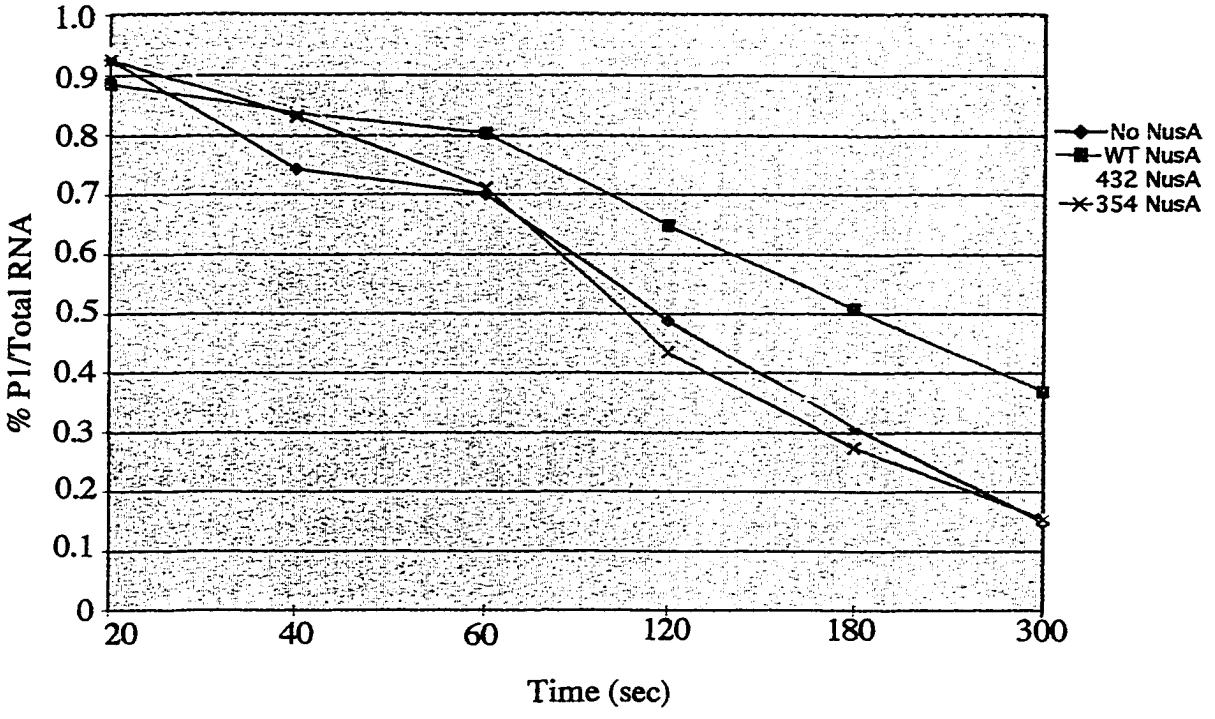


Figure 7: Effect of NusA deletions on percent of total transcripts paused at P1 over time. The gels of the pausing experiments (containing 150 nM NusA) were analyzed and the total amount of transcripts for each timepoint was determined using a phosphoimager. The truncated NusA proteins had the no affect on the length of the P1 pause in contrast to the full-length NusA.

Truncated NusA proteins are unable to bind to the E. coli α subunit of RNAP.

Liu *et al* had previously reported that the interaction between α and NusA is vital for the control of transcript termination and pausing (Liu *et al.*, 1996). Since the truncated NusA proteins bind to *E. coli* RNAP but are unable to enhance intrinsic termination or pausing, we wanted to test the ability of the truncated NusAs to bind the α subunit. To determine this we immobilized the full-length and truncated GST-NusA proteins to Glutathione Sepharose and passed a crude cell lysate overexpressing the α subunit over the affinity column as described in Chapter 6. The column was washed so only proteins interacting with NusA were retained and then these associated proteins were eluted and analyzed by SDS PAGE analysis (Figure 8). The α subunit binds to the full-length GST-NusA (lane 2) as expected. Both Δ432 (lane 3) and Δ354 (lane 4) showed reduced binding to α. These results further support the hypothesis that the C-terminal 63 amino acids of *E. coli* NusA are critical for binding the α subunit of RNAP which is necessary for function in enhancing pausing and termination.

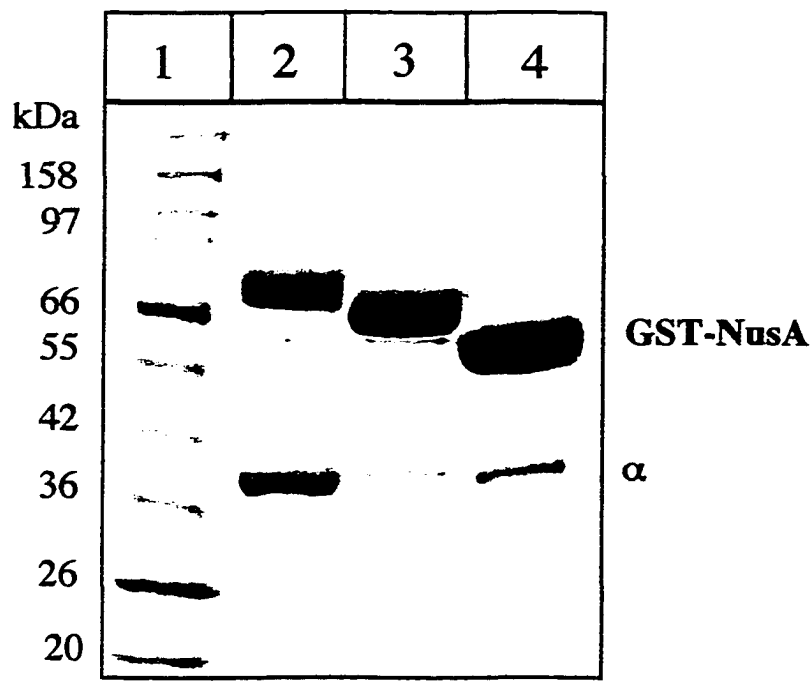


Figure 8: Interactions of the *E. coli* RNA polymerase alpha subunit with immobilized NusA deletion proteins. Full-length GST-NusA (lane 2), Δ432 GST-NusA (lane 3), or Δ354 GST-NusA (lane 4) was immobilized on Glutatione Sepharose. A crude cell lysate which contained the overexpressed α subunit from *E. coli* RNA polymerase was incubated with the immobilized proteins for 30 minutes at 37°C. The resin was washed three times with buffer and then the bound proteins were eluted from the resin with SDS loading buffer and heating at 100°C for 3 minutes. The released proteins were analyzed by SDS-PAGE followed by Coomassie blue staining of the gel.

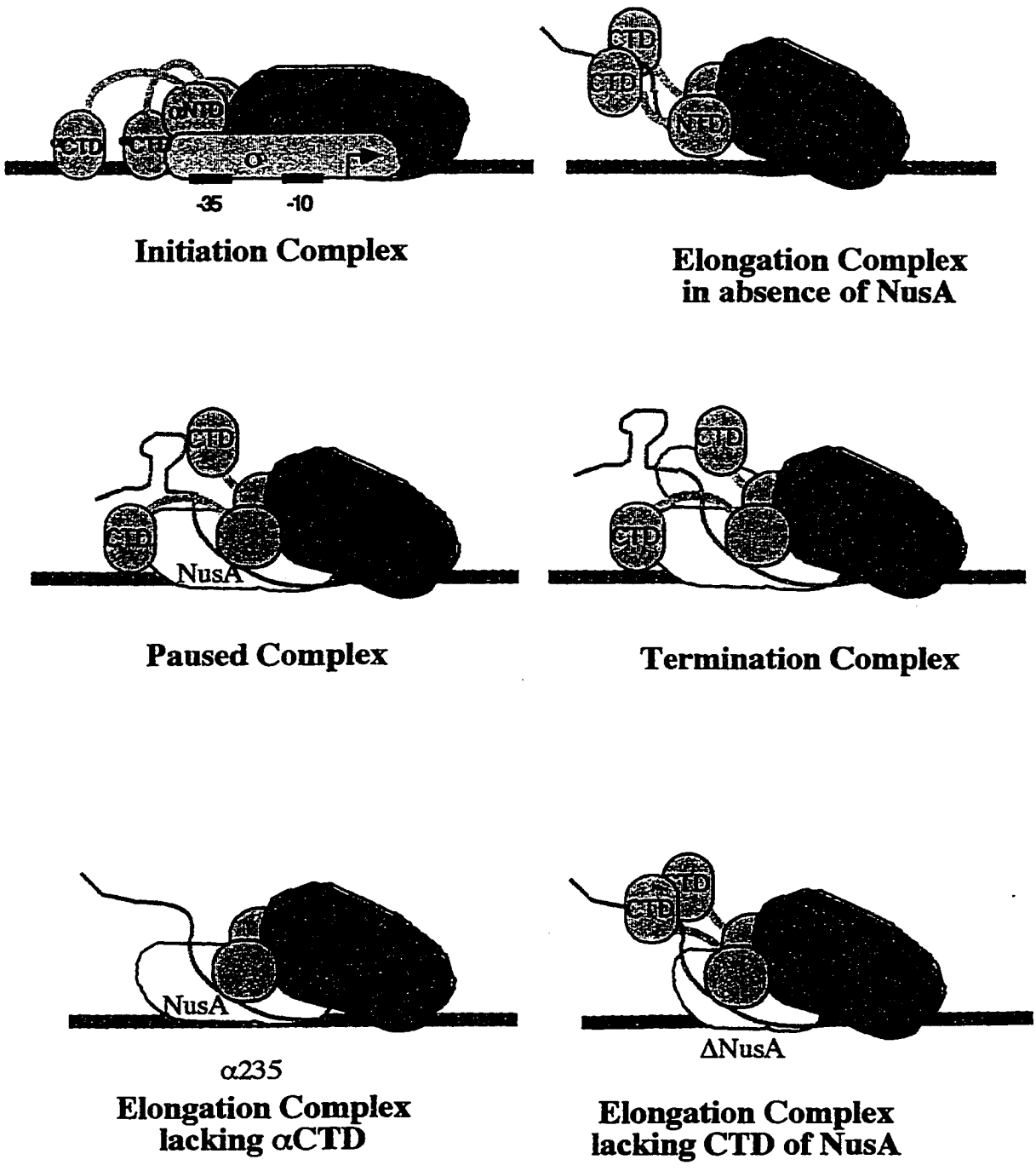


Figure 9: Model for NusA function in pausing and termination.

Discussion

The NusA protein is an elongation factor which plays an important role in the regulation of pausing, termination, and antitermination in *E. coli*. In order to function in termination and pausing, NusA must physically interact with the nascent RNA and the core enzyme (including a critical contact with the αCTD) (Liu & Hanna, 1995a; Liu & Hanna, 1995b; Liu *et al.*, 1996). The specific amino acids on the αCTD necessary for this interaction are reported in Chapter 5. Truncated NusA protein (truncated at amino acids 432 or 354) indicate that the C-terminal 63 amino acids on NusA are involved in the functional interaction with α.

Although the truncated NusA proteins were able to bind to the core enzyme, our results indicate that the C-terminus of NusA is necessary for function in enhancing pausing or enhancing termination at ρ-independent terminators. Using *in vitro* transcription assays, we showed that the C-terminally truncated proteins could only terminate 45% of the transcripts whereas full-length NusA terminated 65% of the transcripts. Likewise, the truncated NusA proteins were unable to enhance pausing at the P1 pause site. When assayed in an *in vitro* pausing experiment, the addition of either of the truncated NusA proteins had little or no effect on the rate of elongation through the P1 pause, compared to full-length NusA. Using *in vitro* binding experiments, we show that the inability of the truncated NusA proteins to function correlates with to the loss of the α binding site. These finding further support our hypothesis from the previous chapter that the NusA protein from *C. trachomatis* is lacking the *E. coli* binding domain critical for interacting with the α subunit. In addition, these results further suggest a critical functional interaction between the C-terminal domain of the α subunit of RNAP and the elongation factor NusA.

However, the question of how NusA enhances pausing and termination still remains unanswered. We propose a simple model to explain how the loss of the interaction between NusA and αCTD affects function (shown in Figure 9). Our model suggests that in order

for NusA to function it must interact with αCTD and lifts the RNA off of one or both of the αCTDs. It has been shown that in the initiation complex, the αCTD can interact with the DNA template at some promoters (Ross *et al.*, 1993). DNA footprints using RNAP lacking αCTD suggest that when the polymerase enters the elongation phase, αCTD does not contact the DNA (Hanna personal communication). Using photocrosslinking experiments Liu *et al* reported the RNA contacted the αCTD in the absence of NusA. Upon addition of NusA the RNA no longer contacted the αCTD but crosslinked to NusA (Liu & Hanna, 1995a). In our model we suggest that at pause sites, the interaction of NusA with αCTD abolishes the interactions with the RNA and allows the αCTD to contact the DNA. Interaction of α with the DNA could prevent the polymerase from escaping the pause. This is consistent with the result that NusA cannot enhance pausing or termination with RNAP which lacks the αCTD. This model is also supported with evidence that RNAP lacking one of the αCTD, in the absence of NusA, shows an increase in termination and pausing at intrinsic terminators (unpublished data). Therefore, without this interaction the αCTD remains in contact with the RNA preventing a DNA interaction. Perhaps at terminator sites a second NusA interacts with the second αCTD, this interaction further weakens the already weak DNA:RNA hybrid and causes the release of the transcript. This model is supported by our binding data in Chapter 5 which suggests a molar ration of 1:1 of α to NusA.

Although this model proposes how the functional interaction between NusA and αCTD affects function in pausing and termination many questions remain unanswered. Does NusA recognizes the α monomer or dimer in the elongation complex? Do both of the αCTDs contact the RNA hairpin at pause sites? Does the α contact with NusA induce a conformational change in NusA allowing it to bind the RNA? Further experiments (to be performed by some other graduate student!!) must be performed to answer these questions.

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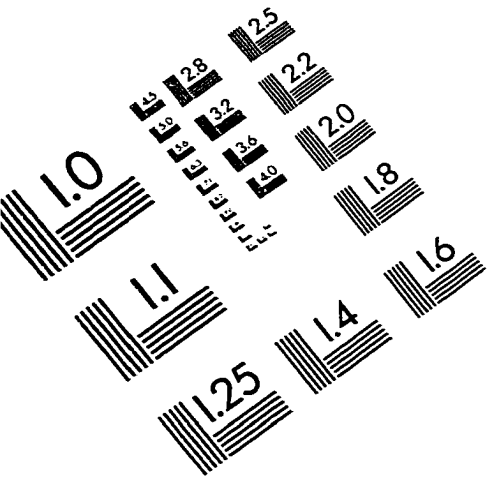
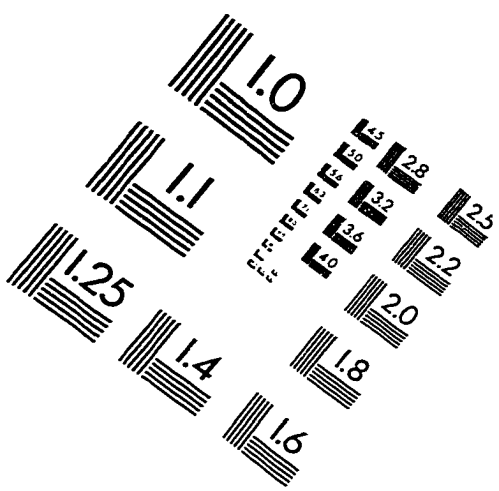
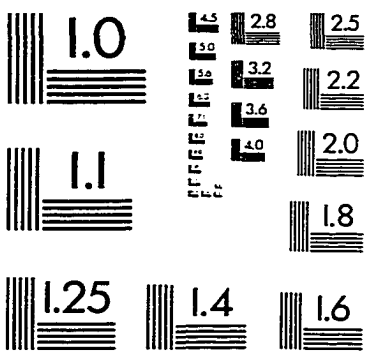
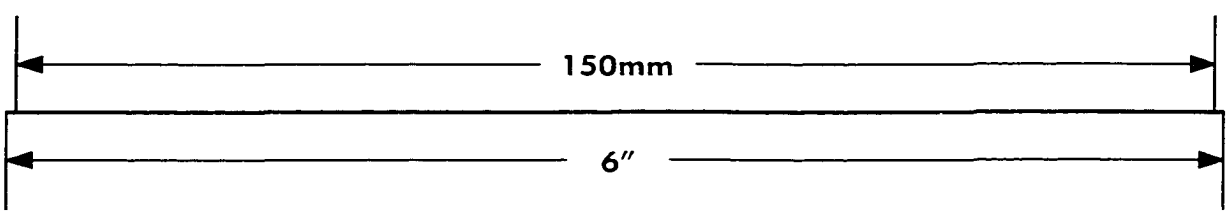
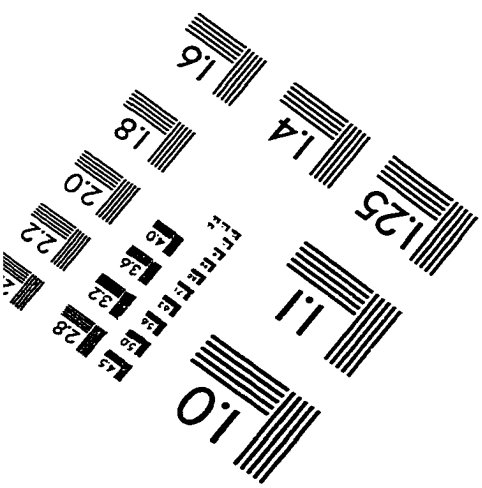
IMAGE EVALUATION TEST TARGET (QA-3)

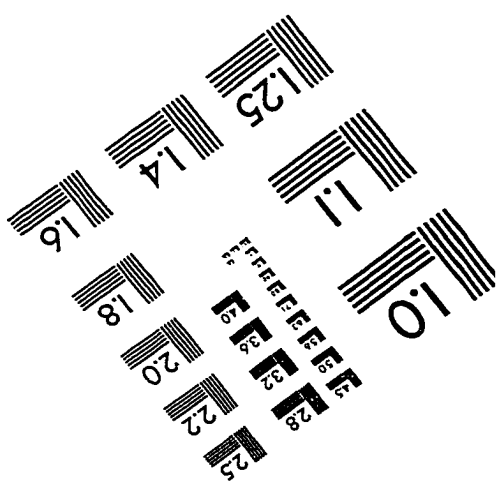












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