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FUNCTIONAL ANALYSIS
OF
THE NINTH SUBUNIT OF YEAST RNA POLYMERASE II, RPB9

By
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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University

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FUNCTIONAL ANALYSIS OF YEAST RNA POLYMERASE II SUBUNIT RPB9

DOCTOR OF PHILOSOPHY (1998)
(Biochemistry)

McMaster University
Hamilton, Ontario

**TITLE: Functional Analysis of the Ninth Subunit of Yeast RNA
 Polymerase II, RPB9**

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NUMBER OF PAGES: xii, 120

ABSTRACT

RNA polymerase II is the eukaryotic enzyme that synthesizes mRNA. It is a complex enzyme that is highly regulated by many protein factors throughout transcription. RNA polymerase II comprises twelve subunits, each of which likely plays a specific role in the function of the enzyme. The ninth subunit, RPB9, is involved in the initiation and elongation stages of transcription. In yeast, deletion of RPB9 results in sensitivity to high and low temperatures, as well as to the drug 6-aza-uracil. RPB9-deficient strains exhibit an alteration in the selection of transcript start sites, with an upstream shift of the start site window. The RNA polymerase II isolated from RPB9-null strains is unable to recognize intrinsic pause sites during elongation. The polymerase molecules that do arrest cannot resume transcribing upon stimulation by the elongation factor TFIIIS.

RPB9 is a small polypeptide, containing two zinc-binding regions (Zn1 and Zn2) connected by an intervening sequence (linker region). This study presents the results of a mutational analysis designed to assign the various functions of RPB9 to regions within the subunit. The Zn1 region was shown to be required for the start site selection activity of RPB9. When expressed in an RPB9-null yeast strain, the Zn1 region was found to be sufficient to restore start site selections to the wildtype pattern. Using a gel shift assay and purified proteins, an RNA polymerase II binding region was located to a conserved sequence (D---DPTLPR) within the linker region. The elongation activity of RPB9 was ascribed to the Zn2 region. Conserved charged residues within Zn2 were essential for the ability of RPB9 to mediate the reactivation of RNA polymerase II upon stimulation by

TFIIS. This region of Zn2 is analogous to a charged flexible loop within the zinc ribbon domain of TFIIS. These observations provide the basis for a preliminary model of RPB9 interactions with RNA polymerase II.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Aled Edwards, for his advice and guidance throughout my degree. As well, I extend my appreciation and gratitude to Stephen Orlicky, Don Awrey and David Jansma for their assistance with particular experiments, as well as their friendships and the many scientific discussions.

I would like to thank Chris Koth, Derek Ceccarelli and Kenji Miyata for their friendships, and for sharing all the grad school experiences with me. I would also like to thank all the members of the Edwards, Frappier and Friesen labs for making the lab environment a fun one.

I thank my parents, my sisters and my brother for their support and encouragement during my studies. I thank Frank for all his love and inspiration.

TABLE OF CONTENTS

	Page
CHAPTER 1 INTRODUCTION	1
1.1 General Overview	1
1.2 RNA Polymerase Subunit Architecture	3
1.3 Yeast RNA Polymerase II Subunit Composition and Structure	4
1.4 Transcription Initiation and Start Site Selection	8
1.5 Transcription Elongation	12
1.6 Transcription Elongation Factor TFIIS	15
1.7 RNA Polymerase II Subunit RPB9	18
1.8 Summary of This Research	20
CHAPTER 2 MATERIALS AND METHODS	22
2.1 Materials	22
2.2 Methods	26

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vii

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LIST OF FIGURES

Figure	Title	Page
1	Schematic Representation of the Sequence of RPB9 from <i>S. cerevisiae</i>	33
2	Homology of the Zn2 Region of RPB9 to the Zinc Ribbon Region of TFIIS	36
3	Model of the Zn Ribbon Domain from the Transcription Factor TFIIS	37
4	Purification of RNA Polymerase II $\Delta 9$	41
5	Purification of GST-RPB9 Fusion Proteins	43
6	Binding Assay with GST-RPB9 and RNA Polymerase II $\Delta 9$	46
7	Identification of Components of Upper and Lower Complexes in Binding Assay	48
8	Competition for Binding to RNA Polymerase II $\Delta 9$ with Unlabeled GST-RPB9	49
9	Binding Assay with GST-RPB9 Truncation Mutants	52
10	Binding Assay with Representative Mutants from the Zn1, Linker, and Zn2 Regions of RPB9	53
11	Readthrough Assay with Wildtype and $\Delta 9$ RNA Polymerase II	56
12	Readthrough Assay with $\Delta 9$ RNA Polymerase II and Representative Mutants from the Zn1, Linker, and Zn2 Regions of RPB9	59
13	Transcript Cleavage Assay with Wildtype and $\Delta 9$ RNA Polymerase II	63

14	Transcript Cleavage Assay with $\Delta 9$ RNA Polymerase II and Representative Mutants from the Zn1, Linker, and Zn2 Regions of RPB9	65
15	Complementation of Phenotype by Various RPB9 Mutants	69
16	Primer Extensions of the ADH1 Transcript from Wildtype and Deletion Strains of <i>S. cerevisiae</i>	73
17	Primer Extension of the ADH1 transcript from a Yeast Strain Carrying the Zn1 Allele of RPB9	75

LIST OF TABLES

Table	Title	Page
1	List of Truncation, Deletion and Point Mutants of RPB9	39
2	Sequence of Flexible Loop from Zinc Ribbon Domain	60
3	Summary of <i>in vitro</i> Analyses of Mutant GST-RPB9 Fusion Proteins	66
4	Summary of <i>in vivo</i> Analyses of RPB9 Mutant Alleles	76

ABBREVIATIONS

ATP	adenosine 5'-triphosphate
CTD	carboxyl terminal domain
CTP	cytosine 5'-triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GTP	guanosine 5'-triphosphate
GST	glutathione S-transferase
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HMK	heart myosine kinase
IPTG	isopropyl- β -D-thiogalactoside
kDa	kilodalton
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
Pol II	RNA polymerase II
Pol II $\Delta 9$	RPB9-deficient RNA polymerase II

RNA	ribonucleic acid
RPB9	ninth subunit of RNA polymerase II
SDS	sodium dodecylsulfate
TAF	TBP associated factor
Tris	tris(hydroxymethyl)aminomethane
UTP	uridine 5'-triphosphate

CHAPTER 1 INTRODUCTION

1.1 General Overview

DNA-dependent RNA polymerases catalyze the synthesis of RNA from a DNA template. These enzymes synthesize the RNA transcript in a 5' to 3' direction, catalyzing the formation of a phosphodiester bond between the 3' hydroxyl group of the nascent RNA chain and the α -phosphate group of the incoming nucleotide tri-phosphate. The resulting RNA transcripts are used as templates for protein synthesis, or as structural or catalytic RNA moieties in ribonucleoproteins.

RNA polymerases are complex enzymes; they catalyze the synthesis of RNA in a highly conserved process while interacting with a wide range of regulatory proteins. Their catalytic role is known, but the specifics of how they accomplish RNA synthesis are not well understood. Furthermore, little is known about how the various transcription factors interact with RNA polymerase in order to directly influence the transcription reaction. The intricacy of the transcription process is underscored by the complexity of the bacterial and eukaryotic enzymes. Both classes of RNA polymerases comprise multiple subunits, and each subunit plays a distinct role in RNA polymerase function.

The mechanism of RNA synthesis is highly conserved among all organisms, as indicated by sequence and structural homologies. Bacterial, eukaryotic and archael RNA polymerases are multisubunit molecules; the two largest subunits of each class of enzyme

share stretches of conserved sequences (Allison *et al*, 1985); (Langer *et al*, 1995). In each case, these conserved subunits compose the catalytic core of the enzyme. Structural similarities have been revealed by the solution of the structures of bacterial and yeast RNA polymerases (Darst *et al*, 1989); (Darst *et al*, 1991); (Schultz *et al*, 1993); (Jensen *et al*, 1998). The conservation of structural features implies that prokaryotes and eukaryotes have maintained a common transcription mechanism through the preservation of the general protein architecture.

The eukaryotic and archaeobacterial RNA polymerases are more complex than their eubacterial counterparts, reflecting the greater complexity of the eukaryotic and archaeal transcription processes. The eubacterial enzymes are composed of 4 core subunits, while the eukaryotic and archaeal enzymes contain between 12 and 15 subunits (Thuriaux and Sentenac, 1992); (Langer *et al*, 1995). The eukaryotic enzymes are categorized as I, II, or III, according to the various classes of genes that they transcribe (Young, 1991). The yeast RNA polymerases are the best characterized of the eukaryotic enzymes, and serve as a model for eukaryotic transcription systems.

Although it is the RNA polymerase that synthesizes the RNA chain, there are numerous protein factors that associate with the polymerase to modulate its activity throughout transcription. The transcription process can be divided into the stages of initiation (Conaway and Conaway, 1993), elongation (Kane, 1994) and termination (Reines, 1994). A different group of proteins interacts with the polymerase to influence events at each stage. Specific factors locate the polymerase at the promoter, help select

the transcript start site, direct the enzyme to clear the promoter, stimulate the active polymerase to elongate through pause sites, and modulate termination of transcription.

This study focuses primarily on yeast RNA polymerase II, and specifically its ninth subunit, RPB9. The enzyme is a complex molecule in which 12 subunits assemble to form a single unit that contains the transcriptional catalytic activity. This single catalytic unit must interact with the many proteins that moderate the different stages of transcription. Each of the subunits must contribute to the catalytic and regulatory functions of the enzyme through specific protein and DNA contacts. RNA polymerase II uses RPB9 to exert an influence on regulatory events in both initiation and elongation. RPB9 has been shown to play a role in transcript start site selection (Hull *et al*, 1995); (Furter-Graves *et al*, 1994); (Sun *et al*, 1996). It has also been demonstrated to mediate the response between RNA polymerase II and the transcription elongation factor TFIIS (Awrey *et al*, 1997). TFIIS stimulates RNA polymerase II to transcribe through sequences that cause the polymerase to stall.

1.2 RNA Polymerase Subunit Architecture

The *E. coli* RNA polymerase is the best characterized of the eubacterial RNA polymerases. It consists of four core subunits: β , β' , and two copies of the α subunit. The β' subunit is the largest, and binds the DNA template (Nudler *et al*, 1996). It also contains the catalytic center of the enzyme (Zaychikov *et al*, 1996). The second largest subunit, β , makes contacts with the nascent transcript (Markovtsov *et al*, 1996), the DNA template (Nudler *et al*, 1996), and contains the nucleotide binding site (Grachev *et al*,

1989). These two subunits share regions of homology with the two largest subunits in the eukaryotic enzymes. These regions are likely to contain residues that are critical for the basic transcription reaction. The α subunit is present as a dimer, and plays a role in assembling the enzyme. The structure of the N-terminal domain of this subunit has been solved by X-ray crystallography (Zhang and Darst, 1998). This region is sufficient for both subunit dimerization and assembly of the polymerase. The α subunit is also involved in sequence-specific binding to DNA (Ross *et al*, 1993); (Blatter *et al*, 1994).

The eukaryotic RNA polymerases are more complex, and contain between 12 to 15 subunits (Thuriaux and Sentenac, 1992). They can be divided into three classes, RNA polymerase I, II and III (or A, B and C) (Roeder and Rutter, 1969), based on the class of gene each type transcribes (Young, 1991). Class I enzymes transcribe the large rRNA, class II enzymes transcribe pre-mRNA and small nuclear RNA, and class III enzymes transcribe the small rRNA and tRNA. The majority of the mass of the eukaryotic RNA polymerases is contained within a core region that has homology to bacterial enzymes (Allison *et al*, 1985). The remaining subunits are either shared with other classes of eukaryotic RNA polymerases or are specific to each particular class of RNA polymerase (Sentenac *et al*, 1992).

1.3 Yeast RNA Polymerase II Subunit Composition and Structure

1.3.1 Core Subunits

Yeast RNA polymerase II possesses 12 subunits (RPB1 through to RPB12) (Sentenac *et al*, 1992). The three largest subunits form the catalytic core of the enzyme,

and have some homology with the *E. coli* RNA polymerase (Allison *et al*, 1985). RPB1 has eight regions of homology (A through H) shared with the β' subunit of the *E. coli* RNA polymerase. RPB2 possesses nine conserved stretches (A through I) that are homologous to regions of the *E. coli* β subunit. These stretches of homology are also shared with the two largest subunits of RNA polymerases I and III, and likely form the basis of a conserved reaction center. RPB1 binds the DNA template (Horikoshi *et al*, 1985); (Carroll and Stollar, 1983), and RPB2 contains the nucleotide-binding site (Riva *et al*, 1987).

RPB3 has some limited sequence homology with the α subunit of *E. coli* RNA polymerase (Kolodziej and Young, 1989), as well as with RPB11, and two subunits common to RNA polymerases I and III (Woychik and Young, 1993). The RPB3 and RPB11 homologues from *Saccharomyces pombe* form a heterodimer (Kimura *et al*, 1997); (Ishiguro *et al*, 1998); (Yasui *et al*, 1998) which is suggested to play a role in enzyme assembly analogous to that of the α_2 homodimer of *E. coli* RNA polymerase (Zhang and Darst, 1998).

RPB1 also possesses a carboxy terminus domain (CTD) that is essential for yeast viability (Nonet *et al*, 1987a). This domain is unique to class II polymerases, and is composed of multiple heptapeptide repeats of the consensus sequence YSPTSPS. It is glycosylated (Kelly *et al*, 1993) and undergoes phosphorylation prior to transcription elongation (Cadena and Dahmus, 1987); (Payne *et al*, 1989). The exact role of the CTD is not known, but it is involved in activation of transcription initiation (Nonet *et al*,

1987a) and in recruitment of transcript-processing machinery to the transcription complex (McCracken *et al*, 1997).

1.3.2 Common Subunits

There are five subunits that are common to all three classes of eukaryotic RNA polymerases: RPB5, RPB6, RPB8, RPB10 and RPB12. These common subunits are all essential for viability and each is encoded by a single gene (Woychik *et al*, 1990); (Carles *et al*, 1991). Specific roles for these subunits are not known.

1.3.3 RNA Polymerase II Unique Subunits

There are four subunits that are specific to RNA polymerase II. Two of the subunits, RPB4 and RPB7, form a complex that dissociates from the enzyme (Edwards *et al*, 1991). RPB4 is nonessential (Woychik and Young, 1989), but RPB7 is required for viability (McKune *et al*, 1993). Disruption of the *RPB4* gene results in an enzyme that is lacking both subunits. This implies that the RPB4/RPB7 complex associates with the remainder of the polymerase via RPB4. The $\Delta 4/7$ enzyme is defective in promoter-specific initiation (Edwards *et al*, 1991), indicating that the RPB4/RPB7 complex is involved in promoter recognition and initiation. A recent structure of yeast RNA polymerase II containing the RPB4/RPB7 heterodimer demonstrates that the 4/7 complex alters the size of the active site cleft (Jensen *et al*, 1998). This result indicates that 4/7 may play a role in the closing of the cleft after binding of the DNA template.

RPB9 and RPB11 are also unique to RNA polymerase II, although both have some homology with subunits of RNA polymerases I and III (Nogi *et al*, 1993); (Christophe Carles, personal communication); (Woychik and Young, 1993). As

mentioned above, RPB11 forms a heterodimer with RPB3 that is analogous to the α_2 dimer of *E. coli* polymerase, and is likely involved in enzyme assembly (Zhang and Darst, 1998). RPB9 plays a role in both initiation and elongation. Several *in vivo* and *in vitro* studies have demonstrated that RPB9 is involved in transcript start site selection (Furter-Graves *et al*, 1994); (Hull *et al*, 1995); (Sun *et al*, 1996). RPB9 also affects the ability of the polymerase to recognize pause sites within a template and to respond to TFIIIS to transcribe through those pause sites (Awrey *et al*, 1997). A more detailed description of RPB9 is given later in the text.

1.3.4 RNA Polymerase II Structure

A three-dimensional structure of yeast $\Delta 4/7$ RNA polymerase II has been solved to 16 Å (Darst *et al*, 1991). The enzyme possesses a 25 Å cleft that forms the “palm” of a hand-like structure. The cleft is surrounded by protrusions similar to a “thumb” and “fingers”. One model proposes that the DNA template occupies this cleft, held in place by the “thumb” projection. Similar structures are seen with two other multi-subunit RNA polymerases whose structures have been solved. Both *E. coli* RNA polymerase (Darst *et al*, 1989) and yeast RNA polymerase I (Schultz *et al*, 1990) (solved to 23 Å and 30 Å, respectively) display the hand-like configuration. The RPB9 homologue of RNA polymerase I, subunit A12, has been localized to the floor of the cleft using immunolocalization and electron microscopy techniques (Patrick Schultz, personal communication). The conservation of the polymerase structural features suggests that these characteristics are essential for RNA polymerase function. It is possible that the homologous regions of the core subunits form the architecture of this conserved structure.

1.4 Transcription Initiation and Start Site Selection

Eukaryotic transcription initiation requires many transcription factors to direct the polymerase to the promoter and regulate the transcription of specific genes. Eukaryotic RNA polymerases are unable to recognize and bind to the promoter by themselves, unlike prokaryotic polymerases that can directly recognize promoter elements through their associated sigma subunit. Thus, initiation by eukaryotic RNA polymerase requires a set of general transcription factors that bind to the promoter and recruit the polymerase to form a pre-initiation complex capable of initiating transcription (Conaway and Conaway, 1993). Regulation of eukaryotic transcription initiation is governed by specific transcription factors that bind to DNA elements at certain promoters to activate or repress transcription of specific classes of genes. A summary of the events involved in transcription initiation by RNA polymerase II will be given here, with particular focus on the factors that determined the site from which transcription begins.

The majority of eukaryotic class II promoters require the minimal elements of a TATA sequence and an initiator site for basal level transcription by RNA polymerase II (reviewed in (Struhl, 1989) and (Guarente, 1987)). The TATA element is defined by the consensus sequence TATAA, and is located upstream of the transcript start site. The initiator sequence identifies the location where synthesis of the transcript will begin, and is located immediately adjacent to the transcript start site. Several distinct initiator consensus sequences have been identified in eukaryotes. In mammalian promoters the initiator site is located 25 to 30 bases downstream of the TATA element. However, in

yeast, the spacing between the two elements can be between 40 and 120 bases, and several initiator sites may be utilized within a single promoter.

In addition to the basal elements, many eukaryotic promoters contain DNA sequences that bind specific regulatory proteins. In higher eukaryotes, sequences that are required for increased transcription are termed enhancer elements and can be located upstream or downstream of the basal promoter elements (Guarente, 1987). In yeast, UAS (Upstream Activator Sequences) elements that bind transcriptional activators are located 100 to 1500 bases upstream of the initiator site. The transcriptional activator proteins that bind to these sequences increase transcription levels of the genes in which they are located by enhancing the rate of transcription initiation or stabilizing the formation of the pre-initiation complex. In addition to DNA elements that lead to activation of transcription, some promoters contain upstream repressor sequences (URS) that bind negative regulator proteins. Binding of repressor proteins at these URS sites leads to a decrease in transcription of particular genes.

Five general transcription factors have been identified that are required for transcription by RNA polymerase II from a basal class II promoter *in vitro*: TBP, TFIIB, TFIIE, TFIIF and TFIIH (Sayre *et al*, 1992). These factors assemble with RNA polymerase II at the promoter to form a pre-initiation complex that enables polymerase to initiate transcription.

The first event required for assembly of the complex is the binding of TBP (Tata Binding Protein) to the consensus TATA sequence. *In vivo*, TBP, together with at least seven TAFs (TPB associated factors), forms the multi-subunit transcription factor TFIID

(Zhou *et al*, 1992). *In vitro*, TBP alone, rather than the complete TFIID complex, is sufficient to begin the formation of the pre-initiation complex (Kambadur *et al*, 1990).

Once TBP is positioned at the promoter, TFIIB is incorporated into the complex. TFIIB is a monomeric factor (Ha *et al*, 1991); (Pinto *et al*, 1992), which once it is located at the promoter, recruits RNA polymerase II and TFIIF (Buratowski *et al*, 1989). TFIIB acts as a bridge between TBP and RNA polymerase through interactions with both proteins as well as the DNA (Tschochner *et al*, 1992); (Buratowski and Zhou, 1993); (Malik *et al*, 1993). TFIIF helps stabilize the binding of the polymerase at the promoter. TFIIF has been identified as a heterodimeric factor in mammalian systems (Burton *et al*, 1988), and a heterotrimeric factor in yeast (Henry *et al*, 1992).

After the incorporation of RNA polymerase-II, the factors TFIIE and TFIIH assemble to complete the pre-initiation complex. TFIIE is a heterodimer (Sayre *et al*, 1992), and TFIIH is multimeric factor with associated ATPase and kinase activities ((Flores *et al*, 1992); (Svejstrup *et al*, 1994); (Schaeffer *et al*, 1993)). Isolation of a free holoenzyme complex containing RNA polymerase II, transcription factors TFIIB, TFIIF and TFIIH, and several associated factors termed SRBs, indicates that at least part of the pre-initiation complex may assemble prior to binding at the promoter (Koleske *et al*, 1992).

The formation of the pre-initiation complex at the promoter precedes transcript synthesis. Transcription initiation is dependent on ATP hydrolysis (Sawadogo and Roeder, 1984); (Cai and Luse, 1987) and phosphorylation of the CTD tail of RPB1 (Cadena and Dahmus, 1987); (Payne *et al*, 1989), and has a requirement for nucleotide

substrates. Although the position of the TATA element determines where the pre-initiation complex will assemble, it does not influence the exact start site of transcription (Eisenmann *et al*, 1989). Transcript synthesis begins at an initiator site. As mentioned above, in yeast the start site for transcription occurs within a window located approximately 40 to 120 bases downstream of the TATA element, and can begin at several different initiator sites within the same promoter. This results in a set of transcripts with heterologous 5' ends produced from a single promoter.

Protein components within the pre-initiation complex determine precisely which initiator sites will be used and the frequency of use for each site. TFIIB and RNA polymerase II are the primary factors that directly influence the selection of transcript start sites. Replacement of *Saccharomyces cerevisiae* TFIIB and RNA polymerase II in an *in vitro* transcription system with *Schizosaccharomyces pombe* homologues switches the start site selection to reflect a *pombe*-like transcript pattern (Li *et al*, 1994). TFIIB (SUA7) and RPB1 (SUA8) define the downstream boundary of transcription start sites, as mutations in either protein result in a downstream shift of start site preferences (Hekmatpanah and Young, 1991); (Pinto *et al*, 1992); (Berroteran *et al*, 1994). RPB9 is responsible for defining the upstream limit of the start site window; disruptions of the *RPB9 (SHI)* gene result in an upstream shift of transcript start sites (Furter-Graves *et al*, 1994); (Hull *et al*, 1995); (Sun *et al*, 1996). RPB9 mutants suppress start site effects of TFIIB mutants (Sun *et al*, 1996), indicating there is a functional interaction between the two proteins within the pre-initiation complex.

Two additional protein factors, TGF1 and SSU72, indirectly influence transcript start sites. TGF1 is the largest subunit of yeast TFIIF, and like RPB9, is a suppressor of start site mutants of TFIIB (Sun and Hampsey, 1995). SSU72 is a protein of unknown function that was identified through a genetic screen for factors that influence transcription start site. Mutations in this protein enhance the downstream shift in transcript start sites caused by TFIIB mutants (Sun and Hampsey, 1996). TGF1 and SSU72 do not affect start site selection in the presence of wildtype TFIIB, indicating that these proteins exert their influence on transcript start site preferences via an interaction with TFIIB.

1.5 Transcription Elongation

Once the RNA polymerase has initiated RNA synthesis and cleared the promoter region, the polymerase moves into the elongation phase of transcription. The active transcription unit is referred to as the ternary complex, which comprises the RNA polymerase, the engaged template DNA, and the nascent RNA chain (Kerppola and Kane, 1991). Elongation is a processive event in which the productivity of a ternary complex is measured as its ability to produce a full-length transcript. Although the exact mechanism of chain elongation is not known, several models have been proposed to describe the process by which RNA polymerase travels along the template. As is the case with initiation, this stage of transcription is regulated both by sequences within the DNA template and by protein factors.

Several models have been proposed to describe the mechanism by which RNA polymerase transverses the DNA template (reviewed in (Chamberlin, 1995)). One model describes the movement of polymerase along the template as monotonic, with the footprint of the polymerase on the template maintained at a uniform size as the polymerase progresses. The opened region of the template protected by the polymerase is termed the transcription bubble, and within this region a 12 basepair DNA:RNA hybrid is formed between the nascent RNA chain and the melted (Yager and von Hippel, 1991). Factors that influence the stability of the hybrid, such as RNA hairpins in the transcript or dA:rU rich regions in the hybrid, affect the productivity of the ternary complex.

A second model favours a discontinuous mode of elongation, with the movement of the polymerase along the template described as an “inchworm” motion (Chamberlin, 1995). The region of the template protected by the RNA polymerase varies as the front and rear edges of the polymerase move forward alternately along the DNA. This model proposes that the polymerase have two DNA binding sites to maintain processivity as the enzyme moves down the template. An RNA binding site on the polymerase secures the nascent transcript in the ternary complex, with only the last 3 bases of the transcript involved in a short hybrid duplex with the DNA template (Rice *et al*, 1991).

Biochemical studies suggest that elongation may occur through a combination of monotonic and discontinuous mechanisms. The conversion between the two modes occurs in response to particular attenuating sequences within the template (Nudler *et al*, 1994). Ternary complexes progress along an engaged template with the front edge of the polymerase and the catalytic site advancing monotonically until particular sequences are

encountered in the template. These regulatory sequences cause the transcribing polymerase to convert to the inchworm mode of elongation. The front edge of the polymerase stalls while the catalytic site continues to advance with addition of each nucleotide. The front edge of the polymerase may then advance on the template by 7 to 10 nucleotides in a single step.

The attenuating sequences within the template DNA affect the overall rate of elongation by causing the ternary complex to pause or arrest. In either case, the polymerase maintains contacts with the template and transcript, keeping the ternary complex intact (Kerrpola and Kane, 1991). A paused ternary complex halts at an attenuating sequence, but can then resume transcribing without additional protein factors. Release from the paused conformation may be due to the build up of internal strain due to discontinuous elongation by the polymerase (Nudler *et al*, 1994). The conversion back to the active conformation appears to be time dependent (Gu and Reines, 1995).

Sequences in the DNA template can also induce the ternary complex to arrest. An arrested complex is one in which the polymerase has halted and cannot spontaneously revert to an elongating form (Reines *et al*, 1989). This elongation incompetent state may be due to some sort of conformational rearrangement of the polymerase in the arrested complex. The newly synthesized 3' end of the RNA appears to be relocated in the arrested complex (Markovtsov *et al*, 1996); (Nudler *et al*, 1997). Photoreactive nucleotides incorporated at the 3' end of the transcript crosslink to different regions of the polymerase depending on whether the ternary complex is in an arrested or elongating state. Arrested ternary complexes are extremely stable (Chamberlin, 1995); conversion

of an arrested complex to an elongating one requires the activity of elongation transcription factors.

General transcription factors interact with the elongating RNA polymerase to influence the rate of elongation. There are two classes of transcription factors that act on RNA polymerase II. The first class includes the factors TFIIF, ELL, and elongin (Bengal *et al*, 1991); (Shilatifard *et al*, 1996); (Bradsher *et al*, 1993). These factors increase the productivity of the polymerase by binding to the ternary complex to prevent or minimize the formation of paused complexes. They also increase the rate of conversion of polymerase from a paused state to an elongating state.

The second class of RNA polymerase II transcription elongation factors includes TFIIS. The analogous bacterial factors GreA and GreB act on the *E. coli* polymerase. These factors induce arrested ternary complexes to resume elongation. They do so by stimulating the polymerase to cleave the transcript at the 3' end (Reines, 1992); (Borukhov *et al*, 1993). The length of the cleaved products varies, and is dependent on the sequence at which the polymerase has arrested (Izban and Luse, 1993); (Borukhov *et al*, 1993); (Nudler *et al*, 1994). Cleavage occurs in such a way that the polymerase maintains the correct register between transcript and template, allowing the polymerase to continue elongating.

1.6 Transcription Elongation Factor TFIIS

The eukaryotic transcription elongation factor TFIIS stimulates RNA polymerase II to cleave the 3' end of nascent transcripts. This cleavage activity allows the polymerase

to transcribe through sites that normally induce elongation arrest. TFIIS homologues have been identified in species ranging from yeast to man. The protein is composed of three protease-resistance domains (I, II and III) (Morin *et al*, 1996); structures have been solved for two of these domains (Olmsted *et al*, 1998); (Morin *et al*, 1996);(Qian *et al*, 1993); (Qian *et al*, 1993). Regions of TFIIS that are involved in binding to RNA polymerase II (Awrey *et al*, 1998) and induction of the cleavage response have been identified (Jeon *et al*, 1994).

The N-terminal domain of TFIIS consists of approximately 100 residues, and may be important in the regulation of TFIIS activity. This domain is homologous to the N-terminal region of the largest subunit of the transcription elongation factor elongin (Aso *et al*, 1996). Both TFIIS and elongin A associate with the RNA polymerase II holoenzyme via this domain (Pan *et al*, 1997). Also, sites within this region are phosphorylated *in vivo* (Horikoshi *et al*, 1985). Despite the conservation of Domain I among elongation factors, it is not required for the transcriptional activation of RNA polymerase by yeast TFIIS (Nakanishi *et al*, 1992); (Nakanishi *et al*, 1995).

The remaining two regions are highly conserved amongst all TFIIS homologues, and both are required for the transcriptional activity of TFIIS. An NMR structure has been solved for the Domain II of yeast TFIIS, which comprises the central 110 amino acids of the protein (Morin *et al*, 1996). This domain forms a three-helix bundle that is stabilized by a central hydrophobic core. The region of TFIIS that is responsible for binding to RNA polymerase II has been mapped to residues on the third helix of the

bundle (Awrey *et al*, 1998). This interaction occurs via conserved regions of the largest subunit of RNA polymerase II (Wu *et al*, 1996).

The structure of Domain III from human and yeast TFIIS has also been solved by NMR techniques (Qian *et al*, 1993); (Qian *et al*, 1993); (Olmsted *et al*, 1998). This domain has a conserved zinc-chelating motif, $-CX_2CX_{24}CX_2C-$, and forms a three-stranded anti-parallel β -sheet with the four cysteines coordinating the zinc ion. The loop connecting the first and second strands of the sheet is highly flexible and contains conserved charged residues. These charged residues are essential for the transcriptional activity of TFIIS (Jeon *et al*, 1994). This structure was the first to depict this particular zinc-chelating fold, which has been termed the zinc ribbon. The $-CX_2CX_nCX_2C-$ motif is common in eukaryotic transcription-associated proteins; transcription factors TFIIB and TFIIE, as well as RNA polymerase II subunits RPB1, RPB2, RPB9 and RPB12 all contain this motif. The structures of this motif from TFIIB (Zhu *et al*, 1996) and the C-terminus of RPB9 (Wang *et al*, 1998) have since been solved, and both also adopt the zinc ribbon fold.

Although TFIIS binds to RNA polymerase II through interactions with RPB1, it has a functional interaction with RPB9 that is essential for the ability of TFIIS to stimulate elongation readthrough and transcript cleavage (Awrey *et al*, 1997). RNA polymerase II lacking RPB9 does not respond to TFIIS in *in vitro* readthrough and cleavage assays. In addition, yeast strains that are deficient for either TFIIS (Hubert *et al*, 1983) or RPB9 (David Jansma, personal communication) are sensitive to the drug 6-azauracil, providing evidence for a functional interaction between the two *in vivo*.

1.7 RNA Polymerase II Subunit RPB9

The ninth subunit of RNA polymerase II, RPB9, is a small protein of approximately 14 kDa in size. The majority of the protein is composed of two zinc-binding regions, which are defined by the $-CX_2CX_nCX_2C-$ motif. The C-terminal zinc domain has a high degree of homology (30% identity) with the zinc ribbon domain from TFIIIS (Kaine *et al*, 1994). The subunit is highly conserved, and homologues have been cloned from yeast (Woychik *et al*, 1991), archaeobacteria (Langer and Zillig, 1993); (Kaine *et al*, 1994), *Drosophila* (Harrison *et al*, 1992), plants (Hull *et al*, 1995) and humans (Acker *et al*, 1993). In addition, analogous subunits have been identified as components of the eukaryotic RNA polymerases I and III (Nogi *et al*, 1993); (Christophe Carles, personal communication). The high degree of conservation of this subunit is underlined by the ability of the human homologue to partially substitute for yeast subunit *in vivo* (McKune *et al*, 1995). However, the functional conservation is not universal; *Drosophila* RPB9 is not functional in the yeast transcription system (Hull *et al*, 1995).

The conservation of this subunit indicates that its role in transcription is critical. Mutations that result in disruption of RPB9 in *Drosophila* are lethal (Harrison *et al*, 1994). Flies that are homozygous for an interrupted *RpIIIS* gene do not survive beyond the late larval stage. Survival rates of heterozygous flies are low, and are highly dependent on temperature. Deletion of RPB9 in yeast is not lethal, but results in slow growth at optimal temperature, as well as cold- and temperature-sensitive phenotypes (Woychik *et al*, 1991). In addition, these yeast strains exhibit sensitivity to the drug 6-

azauracil (David Jansma, personal communication). This drug lowers the cellular pool of GTP, and is thought to hamper the process of transcription elongation (Exinger and Lacroute, 1992).

Several studies have demonstrated that RPB9 is involved in the initiation of transcription. In particular, RPB9 helps to select the transcript start sites (Furter-Graves *et al*, 1994); (Hull *et al*, 1995); (Sun *et al*, 1996). Deletion or disruption of RPB9 in yeast leads to an upstream shift in the start site preferences at the majority of promoters both *in vivo* and *in vitro* (Furter-Graves *et al*, 1994); (Hull *et al*, 1995). This shift changes the pattern of the normal start site usage such that the upstream sites are used at higher frequency than downstream sites. In addition, new sites located farther upstream than the normal start sites may be used. RPB9 determines the span of particular start sites to be used through an interaction with TFIIB (Sun *et al*, 1996). Mutations in RPB9 suppress the downstream shift in transcript start site caused by mutations in TFIIB. Addition of wildtype RPB9 to each of these systems restores wildtype start site preferences.

RPB9 also plays a role in transcription elongation and response to the transcription factor TFIIS (Awrey *et al*, 1997). This subunit affects the ability of the polymerase to recognize attenuating sequences in the DNA. RNA polymerase II purified from *RPB9* null yeast strains recognizes intrinsic pause sites with much lower frequency than wildtype RNA polymerase II in *in vitro* elongation assays. Furthermore, this $\Delta 9$ enzyme is unable to respond to stimulation by TFIIS in assays measuring either transcript cleavage or reactivation of elongation at arrest sites. Again, each of these activities is restored by the addition of recombinant RPB9 to the transcriptional assays.

The two zinc-binding regions of RPB9 conform to the $-CX_2CX_nCX_2C-$ motif that is conserved in many eukaryotic transcription proteins. This motif from both TFIIS (Qian *et al*, 1993) and TFIIB (Zhu *et al*, 1996) has been shown to adopt the zinc ribbon fold. The C-terminal region of RPB9 has sequence similarity to the zinc ribbon domain of TFIIS, and thus it is likely that it also possesses the ribbon conformation. The NMR structure of the C-terminal zinc-binding region of RPB9 from *Thermococcus celer* (Wang *et al*, 1998) reveals that this domain does assume the ribbon fold. This RPB9 domain is a four-stranded antiparallel β -sheet, with the additional strand being contributed by residues that are N-terminal to the first pair of conserved cysteines. There is a flexible loop located between the second and third strands of the sheet that is analogous to the loop in the TFIIS domain. This region contains charged residues that are essential for TFIIS activity (Jeon *et al*, 1994), and are conserved in many of the RPB9 homologues (Kaine *et al*, 1994). This region is likely to play an important part in the function of RPB9.

1.8 Summary of This Research

RPB9 is an important component of the transcription initiation and elongation machinery. This subunit is involved in selecting transcript start sites as well as responding to activation by TFIIS at arrest sites. The mechanisms by which RPB9 accomplishes these activities are not known. In addition, the subunit is composed mainly of two zinc-binding regions, one of which adopts a zinc ribbon fold. This study attempts to ascribe the functions of RPB9 to the regions within the protein. The research

presented here is a mutational analysis of the RPB9 subunit of RNA polymerase II from *Saccharomyces cerevisiae*. *In vivo* start site and growth phenotype assays and *in vitro* elongation assays are used to define roles for the regions of RPB9. Areas of the protein required for accurate start site selection and for the response of RNA polymerase II to TFIIS are identified. A gel mobility shift assay was developed to identify the residues of RPB9 involved in protein-protein contacts with the remainder of the polymerase.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Purification of Wildtype and $\Delta 9$ RNA Polymerase II

Wildtype and RPB9-deficient RNA polymerase II were purified from baker's yeast and the WY9 yeast strain, respectively, using a combination of ion exchange and immunoaffinity chromatography. They were purified using the protocols described in (Edwards *et al*, 1990) and (Awrey *et al*, 1997). The final purified protein preparations had a protein concentration of between 0.4 and 1.2 milligrams per millilitre as determined by the Biorad protein assay (Biorad), and were stored at -70°C in 10 mM Tris-HCl pH 7.9, 40 mM ammonium sulphate, 10% (v/v) glycerol, 2.5 mM DTT, 100 μ M EDTA, and 10 μ M ZnCl₂.

2.1.2 Purification of TFIIS

Recombinant wildtype yeast TFIIS was obtained from Dr. Don Awrey. It was purified as a 6-histidine-tagged fusion protein from *E. coli* using a combination of ion exchange and metal affinity chromatography as described in (Awrey *et al*, 1997). The final purified protein used in this study was concentrated to between 1 and 3 milligrams per milliliter and stored at -70°C in 20 mM HEPES pH 7.5, 50 mM NaCl, 10 mM DTT, 10% (v/v) glycerol, 10 μ M ZnCl₂, 1 mM benzamidine, and 1 mM PMSF.

2.1.3 Cloning of RPB9 Mutants

The individual open reading frames for the various RPB9 mutants were generated using PCR amplification. The original *Hin* dIII fragment was used as the template for each reaction (Woychik *et al*, 1991). Each coding region, including wildtype, was generated with a 5' *Bam* HI site immediately upstream of the start codon and 3' *Eco* RI site immediately downstream of the stop codon. Mutants were constructed from two fragments in the following manner. One fragment was generated containing the required mutation through the use of the appropriate mutagenic oligonucleotide primers. The second fragment was generated to contain the remaining wildtype sequence. The two fragments were gel purified, phosphorylated with polynucleotide kinase (New England Biolabs), and ligated using T4 DNA ligase (New England Biolabs). A second round of PCR amplification was done on the ligated product to produce a complete RPB9 coding region containing the specific mutation with the necessary restriction site at either end. These fragments were digested with the *Bam* HI and *Eco* RI restriction endonucleases (New England Biolabs) and inserted into the pGEX-2TK vector (Pharmacia), which encodes the heart myosine kinase (HMK) recognition sequence directly upstream of the *Bam* HI restriction site. *E. coli* DH5 α cells were transformed with the resulting plasmids and individual colonies were isolated. Plasmid DNA was recovered using a small-scale plasmid isolation protocol (Sambrook *et al*, 1989) and sequenced to confirm the incorporation of each particular mutation. Sequencing was done according to manufacturer's directions using the T7 Sequenase kit (Pharmacia). These pGEX-2TK RPB9 constructs were then used to express the various GST-RPB9 fusion proteins in *E.*

coli. They were also used to amplify the mutated RPB9 coding regions for use in construction of the RPB9 yeast expression plasmids.

2.1.4 Expression and Purification of GST-RPB9 Fusion Proteins

The *E. coli* strain HB101 was transformed with each of the pGEX-2TK RPB9 plasmids for expression of the GST-RPB9 mutant proteins. The fusion proteins were expressed and purified using glutathione-affinity chromatography as described in (Awrey *et al*, 1997). HB101 cells containing the pGEX-2TK RPB9 plasmids were grown to A_{600} of 0.5 at 37°C. The GST-RPB9 protein expression was induced at 30°C by the addition of 0.5 mM IPTG. The cells were harvested 3 hours post-induction by centrifugation, resuspended in 50 mM HEPES pH7.5, 10% (w/v) sucrose, 100 mM NaCl, 10 mM DTT, 1 mM benzamidine, and 1 mM PMSF, frozen, thawed, and then lysed by sonication at 4°C. All subsequent steps were performed at 4°C. The lysate was clarified by centrifugation at 55 000 X g for 30 minutes in a Beckman SW28 rotor. The supernatant was flowed through a DE52 column (2.5 X 2 cm; Whatman) and loaded directly onto a glutathione-4B-Sepharose column (2.5 X 3 cm; Pharmacia) previously equilibrated with Buffer A (10 mM sodium phosphate, 1.8 mM potassium phosphate, 2.7 mM KCl, 140 mM NaCl, 10 mM DTT, 1 mM benzamidine, and 1 mM PMSF). The glutathione column was washed with 10 column volumes of Buffer A followed by 5 column volumes of Tris buffer (50 mM Tris pH 8.0, 10 mM DTT, 1 mM benzamidine, and 1 mM PMSF). The GST-RPB9 fusion protein was eluted from the column with Tris buffer containing 10 mM glutathione. The purified protein was dialyzed against 5 mM HEPES pH 7.5, 100 mM NaCl, 10% (v/v) glycerol, 10 mM DTT, and 10 μ M ZnCl₂ and stored frozen at -70°C.

The final preparation for each fusion protein had a protein concentration of between 0.15 and 1.5 milligrams per millilitre as determined by the Biorad protein assay (Biorad).

2.1.5 Yeast Strains

The RPB9-deficient strain used to purify pol II $\Delta 9$ for the *in vitro* assays was the WY9 strain, obtained from Dr. Nancy Woychik. The yeast strains used in the *in vivo* assays were constructed and provided by David Jansma. The wildtype strain used was YF2221, the Δ RPB9 strain used was YF2230, the Δ TFIIS strain used was YF2222, and the Δ RPB9/ Δ TFIIS strain used was YF2234. **WY9:** *MAT α ura3-52 his3 δ 200 leu2-3 leu2-112 lys2 δ 201 ade2-1 RPB9 δ 1::HIS3*; **YF2221:** *MAT α ura3-52 his3-11 his3-15 leu2-3 leu2-112 ade2-1 can1-100 ssd1-d2 trp1::hisG-URA3-hisG*; **YF2222:** *MAT α ura3-52 his3-11 his3-15 leu2-3 leu2-112 ade2-1 can1-100 ssd1-d2 trp1-1 ppr2::hisG-URA3-hisG*; **YF2230:** *MAT α ura3-52 his3-11 his3-15 leu2-3 leu2-112 ade2-1 can1-100 ssd1-d2 trp1::hisG-URA3-hisG RPB9::HIS3*; **YF2234:** *MAT α ura3-52 his3-11 his3-15 leu2-3 leu2-112 ade2-1 can1-100 ssd1-d2 trp1-1 ppr2::hisG-URA3-hisG RPB9::HIS3*.

2.1.6 Yeast Expression Plasmid

The yeast expression plasmid pRS314RPB9 containing the *RPB9* open reading frame plus approximately 500 basepairs upstream and 2200 basepairs downstream was obtained from Dr. Rolf Furter (Furter-Graves *et al*, 1994). This plasmid was adapted by the insertion of a *Bam* HI restriction site immediately upstream of the start codon, and an *Eco* RI restriction site immediately downstream of the stop codon, creating the plasmid pRS314RPB9BE. These sites were inserted using the Quikchange protocol and *Pfu* I DNA polymerase (Stratagene). Incorporation of these restriction sites allowed for the

insertion of each of the previously constructed *rpb9* open reading frames into pRS314RPB9BE. The resulting plasmids containing each of the mutant *rpb9* alleles under control of the endogenous *RPB9* promoter were transformed into the various yeast strains to determine their effects on growth phenotype and initiation start site selection preferences.

2.2 Methods

2.2.1 Radiolabeling of GST-RPB9 Fusion Proteins

The wildtype and mutant RPB9 proteins were expressed as GST-RPB9 fusion proteins containing the HMK recognition sequence located between the GST and RPB9 regions. This allowed for phosphorylation of the fusion proteins for use in a gel mobility shift assay. GST-RPB9 fusion proteins were phosphorylated using 100 units of HMK (Sigma) per nanomole of fusion protein. Prior to addition to the phosphorylation reaction, the appropriate amount of HMK was pre-incubated in 40 mM DTT for 10 minutes at room temperature. The pre-treated HMK was incubated with GST-RPB9, 50 μ M ATP and 0.15 μ M γ -³²P-ATP in 20 mM HEPES, 50 mM NaCl and 10 mM MgCl₂ for 1 hour at room temperature. The reaction mixture was then dialyzed against 5 mM HEPES, 50 mM NaCl, 10 mM DTT, and 10% (v/v) glycerol to remove the unincorporated radiolabel. The final solution containing the radiolabeled-RPB9 was separated on an SDS polyacrylamide gel and the total counts per lane were quantified by phosphorimager. The percentage of total counts attached to the GST-RPB9 fusion

protein was calculated in order to determine the specific activity of each radiolabeled GST-RPB9 construct.

2.2.2 Binding Assay between GST-RPB9 and pol II $\Delta 9$

A gel mobility shift assay was used to identify and characterize an interaction between GST-RPB9 and pol II $\Delta 9$. To determine if the GST-RPB9 fusion proteins could assemble with pol II $\Delta 9$, each of the ^{32}P -GST-RPB9 constructs were incubated with pol II $\Delta 9$ and then resolved by non-denaturing gel electrophoresis. 1 picomole of ^{32}P -radiolabeled GST-RPB9 (or the various mutants) was incubated with 0.03 to 5.0 picomoles of pol II $\Delta 9$ in 60 mM Tris pH 8.0, 10 mM ammonium sulphate, 5 mM magnesium acetate, 4 mM spermidine, 1.5 mM DTT, 10% (v/v) glycerol and 300 $\mu\text{g/ml}$ BSA. The reactions were mixed on ice in a total volume of 15 μl , then incubated for 15 minutes at room temperature. The complexed GST-RPB9 and pol II $\Delta 9$ were resolved on a non-denaturing 5% polyacrylamide gel by electrophoresing at 200 volts for 1.5 to 2 hours at 4°C. The amount of complexed radiolabeled GST-RPB9 was quantified with a phosphorimager (Molecular Dynamics).

The binding of radiolabeled GST-RPB9 to pol II $\Delta 9$ was tested for reversibility by competition for binding with unlabeled (cold) GST-RPB9. 1 picomole of ^{32}P -GST-RPB9 was pre-mixed with 1 to 10 picomoles of cold GST-RPB9 prior to the addition of 0.5 picomoles of pol II $\Delta 9$ in the binding reaction described above. The reactions were incubated, electrophoresed, and quantified as described above.

2.2.3 Transcript Elongation Readthrough Assay

The transcript elongation assay was performed using the conditions described in (Christie *et al*, 1994). Briefly, purified RNA polymerase II is allowed to transcribe on a 3'-dC-tailed template containing a portion of the human histone H3.3 gene. This template contains the pause sites TII, TIb, and TIa, which causes a percentage of the polymerase molecules to stall, producing a truncated transcript. Addition of TFIIS can stimulate some of the paused polymerase molecules to read through the pause sites and transcribe to the end of the template. In this study, 0.17 picomoles of wildtype pol II or 0.17 picomoles of pol II $\Delta 9$, alone or in the presence of either 0.83, 17, or 50 picomoles of each of the GST-RPB9 fusion proteins, were assembled on the tailed template. The transcripts were pulse-labeled at the 5' end using 0.8 mM unlabeled UTP, ATP and GTP and a limiting amount of α -³²P-CTP for 75 seconds in a total volume of 10 μ l. The transcript pulse-labeling was then chased for an additional 75 seconds by diluting the reaction 10-fold with reaction buffer containing 0.1 mM non-radioactive CTP. This allowed the polymerase to transcribe to one of the intrinsic pause sites within the template, or to the end of the template to produce full-length transcript. The reaction volume was divided in half, and 0.43 picomoles of TFIIS were added to one half of the reaction. Both the aliquots of the reaction were incubated for 10 minutes, and then stopped. All of the reactions were carried out at 30°C. The transcripts within the reaction mixtures were precipitated with ethanol and then resolved by electrophoresis in a 6% polyacrylamide, 8.3 M urea, TBE gel. The total counts incorporated into the transcripts paused at the TIa site and the full-length transcripts were quantified for each reaction by

phosphorimager. The percentage of transcripts paused at the T1a site was calculated from the total transcripts that reached either the T1a site or the end of the template (runoff transcripts), using the formula $\%T1a = (T1a \text{ counts} / (T1a \text{ counts} + \text{runoff counts})) * 100\%$.

2.2.4 Transcript Cleavage Assay

The transcript cleavage assay is similar to the readthrough assay, but it is designed to examine the ability of RNA polymerase II to cleave the nascent transcript when stalled at a transcription pause site. Paused polymerase complexes stalled on the 3'-tailed human H3.3 template were generated as for the readthrough assay. Free nucleotides were removed from the reaction by the purification of the ternary complexes through a spin column. TFIIS was then incubated with the paused polymerase, stimulating the wildtype polymerase to cleave the 3' end of the transcript. For this study, these reactions were performed as described in (Christie *et al*, 1994). 1.3 picomoles of wildtype or $\Delta 9$ pol II were assembled on the tailed H3.3 template as for the readthrough assay described above. After a 90 second chase, the reactions were spun successively through two pre-equilibrated Bio-gel 30 spin columns (Biorad). The resulting purified ternary complexes were split into six aliquots for equilibration with the various GST-RPB9 constructs. The equivalent of 0.21 picomoles of pol II $\Delta 9$ from the original reaction was mixed with 1.05, 21 or 63 picomoles of each of the GST-RPB9 constructs and incubated for 5 minutes at 30°C. 1.05 picomoles of TFIIS were added to each reaction, and the incubation was continued at 30°C. Samples were taken at 0, 1, 5 and 10 minutes after the addition of TFIIS. Nucleotides were added back to the remainder of each reaction and incubated for an additional 10 minutes at 30°C. The reactions were stopped and the RNA products

were resolved as for the readthrough assay. The transcripts were visualized by exposing the gels to autoradiographic film.

2.2.5 Growth Phenotype Assays

The RPB9-deficient yeast strain has specific altered growth characteristics. This strain grows slowly, even at the growth optimum of 30°C. As well, it is sensitive to high and low temperature extremes, and to the drug 6-azauracil. Expression of wildtype RPB9 can correct these defects. Transforming the various yeast strains with the RPB9 yeast expression plasmids tested the ability of each of the RPB9 constructs to complement growth phenotypes. The yeast strains YF2222, YF2230, and YF2234 were transformed with each of the pRS314RPB9BE constructs, or with pRS314 as a negative control. YF2221 was transformed with pRS314 to serve as a wildtype control. To test for complementation of cold and temperature sensitivity, transformed yeast strains were grown on solid synthetic complete yeast medium lacking tryptophan. Suspensions containing approximately 10000, 2000, 400 and 80 cells were spotted onto plates and grown at 12°C, 30°C, or 37°C for 2 to 6 days. To measure ability to correct sensitivity to 6-azauracil, the transformed strains were grown on solid synthetic complete yeast medium lacking tryptophan and uracil, and containing 50 µg/ml 6-azauracil. Suspensions containing approximately 10000, 2000, 400 and 80 cells were spotted onto plates and grown at 30°C for 3 to 8 days. Each mutant construct was compared to wildtype RPB9 with respect to ability to restore growth characteristics.

2.2.6 Primer Extension Assay

Primer extension assays were performed to measure transcription start site usage in the various yeast strains. The *ADHI* gene was used as the indicator gene as ADHI expression differs significantly between wildtype and $\Delta 9$ yeast strains with respect to start site usage (Hull *et al*, 1995). Yeast strains YF2221, YF2222, YF2230 and YF2234 were grown in YP liquid medium with 2% glucose. YF2230 transformed with each of the pRS314RPB9BE constructs was grown in synthetic complete liquid medium lacking tryptophan. All cultures were grown at 30°C to an optical density at 600 nm between 0.2 and 1.0. For each of the yeast strains to be analyzed, 5×10^7 cells were harvested and total RNA was isolated using the RNeasy protocol (Qiagen). The primer used for these experiments, 5'-AGAAGATAACACCTTTTGGAG-3' (Dalton Chemicals), anneals to nucleotides 37 to 17 in the *ADHI* gene. The primer was radiolabeled at the 5' end by phosphorylating with polynucleotide kinase (New England Biolabs) and γ -³²P-ATP. For each primer extension reaction, 15 μ g of total RNA from the appropriate yeast strain was annealed with 0.4 picomoles of the 5' radiolabeled primer for 45 minutes at 52°C. Reverse transcription from the annealed primer was done with MMLV reverse transcriptase (Gibco BRL) according to manufacturer's instructions. The reverse transcripts were collected by ethanol precipitation and run on a 6% polyacrylamide, 8.3 M urea, TBE gel at 2000 volts for 2 to 3 hours. The reverse transcripts were visualized by exposing the gels to autoradiographic film and phosphor screens.

CHAPTER 3 RESULTS

3.1 Rationale for the Design of RPB9 Mutants

3.1.1 Rationale

The goal of this study is to assign the known functions of the RNA polymerase II subunit RPB9 to particular regions, and if possible, to particular residues of the subunit. RPB9 contributes significantly to RNA polymerase II function at the initiation and elongation phases of transcription. Deletion of RPB9 results in altered start site selection during transcription initiation both *in vivo* and *in vitro* (Furter-Graves *et al*, 1994); (Hull *et al*, 1995); (Sun *et al*, 1996). In addition, the RPB9-deficient polymerase is dysfunctional in particular assays that measure pause-site recognition and response to transcription factor TFIIS *in vitro* (Awrey *et al*, 1997). Although RPB9 is not essential in yeast, loss of RPB9 function results in temperature- and cold-sensitive phenotypes (Woychik *et al*, 1991); (Sun *et al*, 1996). Our aim was to design a set of mutants that would allow for the assignment of specific functions to the various regions of RPB9.

We wished to put the functions of RPB9 into the context of the RPB9 structure. At the start of the study the structure of RPB9 was not known. However, sequence analysis revealed that RPB9 is composed of three distinct regions (Figure 1). RPB9 contains two zinc-binding regions as characterized by the $-CX_2CX_nCX_2C-$ motif, located at either end of the protein (termed Zn1 and Zn2, respectively). Connecting the two zinc-

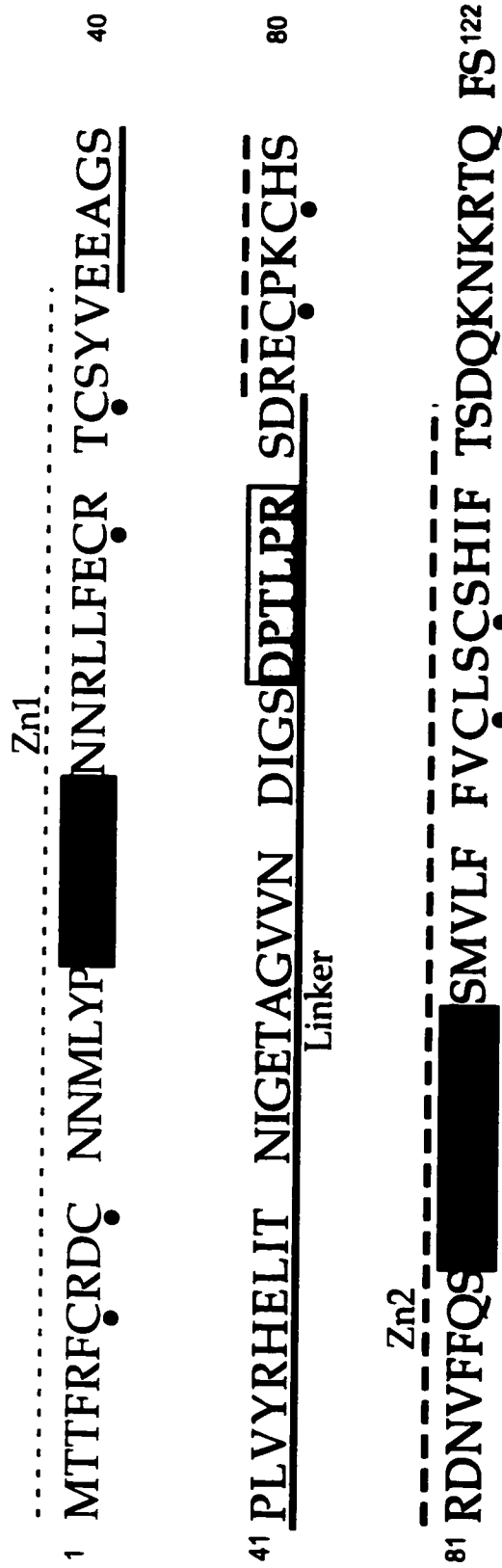


Figure 1. Schematic Representation of the Sequence of RPB9 from *S. cerevisiae*. The amino acid sequence of yeast RPB9 is shown. The zinc ribbon regions, Zn1 and Zn2, are indicated with a light or dark hatched line, respectively. In both ribbon regions the conserved cysteines are indicated with a solid dot, and the residues analogous to the charged loop in the TFHS zinc ribbon structure are contained within the darkly shaded boxes. The linker region that connects the two ribbon regions is underlined, and the conserved stretch of amino acids in this region are within the lightly shaded box.

binding domains is a conserved stretch of six amino acids that is highly conserved among eukaryotic RPB9 homologues (termed the linker region) (Kaine *et al*, 1994); (Hull *et al*, 1995). We surmised that each of the known functions of RPB9 could be ascribed to one or more of these regions of RPB9. This hypothesis formed the basis of the mutagenesis study.

We set out to isolate each of the functions of RPB9 by individually targeting each of the defined regions for mutagenesis. In particular, we wished to mutate amino acids thought to play a functional, rather than a structural role. Since we did not have direct structural information, we had to rely on sequence similarities among RPB9 homologues as well as between the RPB9 zinc-binding regions and other zinc-chelating domains. The structural homology between Zn1, Zn2, and domains from other proteins that contain the same zinc-chelating motif allowed us to define which residues were likely not to be involved in maintaining the structure of these domains. The assignment of structural residues within the linker region was more difficult, and was more dependent on homology among RPB9 homologues.

The linker region connecting the two zinc-binding domains had no obvious domain structure. However, as mentioned above, a stretch of six amino acids with the sequence DPTLPR is conserved among eukaryotic homologues. This region is also flanked by conserved charged residues. Although it was not apparent as to whether these residues are important in a structural or functional sense, their conservation implies that they are critical residues.

As well as the homology shared between RPB9 proteins, the Zn2 region of RPB9 has considerable homology (30% identity) with the zinc ribbon domain of TFIIS (Figure 2). The zinc-binding motif $-CX_2CX_nCX_2C-$ has been shown to form a zinc ribbon fold in other transcription-related proteins (Qian *et al*, 1993a); (Zhu *et al*, 1996). Thus, it seemed extremely likely that the C-terminal zinc-binding region of RPB9 forms a zinc ribbon fold similar to that of TFIIS. The arrangement of hydrophobic and charged residues in the N-terminal zinc-binding domain of RPB9 is similar to that of TFIIS and the C-terminal domain of RPB9. Due to the prevalence of this motif in transcription-related proteins, and its propensity to form the zinc ribbon conformation, we postulated that this zinc-binding N-terminal domain of RPB9 also forms a zinc ribbon fold. Mutants were therefore designed on the assumption that the two zinc-binding regions of RPB9 adopted the zinc ribbon structure.

The TFIIS zinc ribbon domain forms a three-stranded antiparallel β -sheet with the conserved cysteines coordinating a Zn^{+2} ion. The first and second strands of the sheet are connected by an extended flexible loop (Figure 3). Charged residues within this loop have been shown to be essential for TFIIS activity (Jeon *et al*, 1994), and are conserved in RPB9. A recent NMR structure of the C-terminal domain of RPB9 from *Thermococcus celer* demonstrates that the Zn2 region of RPB9 does indeed form a zinc ribbon fold with an extended loop similar to that of TFIIS (Wang *et al*, 1998).

3.1.2 Design of RPB9 Mutants

We set out to allocate specific functions to the three defined regions of RPB9 using mutagenesis. We designed mutations that would independently target each of the

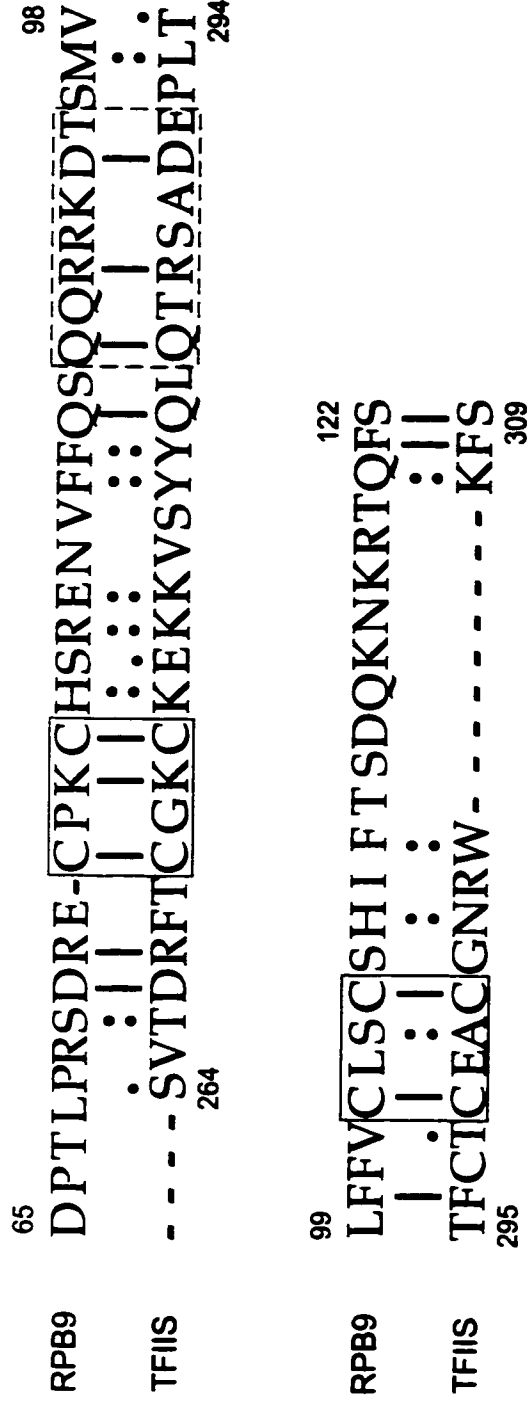


Figure 2. Homology of the Zn2 Region of RPB9 to the Zinc Ribbon Region of TFIIS. The amino acid sequence from the Zn2 region of RPB9 from *S. cerevisiae* is aligned with the sequence of the homologous zinc ribbon region of TFIIS from *S. cerevisiae*. Fourteen of the forty-six residues from the TFIIS sequence are identical in the RPB9 sequence. The two sets of paired cysteine residues that define the zinc chelating domain are enclosed within the solid boxes. The residues that correspond to the charged loop of the TFIIS zinc ribbon are contained within the dashed box. A perpendicular line indicates an identical residue, two dots indicate a highly conserved amino acid substitution, and a single dot indicates a moderately conserved amino acid substitution.

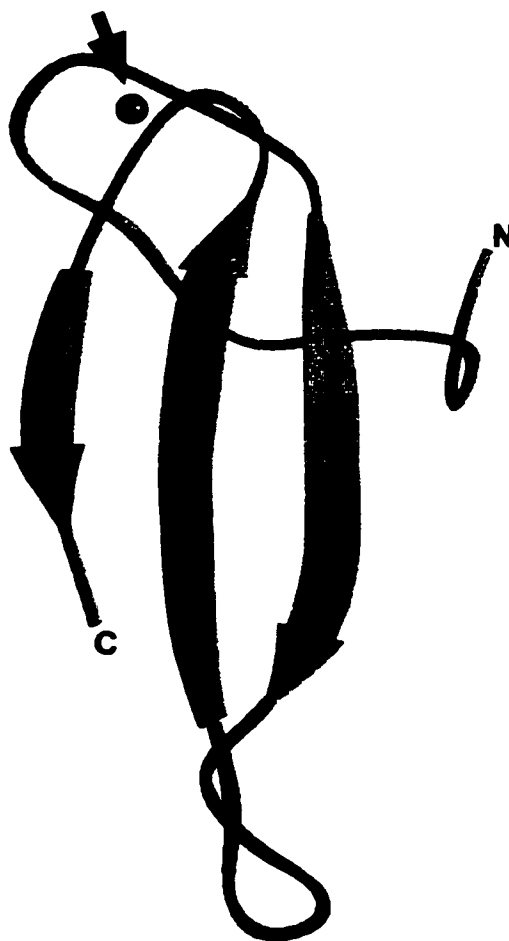


Figure 3. Model of Zn Ribbon Domain from the Transcription Factor TFIIIS. A ribbon diagram depicting the Zn ribbon domain from human TFIIIS was generated from the PDB coordinates using the program Ribbons. The domain forms a three-stranded β -sheet that coordinates a Zn^{+2} ion. It contains a flexible loop with charged residues that are required for TFIIIS function. The three strands are labeled ($\beta 1$, $\beta 2$, $\beta 3$), as are the N- and C- termini of the domain. The Zn^{+2} ion is indicated with the arrow, and the flexible loop located between the first and second strands is highlighted in black.

three regions. The mutations included truncations, deletions, and amino acid substitutions (refer to Table 1).

The first question that we wished to address was whether the two Zn binding motifs play independent roles in the function of the subunit. Therefore, we designed two truncation mutants such that each encompassed one of the zinc-binding regions and part of the linker region.

We also sought to disrupt the function of each region while maintaining the overall architecture of the protein. To this end, we constructed six deletion mutants, two in each of the three regions of the subunit. Large deletions comprising either most of the region between the pairs of cysteines in Zn1 or Zn2, or the majority of the linker region, were made. With the deletions in the Zn regions, the intent was to keep the zinc coordination centers intact but to remove the remainder of the domain. With the large deletion in the linker region, the two Zn binding regions were left unaltered but most of the connector region was eliminated. These were fairly extensive deletions, so we designed smaller deletions that would be potentially less disruptive. These smaller deletions removed only the putative flexible loop regions in Zn1 or Zn2, or the conserved stretch in the linker region (Figure 1). In most cases, the deleted amino acids were replaced with either three or four Ala residues in order to prevent disruption of the overall domain structure.

Finally, we wished to ascribe functions to individual residues within each of the three regions. Thus the remaining mutants possess single or double amino acid changes involving conserved residues from each of the domains. In particular, the charged

Table 1. List of Truncation, Deletion and Point Mutants within the Three Regions of RPB9

Region of RPB9	Mutation
Zn1	1-47
	Δ 12-27 [*]
	Δ 16-23 [†]
	R5A, F6A
	R8A, D9A
	R17A
	E18A
	D19A
	K20A
	E21A
	R30A
	Y34A
	Linker
Δ 65-70 [†]	
E54A	
D61A	
D65A	
R70A	
D72A	
Zn2	55-112
	K77A
	Δ 80-101 [*]
	Δ 89-95 [†]
	R91A
	R92A
	K93A
D94A	

^{*}The deleted region was replaced with three Ala residues.

[†]The deleted region was replaced with four Ala residues.

residues in the two putative loops and the conserved linker sequence were targeted. All amino acid changes were substituted with alanine.

The set of RPB9 mutants described above were subcloned, expressed and purified for *in vitro* and *in vivo* analyses designed to characterize the different functions of RPB9. The following sections contain descriptions of those assays and the results obtained from them.

3.2 Protein Expression and Purification

3.2.1 Purification of RNA Polymerase II $\Delta 9$

In order to perform *in vitro* analyses with the various RPB9 mutant proteins, we required a source of the RPB9-deficient RNA polymerase II. The RNA polymerase II $\Delta 9$ was purified from the RPB9-deficient *S. cerevisiae* strain, WY9, as described in the Methods section, using immunoaffinity chromatography techniques (Awrey *et al.*, 1997). The resulting enzyme preparation yielded a purified pol II that contained all of the subunits with the exception of RPB9 (Figure 4). When compared to wildtype pol II, the pol II $\Delta 9$ enzyme had a comparable maximal rate of elongation and similar temperature sensitivity in non-specific elongation assays (Rod Weilbaecher, personal communication). However, the pol II $\Delta 9$ enzyme demonstrated a two- to three-fold higher specific activity than the wildtype enzyme in promoter-independent assays. Pol II $\Delta 9$ was also less efficient at recognizing pause sites within a template, and was unable to respond to stimulation by TFIIS (Awrey *et al.*, 1997). This difference between the wildtype and $\Delta 9$ enzymes in responding to TFIIS was utilized in two of the *in vitro*

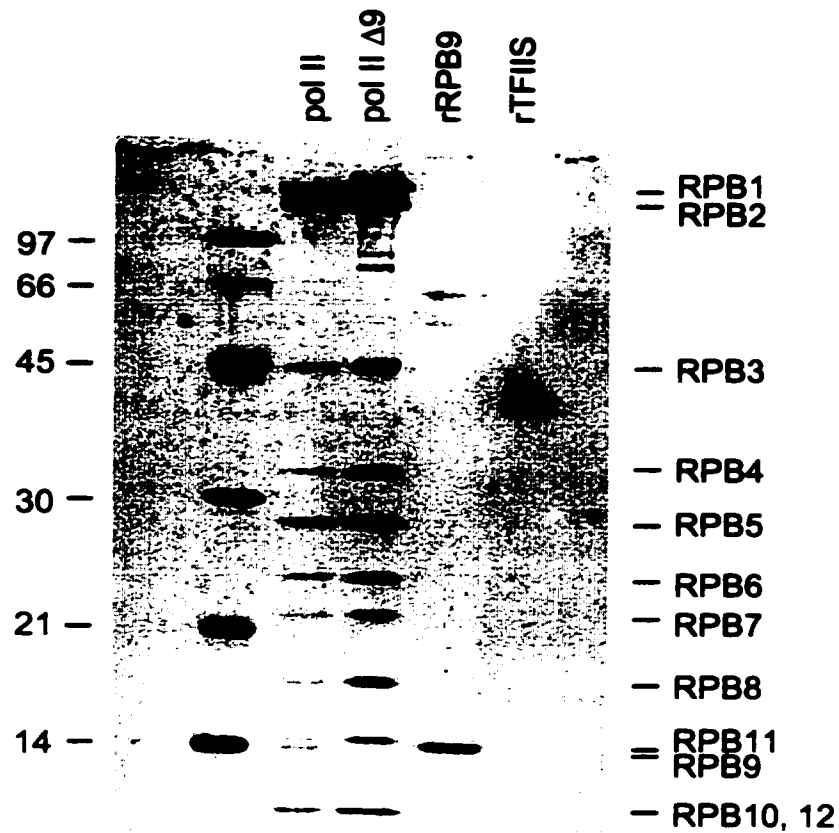


Figure 4. Purification of RNA Polymerase II $\Delta 9$. A Coomassie stained SDS-PAGE gel is displayed that contains purified wildtype (pol II) and RPB9-deficient (pol II $\Delta 9$) RNA polymerase II isolated from *S. cerevisiae*, recombinant yeast RPB9 (rRBP9), and recombinant yeast TFIIS (rTFIIS) isolated from *E. coli*. The content of each lane is indicated, with molecular weight standards in the leftmost lane. Apparent molecular weights of the standards are given (in kilodaltons). The position of each of the twelve pol II subunits is indicated on the right. Details of purification are provided in the Materials and Methods section.

assays used to characterize the RPB9 mutants. These assays are described in following sections.

3.2.2 Expression of RPB9 Mutants for in vitro Analyses

In order to analyze the function of RPB9 *in vitro*, we developed a system to express and purify the yeast RNA polymerase subunit. Our initial attempts to purify recombinant histidine-tagged RPB9 from *Escherichia coli* resulted in the production of highly aggregated protein (Awrey *et al*, 1997). Therefore, to maintain the solubility of the subunit, RPB9 constructs were purified as GST fusion proteins. This approach allowed for the straightforward and rapid purification of highly soluble GST-RPB9 proteins. Results from dynamic light scattering experiments indicated that the fusion proteins were mono-dispersed dimers at a concentration of 3 milligrams per millilitre (data not shown).

Cells expressing the various GST-RPB9 fusion proteins were lysed and clarified with a high-speed spin (refer to Methods section). Clarified lysates were passed over a DE52 column, and then loaded directly onto a glutathione-sepharose column. The GST fusion proteins were eluted with glutathione. The hinge region that connects GST and RPB9 in the fusion proteins proved to be very susceptible to digestion by endogenous proteases. Therefore, the eluates often contained a combination of both GST-RPB9 fusion and GST alone (Figure 5). In the final eluate, the GST-RPB9 fusion and GST together contributed 90-95% of the total protein. 5 to 35% of the GST constructs contained in the final fraction were GST alone. The free RPB9 liberated by the proteolysis was washed off the glutathione-sepharose column, and was not contained in

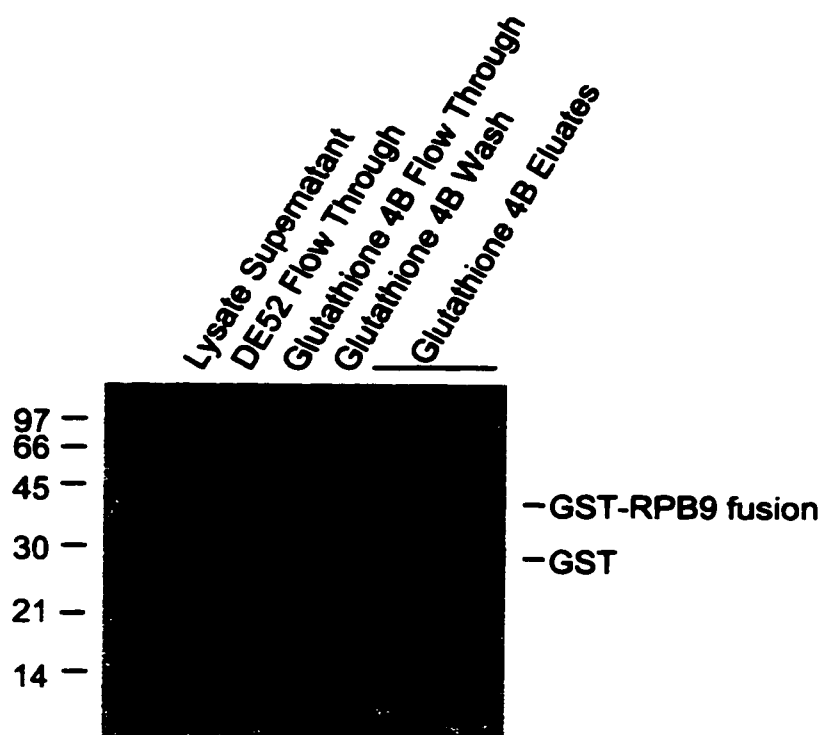


Figure 5. Purification of GST-RPB9 Fusion Proteins. A Coomassie stained SDS-PAGE gel is displayed that shows protein fractions from each step of a typical purification of a recombinant GST-RPB9 fusion protein. The content of each lane is indicated, with molecular weight standards in the leftmost lane. Apparent molecular weights of the standards are given (in kilodaltons). The position of the GST-RPB9 fusion protein is indicated, as well as that for GST alone. Details are provided in the Materials and Methods section. (Glutathione 4B = Glutathione 4B Sepharose).

the final protein fraction. Typical yields were between 1 and 5 milligrams of purified fusion protein per litre of culture.

Initially, fusion proteins were subjected to proteolytic digestion to liberate the RPB9 constructs. Removal of the GST domain caused the RPB9 proteins to aggregate. Thus, to maintain the RPB9 constructs as soluble protein in subsequent experiments, the GST-RPB9 fusion proteins were not cleaved. In this work, all *in vitro* assays with the various RPB9 constructs were performed with GST-RPB9 fusion proteins.

3.2.2 Preparation of Yeast Strains Containing the Various RPB9 Alleles

To perform the *in vivo* analysis on the various forms of RPB9, each of the mutant alleles were subcloned into a low-copy yeast expression plasmid under control of the *RPB9* promoter. The RPB9-deficient yeast strain YF2230 was transformed with each of the *rpb9* alleles and grown on selective complete medium to maintain the expression plasmid. The plasmids with the *rpb9* alleles were also transformed into the TFIIS knockout strain YF2222, and the RPB9/TFIIS double knockout strain YF2234. The transformed yeast strains were used in assays measuring growth phenotype complementation and transcription initiation start site selection. The assays and results are described in later sections.

3.3 Reconstitution of RPB9 with RNA Polymerase II $\Delta 9$

3.3.1 Development of a Gel Mobility Shift Assay between GST-RPB9 and Pol II $\Delta 9$

Addition of GST-RPB9 to *in vitro* elongation assays corrects the inability of pol II $\Delta 9$ to respond to TFIIS (Awrey *et al*, 1997). Also, GST-RPB9 restores correct initiation

start site preferences when added to nuclear extracts prepared from RPB9 null yeast strains (Hull *et al*, 1995). The mechanism of RPB9 action is not known. RPB9 may bind to pol II $\Delta 9$ to form an intact, fully functional enzyme. Alternatively, RPB9 may interact with pol II $\Delta 9$ in a transient manner, temporarily restoring function to transcription complexes. We wished to determine whether GST-RPB9 assembles with the polymerase or acts in a transient fashion. We developed a gel mobility shift assay to measure the reconstitution of the GST-RPB9 constructs with pol II $\Delta 9$. The GST fusion constructs contain a heart myosine kinase (HMK) recognition sequence, allowing for ^{32}P -radiolabeling of the GST-RPB9 fusion proteins. A gel mobility shift assay using purified pol II $\Delta 9$ and radiolabeled GST-RPB9 fusion proteins demonstrated that GST-RPB9 was assembling with pol II $\Delta 9$ to form an intact enzyme.

A titration of pol II $\Delta 9$ with radiolabeled wildtype GST-RPB9 demonstrated that the two proteins form a stable complex (Figure 6). The interaction was found to be saturatable and stoichiometric. 1 pmol of GST-RPB9 was incubated with increasing amounts of pol II $\Delta 9$ and the reactions were resolved by non-denaturing gel electrophoresis. The free GST-RPB9 migrated as a diffuse band toward the anode. As the concentration of pol II $\Delta 9$ increased, two complexes appeared (Figure 6A). The signals from the upper and lower complexes were combined to quantify the total amount of complexed GST-RPB9 (Figure 6B). The binding of GST-RPB9 to pol II $\Delta 9$ saturated at a molar ratio of approximately 1:1 for GST-RPB9: pol II $\Delta 9$. An equilibrium dissociation constant was calculated to be 22 nM.

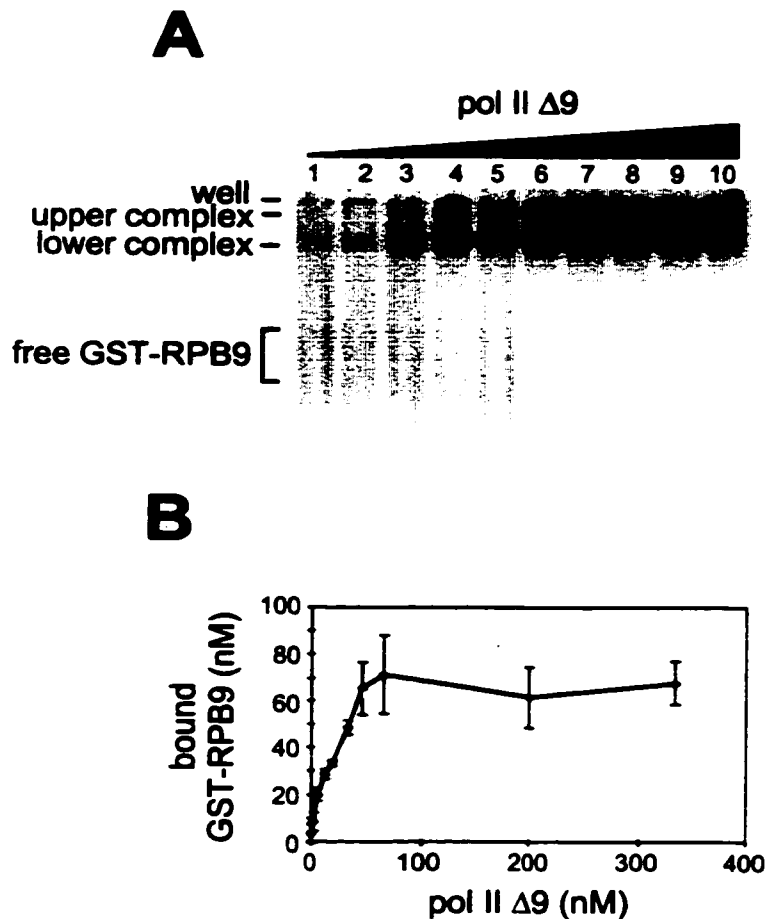


Figure 6. Binding Assay with GST-RPB9 and RNA Polymerase II $\Delta 9$. A. An autoradiogram of a gel mobility shift assay between radiolabeled GST-RPB9 and increasing amounts of RNA polymerase II $\Delta 9$ (pol II $\Delta 9$) is shown. The upper and lower complexes are indicated, as are the positions of the well and unbound GST-RPB9. All lanes contain 1.0 pmol of GST-RPB9. Lanes 1 through 10 contain the following amounts of polymerase: 0, 0.03, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 3.0, and 5.0 pmol, respectively. Details of the assay are given in the Material and Methods section. B. The total amount of bound GST-RPB9 in the combined upper and lower complexes for each concentration of polymerase was plotted from data quantified using the phosphorimager. The error bars represent the standard deviation calculated from the averaging of three experiments.

We had expected to see a single complex form between GST-RPB9 and pol II $\Delta 9$, which would contain a single GST-RPB9 molecule and a single pol II $\Delta 9$ molecule. However, two complexes were observed in the gel mobility shift assay. We used a combination of western blotting directed against RPB1 and autoradiography of a gel mobility shift assay to identify the components of the upper and lower complexes (Figure 7). Both complexes contain pol II $\Delta 9$ (Figure 7A) and radiolabeled GST-RPB9 (Figure 7B), and are distinct from either free pol II $\Delta 9$ or free GST-RPB9. However, the exact nature of the two complexes is not known. GST-RPB9 is a dimer in solution (Dirr *et al*, 1994) and RNA polymerase is also known to dimerize in solution at low salt concentrations (Darst *et al*, 1991). The lower complex is shifted only slightly above the free pol II $\Delta 9$; it is likely that it contains a pol II $\Delta 9$ molecule bound to a single GST-RPB9 dimer. The upper complex may contain a dimer of polymerase with one or both polymerase molecules binding to a GST-RPB9 dimer. It is also possible that it may contain two polymerase molecules that are bound to the two RPB9 molecules within the same GST-RPB9 dimer. Our results do not differentiate between these two possibilities, they merely confirm that both GST-RPB9 and pol II $\Delta 9$ are components of both complexes.

The results of a competition assay using unlabeled GST-RPB9 (Figure 8) determined that the binding interaction was reversible. Increasing amounts of unlabeled fusion protein were included in binding reactions containing constant amounts of radiolabeled GST-RPB9 and pol II $\Delta 9$. At higher levels of cold GST-RPB9, the amount

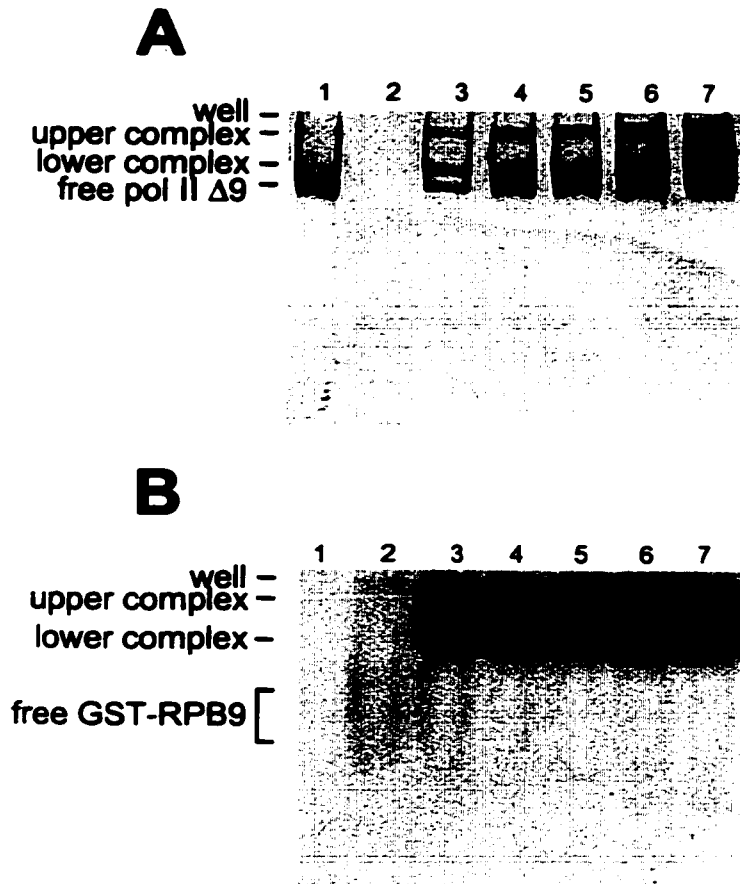


Figure 7. Identification of Components of Upper and Lower Complexes in Binding Assay. A gel mobility shift assay was performed between radiolabeled GST-RPB9 and pol II $\Delta 9$, and then transferred to a nitrocellulose membrane. The membrane was probed with an antibody directed against the largest subunit of pol II $\Delta 9$. The Western (shown in A) was developed with alkaline phosphatase conjugated secondary antibody. After development of the Western, the blot was exposed to film (shown in B). Lane 1 contains 0.5 pmol pol II $\Delta 9$; lanes 2 through 7 contain 1 pmol radiolabeled GST-RPB9 and 0, 0.2, 0.3, 0.5, 0.7, and 1.0 pmol of pol II $\Delta 9$, respectively.

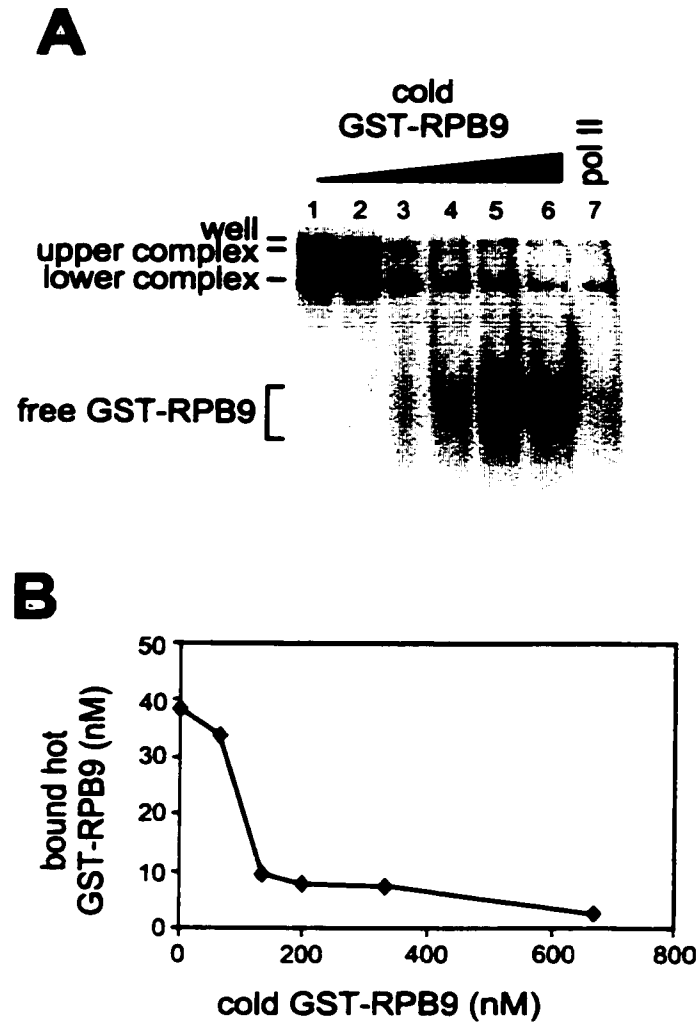


Figure 8. Competition for Binding to RNA Polymerase II $\Delta 9$ with Unlabelled GST-RPB9. A. A gel mobility shift assay was performed with 1.0 pmol of radiolabeled GST-RPB9 and 0.5 pmol of pol II $\Delta 9$. Increasing amounts of unlabelled (cold) GST-RPB9 were added in lanes 1 through 6: 0, 1.0, 2.0, 3.0, 5.0, and 10.0 pmol, respectively. Lane 7 contains 1.0 pmol of radiolabeled GST-RPB9 and 1.0 pmol of wildtype RNA polymerase II. B. The amount of bound radiolabeled (hot) GST-RPB9 in the combined upper and lower complexes in each lane was quantified using a phosphorimager, and plotted against the amount of unlabelled GST-RPB9 used in the competition assay.

of radiolabeled GST-RPB9 in the shifted complexes was significantly reduced. These results demonstrate that the radiolabeled and unlabeled GST-RPB9 are competing for the same binding site on the polymerase, rather than binding in a nonspecific and irreversible manner.

To further investigate the reversibility of the interaction between RPB9 and RNA polymerase II, a binding reaction was performed using wildtype pol II. Radiolabeled GST-RPB9 was incubated with an equimolar amount of wildtype pol II that contains endogenous RPB9. Binding of GST-RPB9 to wildtype pol II was observed, although complete exchange of endogenous RPB9 and radiolabeled GST-RPB9 did not occur (compare lanes 1 and 7 in Figure 8A). This result indicates that there is exchange between the endogenous RPB9 and the GST-RPB9 added to the reaction. As well, the incomplete binding of GST-RPB9 indicates that GST-RPB9 is likely replacing endogenous RPB9 at a specific binding site, rather than binding indiscriminately to the surface of the polymerase.

3.3.2 Binding of RPB9 to RNA Polymerase II is not Mediated Through Zn1 or Zn2

The purpose of this study was to assign function to the various regions of RPB9. The binding experiments described above demonstrated that wildtype GST-RPB9 assembles with pol II $\Delta 9$ to form a stable complex. Therefore, we used this binding assay to identify regions of RPB9 that are involved in mediating the interaction with pol II $\Delta 9$. Each of the GST-RPB9 mutants was tested in the gel mobility shift assay. Binding results for all GST-RPB9 constructs were tabulated and estimates of equilibrium binding constants for fusion proteins that demonstrated binding were calculated (Table 3).

In order to determine if either of the zinc-binding domains could independently bind to pol II $\Delta 9$, we first tested the two truncated mutants, GST-RPB9₁₋₄₇ and GST-RPB9₅₅₋₁₁₂. No detectable binding to pol II $\Delta 9$ was observed for either mutant in this binding assay (Figure 9). This result indicates that neither zinc-binding region is able to bind independently to pol II $\Delta 9$.

The remaining mutants within the Zn binding regions were assayed to confirm that they would not influence binding of GST-RPB9 to pol II $\Delta 9$. Indeed, mutations within Zn1 and Zn2 were found not to affect binding, with three exceptions. Binding assays with the large and small deletion mutants from Zn1 (GST-RPB9 $_{\Delta 12-27}$ and GST-RPB9 $_{\Delta 16-23}$, respectively) demonstrated that these mutants are not capable of binding to pol II $\Delta 9$. Similarly, no binding was seen with the large deletion in Zn2 (GST-RPB9 $_{\Delta 80-101}$). It is probable that these mutations disrupt the global structure of the protein rather than specifically perturb the polymerase-binding interface. Binding to pol II $\Delta 9$ was observed for all of the substitution mutants from the Zn1 region (Figure 10). Likewise, the remaining mutants from the Zn2 region were all able to reassemble with pol II $\Delta 9$, including the small deletion mutant, GST-RPB9 $_{\Delta 89-95}$. Therefore, mutations in either Zn1 or Zn2 did not disrupt binding of GST-RPB9 to pol II $\Delta 9$, with the exception of three deletion mutants. These proteins appear to have been significantly affected in terms of overall activity, as will be described later.

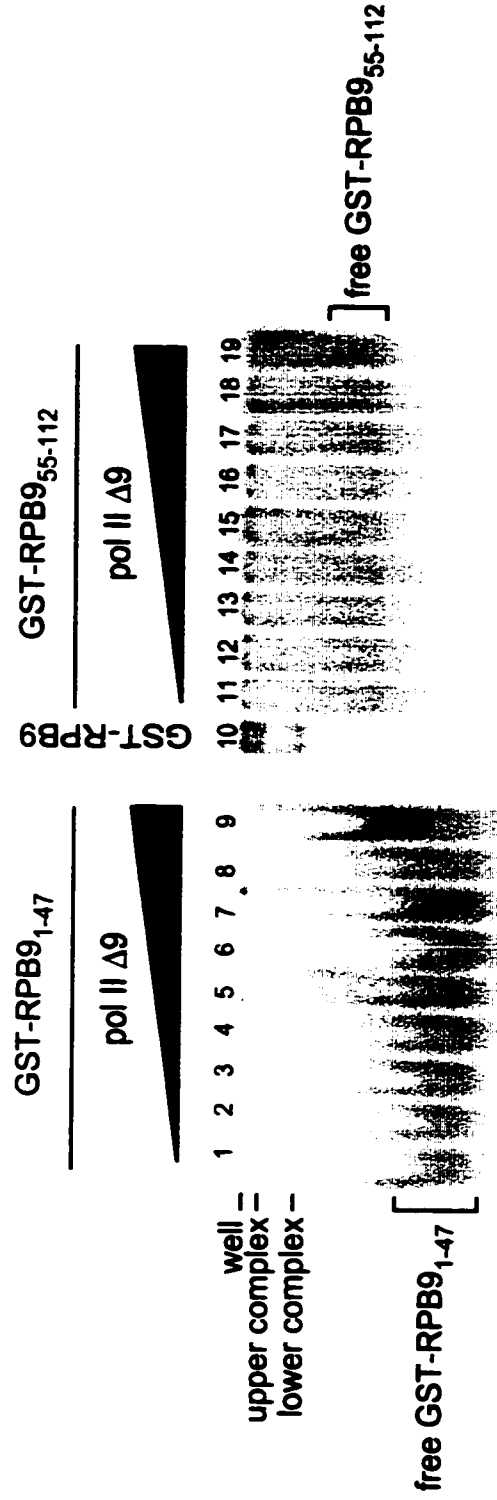


Figure 9. Binding Assay with GST-RPB9 Truncation Mutants. Gel mobility shift assays using GST-RPB9 truncation mutants are shown. All lanes contain 1 pmol of the relevant radiolabeled GST-RPB9 fusion protein. Lanes 1 to 9 and 11 to 19 contain 0, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 3.0, or 5.0 pmol of pol II Δ9, respectively. Lane 10 contains 0.5 pmol of pol II Δ9.

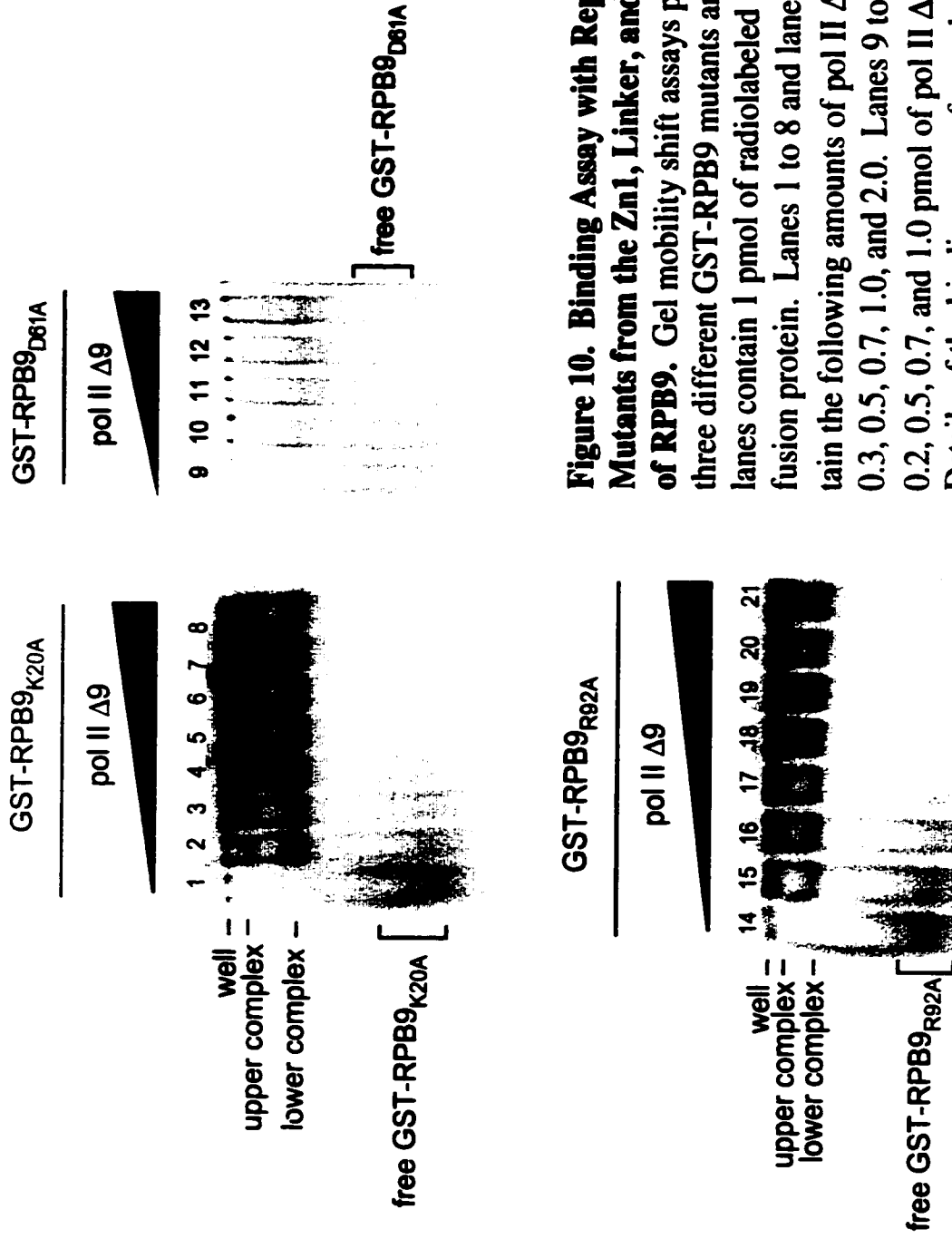


Figure 10. Binding Assay with Representative Mutants from the Zn1, Linker, and Zn2 Regions of RPB9. Gel mobility shift assays performed with three different GST-RPB9 mutants are shown. All lanes contain 1 pmol of radiolabeled GST-RPB9 fusion protein. Lanes 1 to 8 and lanes 14 to 21 contain the following amounts of pol II Δ9: 0.0.1, 0.2, 0.3, 0.5, 0.7, 1.0, and 2.0. Lanes 9 to 13 contain 0, 0.2, 0.5, 0.7, and 1.0 pmol of pol II Δ9, respectively. Details of the binding assay for each specific GST-RPB9 mutant are summarized in Table 2.

3.3.3 Conserved Charges in the Linker Region Mediate Binding of RPB9 to RNA Polymerase II

We have identified specific residues within the linker region that are essential for the interaction of GST-RPB9 with pol II $\Delta 9$. The charged residues in the conserved DPTLPR motif appear to be critical for binding of the subunit to the polymerase. Neither of the deletion mutants, GST-RPB9 $_{\Delta 36-70}$ and GST-RPB9 $_{\Delta 65-70}$ exhibited binding to pol II $\Delta 9$. In addition, the single amino acid substitution mutants, GST-RPB9 $_{D61A}$, GST-RPB9 $_{D65A}$, and GST-RPB9 $_{R70A}$ were unable to assemble with the polymerase (Figure 10). These constructs contain mutations of conserved charges either immediately N-terminal to, or within, the conserved stretch of amino acids in the linker region. The other mutants from the linker region, GST-RPB9 $_{E54A}$ and GST-RPB9 $_{D72A}$, did bind to pol II $\Delta 9$ in this reconstitution assay. Thus, it appears that certain conserved charges within the linker, especially those within the conserved stretch of residues 65 to 70, are required for binding of GST-RPB9 to pol II $\Delta 9$ in this assay.

3.4 Response to TFIIS-Induced Stimulation of Pause Site Readthrough

3.4.1 GST-RPB9 Restores Pol II $\Delta 9$ Response to TFIIS in Readthrough Assay

Attenuating sequences within the DNA template cause elongating ternary complexes to pause or arrest. The transcription factor TFIIS is able to stimulate RNA polymerase II to continue transcribing through such sequences while maintaining the correct register between template and transcript. The human H3.3 template, which

contains characterized pause sites, has been employed in elongation assays to measure the ability of RNA polymerase II to respond to TFIIS.

We performed an *in vitro* readthrough assay that uses a 3' tailed template to measure the ability of RNA polymerase II to respond to stimulation by TFIIS (Christie *et al.*, 1994). This template consisted of a portion of the human histone H3.3 template, which contains the intrinsic pause sites T1a, T1b, and TII. The non-coding strand of the template had a 3' single-stranded extension added to enable the polymerase to initiate transcription without the requirement for the general transcription factors. A limiting amount of radiolabeled CTP was added to pulse-label the 5' end of the transcript (refer to Methods). The elongating polymerase was allowed to transcribe either to one of the attenuating sequences or to the end of the template. The resulting transcripts were resolved and visualized by denaturing gel electrophoresis followed by phosphorimage analysis. The degree of pausing by the polymerase was expressed as the percentage of transcripts arrested at the T1a site out of the total transcripts to reach either the T1a site or the end of the template.

We had previously tested the RPB9-deficient polymerase in the readthrough assay (Awrey *et al.*, 1997). Pol II $\Delta 9$ that had arrested at the T1a site was not stimulated to transcribe through pause sites by TFIIS. That is, the same proportion of $\Delta 9$ polymerase molecules remained stalled at the T1a site in the presence or absence of a five-fold molar excess of TFIIS (Figure 11). In contrast, the wildtype pol II significantly responded to the addition of TFIIS. The percentage of T1a truncated transcripts changed from 48% in the absence of TFIIS to 17% upon the addition of TFIIS. Addition of a five-fold molar

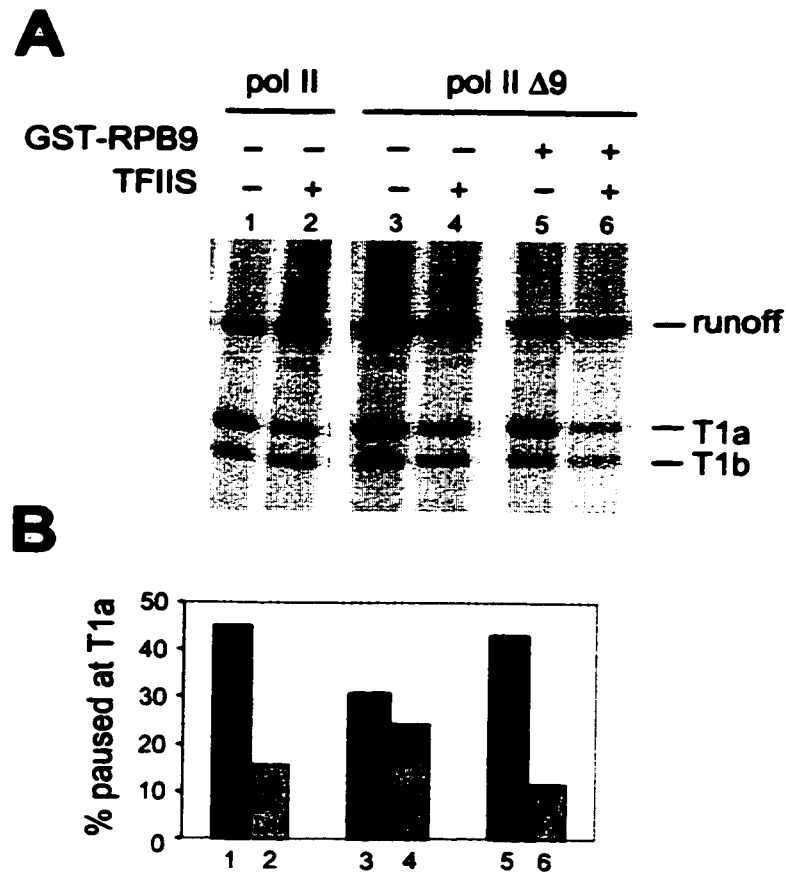


Figure 11. Readthrough Assay with Wildtype and $\Delta 9$ RNA Polymerase II. Readthrough assays were performed on the histone H3.3 template with either pol II or pol II $\Delta 9$. Assays using pol II $\Delta 9$ were performed either with or without a five-fold molar excess of GST-RPB9. Reactions were incubated for 10 minutes post-chase either in the presence or absence of the transcription factor TFIIS. Details of the reaction are given in the Materials and Methods section. Lanes 1 through 6 in part A correspond to bars 1 through 6 in part B. A. An autoradiogram of transcripts separated on a denaturing gel is displayed. The position of the runoff transcript is indicated, as are those for the transcripts paused at the T1a and T1b pause sites. B. This graph represents the percentage of transcripts that remain paused at the T1a pause site. The percentage is calculated from all transcripts that reach either T1a or the end of the template. Transcript levels were quantified on a phosphorimager.

excess of GST-RPB9 to the assay completely restored the ability of pol II $\Delta 9$ to respond to TFIIS; the majority of the complexes paused at the T1a were able to transcribe through the pause site to produce full-length transcript.

3.4.1 Mutations in Zn1 did not Affect Readthrough Response to TFIIS

We used the readthrough assay to identify the region of RPB9 that is required to mediate the functional interaction between pol II and TFIIS. Each of the GST-RPB9 mutants was tested for the ability to restore polymerase response to stimulation by TFIIS. The mutants were assayed at a five, one hundred, and three hundred-fold molar excess over pol II $\Delta 9$ (Table 3). We found that the Zn1 and Zn2 regions were not able to effect this response as independent domains. The two truncation mutants, GST-RPB9₁₋₄₇ and GST-RPB9₅₅₋₁₁₂, were unable to restore pol II $\Delta 9$ response to TFIIS, even at a three hundred: one ratio over pol II $\Delta 9$. This is not surprising, since neither of these mutants assembled with pol II $\Delta 9$ in the binding assay.

The Zn1 region was found not to be involved in mediating the readthrough response to TFIIS, even in the context of the entire subunit. The majority of the mutations within the Zn1 region had no affect on the ability of GST-RPB9 to restore response to TFIIS in this assay, with the exception of the two deletions in this region (mutants GST-RPB9 _{Δ 12-27} and GST- RPB9 _{Δ 16-23}) which completely abolished activity. Again, it is assumed that these mutations significantly disrupted RPB9. All of the Zn1 substitution mutants were able to restore wildtype activity at a five-fold molar excess (Figure 12). Thus, residues within Zn1 are not responsible for mediating the response of pol II $\Delta 9$ to TFIIS.

3.4.3 Linker Region Mutants That Were Deficient for Binding can Restore Readthrough Response at High Concentrations

Although the linker region is important for binding of RPB9 to pol II $\Delta 9$, it does not appear to be directly involved in the stimulation of pol II in response to TFIIS. Mutations within this region affected restoration of readthrough response only to the extent that they affected binding. The mutations in this region that did not affect binding also did not affect the restoration of the readthrough response to TFIIS. GST-RPB9_{E54A} and GST-RPB9_{D72A} could bind to pol II $\Delta 9$; both mutants were able to restore response to TFIIS at a five-fold molar excess (Table 3). The single amino acid substitution mutants from this region that did not recombine with pol II $\Delta 9$ (GST-RPB9_{D61A}, GST-RPB9_{D65A}, and GST-RPB9_{R70A}) were not able to restore response to TFIIS at a 5:1 ratio of fusion protein: pol II $\Delta 9$ (Figure 12). However, these mutants restored activity at higher levels of fusion protein (at a 100:1 ratio). Thus, it seems that single amino acid changes that disrupted binding activity did not abolish the ability to restore readthrough activity. At high levels of fusion protein, which may allow interaction between the GST-RPB9 mutants and pol II $\Delta 9$, readthrough activity is intact. Neither of the two deletion mutants from the linker region could restore readthrough response to TFIIS, even at a 300:1 ratio of GST-RPB9 fusion: pol II $\Delta 9$.

3.4.4 The Putative Flexible Loop in Zn2 is Required for Readthrough Response

The Zn2 region of RPB9 has homology to the zinc ribbon domain of TFIIS (Figure 2). We found that charged residues within this domain that correspond to the

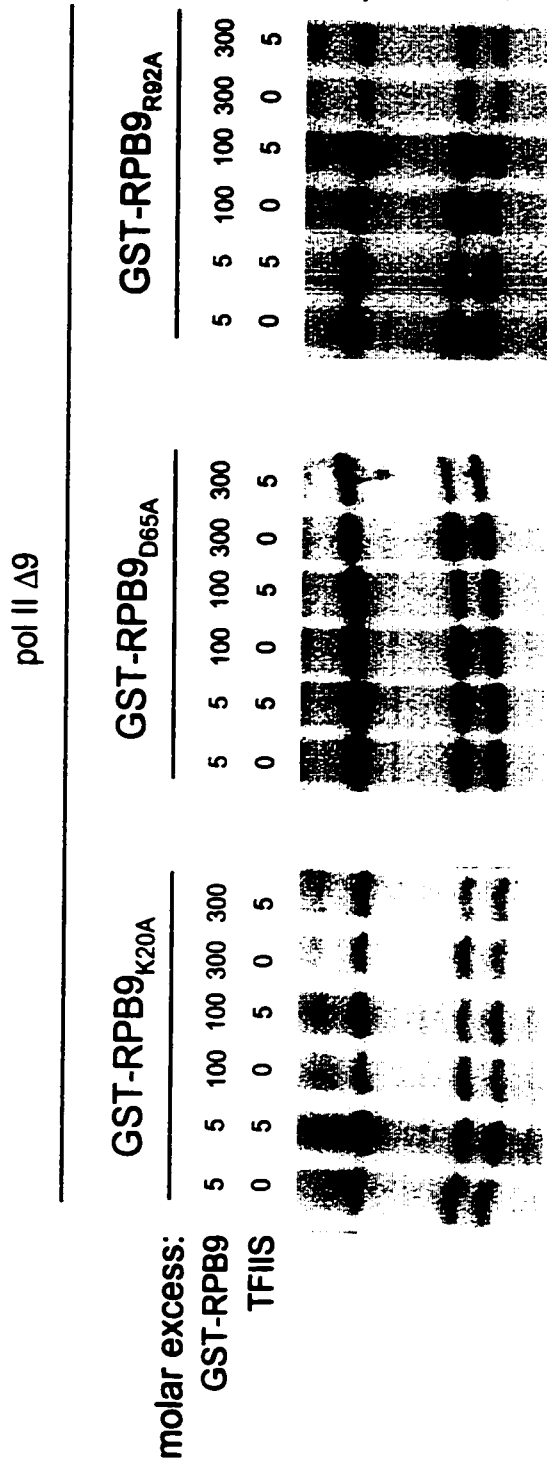


Figure 12. Readthrough Assay with $\Delta 9$ RNA Polymerase II and Representative Mutants from the Zn1, Linker, and Zn2 Regions of RPB9. Readthrough assays performed with pol II $\Delta 9$ and a mutant GST-RPB9 fusion protein from each of the three regions of RPB9 are shown. The assay was performed with a five-, hundred-, or three hundred-fold molar excess of fusion protein over polymerase, and was done in either the presence or absence of five-fold molar excess of TFIIS. Details for each mutant are summarized in Table 2.

flexible loop of TFIIS (Table 2) were required to mediate the stimulation of pol II in the readthrough assay.

Table 2. Sequence of Flexible Loop from Zinc Ribbon Domain

TFIIS	(285)	QTRSADE	(291)
RPB9	(89)	QQRKDT	(95)

Residues R91, R92, K93 and D94 of RPB9 were essential for restoration of the readthrough response to TFIIS (Table 3). GST-RPB9 mutants from the Zn2 region were able to reassemble with pol II $\Delta 9$ in the binding assay. However, the mutant with the deletion of the charged loop, GST-RPB9 $_{\Delta 89-95}$, and mutants with substitutions of the charged residues within the loop did not restore the ability of pol II $\Delta 9$ to respond to TFIIS, even at a 300:1 ratio over pol II $\Delta 9$ (Figure 12). These data suggest that the flexible loop of Zn2 within RPB9 is responsible for mediating the response of RNA polymerase II to TFIIS. Two of the three charged residues in the analogous loops are identical between yeast RPB9 and TFIIS. In fact, the analogous charged loop within TFIIS was found to be essential for TFIIS to stimulate readthrough by RNA polymerase II (Jeon *et al*, 1994); (Awrey *et al*, 1998). Thus, our results indicate that the analogous regions of RPB9 and TFIIS are acting on pol II to effect a stimulation of pause site readthrough.

3.5 Response to TFIIS-Induced Transcript Cleavage

3.5.1 GST-RPB9 Restores Pol II $\Delta 9$ Response to TFIIS in Transcript Cleavage Assay

TFIIS reactivates stalled RNA polymerase II that has arrested at a pause site. TFIIS achieves this effect by inducing the polymerase to cleave the nascent transcript at the 3' end prior to transcribing through a pause site (Reines, 1992). This process appears to be at least a two-step procedure, resulting in the release of two short RNA oligomers when performed in the absence of nucleotide substrates (Reines *et al*, 1992); (Awrey *et al*, 1997). Only the first cleavage event is required for resumption of transcription (Gu *et al*, 1993), but TFIIS mutants that can only stimulate the first cleavage event cannot activate polymerase readthrough activity (Awrey *et al*, 1998). The cleavage event occurs in a manner that allows the polymerase to keep the transcript within the correct frame with respect to the DNA template. The polymerase then continues elongating through the attenuating sequence. This effect of TFIIS on pol II can be measured in an assay similar to the previously described readthrough assay.

We employed a transcript cleavage assay that was based on the readthrough assay performed on the 3' extended H3.3 template (Christie *et al*, 1994). Paused ternary complexes were generated and pulse-labeled as for the readthrough assay, but then were purified away from free nucleotides prior to the addition of an excess of TFIIS to a portion of the reaction. After cleavage of the transcript, the ability of the polymerase to transcribe through the pause site was measured by the addition of nucleotides back to the reaction. The resulting transcripts generated in the presence and absence of TFIIS were compared by denaturing gel electrophoresis and phosphorimager analysis. Transcripts

truncated at T1a site were shortened after incubation with TFIIS to produce the C1 and C2 cleavage products (Awrey *et al*, 1997).

We found that pol II $\Delta 9$, which does not respond to TFIIS in the readthrough assay, was capable of cleaving back to produce the first cleavage product (C1) upon addition of TFIIS, but could not produce the second cleavage product (C2). Addition of a five-fold molar excess of GST-RPB9 to the reaction restored the ability of pol II $\Delta 9$ to produce both cleavage products (Figure 13) as well as the ability to produce full-length transcript upon the addition of nucleotides.

We tested each of the GST-RPB9 constructs for the ability to restore cleavage activity to the $\Delta 9$ polymerase. As pol II $\Delta 9$ can respond to TFIIS stimulation by producing C1 in the absence of GST-RPB9, we looked for the ability of the fusion proteins to stimulate pol II $\Delta 9$ to produce C2 in the presence of TFIIS. Thus, when referring to the ability of particular RPB9 mutants to restore cleavage activity, we are referring to the restoration of the second cleavage event.

3.5.2 Mutants in the Zn1 and Linker Regions Restore Transcript Cleavage at the Same Levels as for Readthrough

We found that Zn1 and the linker regions of RPB9 do not play a direct role in stimulating pol II to cleave the transcript in response to TFIIS. Moreover, the ability of GST-RPB9 to restore transcript cleavage activity in response to stimulation by TFIIS appears to be prerequisite to its ability to restore readthrough response. The results obtained from testing each of the GST-RPB9 mutants in the cleavage assay are similar to those obtained with the readthrough assay (Figure 14). For mutants from either the Zn1

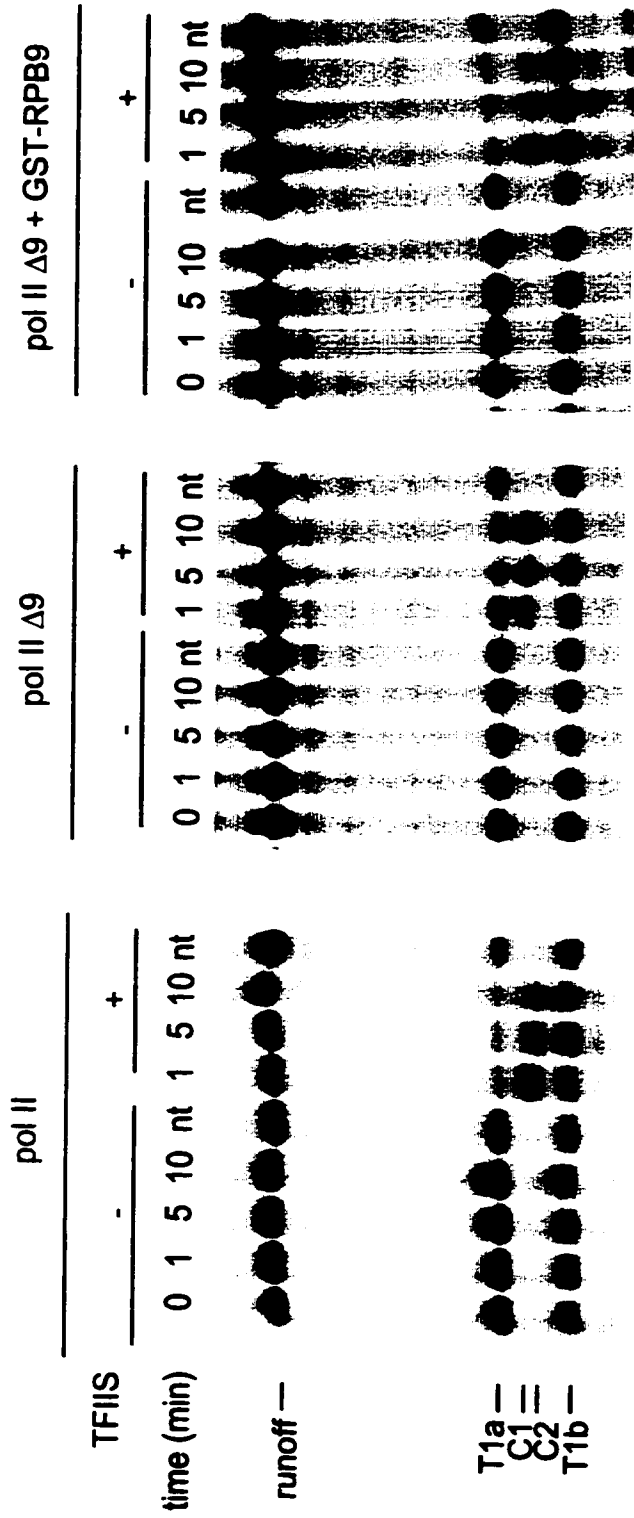


Figure 13. Transcript Cleavage Assay with Wildtype and $\Delta 9$ RNA Polymerase II. A paused ternary complex of pol II on the H3.3 template was generated as for the readthrough assay. Free nucleotides were then removed using a spin column. Complexes were subsequently incubated in the presence or absence of a five-fold molar excess of TFIIIS. Autoradiograms of reactions with pol II, pol II $\Delta 9$, or pol II $\Delta 9$ and a five-fold molar excess of GST-RPB9 are shown. Samples were taken at 0, 1, 5, or 10 minutes after the time of addition of TFIIIS, as indicated. After 10 minutes, nucleotides were added back to reactions, which were then incubated for an additional 10 minutes (lanes labeled nt). Details of the reactions are given in the Materials and Methods section. C1 indicates the position of the first cleavage product, and C2 indicates that for the second cleavage product.

region or the linker region, polymerase cleavage activity was restored at the same levels of fusion protein that restored polymerase readthrough activity (Table 3). Those mutants that could induce pol II $\Delta 9$ to readthrough at a five-fold molar excess also restored wildtype cleavage patterns at a 5:1 ratio of fusion: pol II $\Delta 9$. The binding-deficient mutants that required a 100:1 ratio of fusion: pol II $\Delta 9$ to restore readthrough activity restored cleavage also at a ratio of 100:1. The deletion mutants from these regions that were not able to restore readthrough activity could not induce pol II $\Delta 9$ to produce the second cleavage product. Thus, results for the cleavage assay directly correlate with results from the readthrough for mutants in either Zn1 or the linker region.

3.5.3 Mutants from Zn2 that could not Stimulate Readthrough can Induce Cleavage at High Concentrations

The ability to influence RNA polymerase II to respond to readthrough stimulation by TFIIS has been localized to the Zn2 region of RPB9. This activity appears to be dependent on the ability of RPB9 to stimulate the polymerase to cleave the transcript in response to TFIIS. Our results indicate that cleavage can occur in the absence of readthrough response (Table 3 and Figure 14). Mutations that abolish the ability of RPB9 to mediate stimulation of readthrough do not completely eliminate its ability to induce pol II to cleave the nascent transcript to produce C2 upon addition of TFIIS. Although no stimulation of readthrough was seen for mutants within the flexible loop of Zn2, even at high concentrations of fusion protein, cleavage activity was restored at some level for all mutants in Zn2. For the small deletion mutant and the substitution mutants from the Zn2 region of RPB9 that were unable to restore readthrough response to TFIIS,

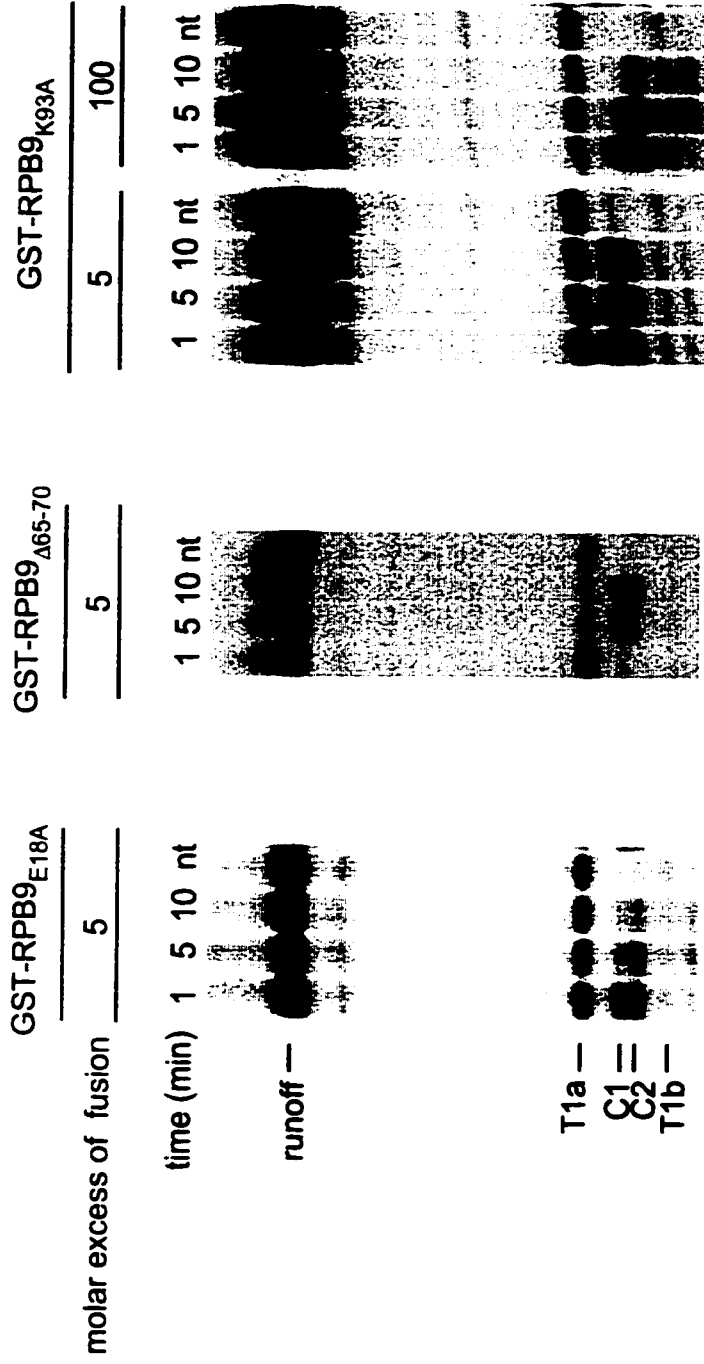


Figure 14. Transcript Cleavage Assay with $\Delta 9$ RNA Polymerase II and Representative Mutants from the Zn1, Linker, and Zn2 Regions of RPB9. Cleavage assays performed with pol II $\Delta 9$ and a mutant GST-RPB9 fusion protein from each of the three regions of RPB9 are shown. The assays were performed with a five-, or hundred-fold molar excess of fusion protein over polymerase, and were done in the presence of five-fold molar excess of TFIIIS. Samples were taken at 0, 1, 5, or 10 minutes after the time of addition of TFIIIS, as indicated. After 10 minutes, nucleotides were added back to reactions, which were then incubated for an additional 10 minutes (lanes labeled nt). Details for each mutant are summarized in Table 2.

Table 3. Summary of *in vitro* Analyses of Mutant GST-RPB9 Fusion Proteins

Fusion Protein	Binding^a	Readthrough	Cleavage
GST-RPB9	22	++	++
GST-RPB9 ₁₋₄₇	—	—	—
GST-RPB9 _{Δ12-27}	—	—	—
GST-RPB9 _{Δ16-23}	—	—	—
GST-RPB9 _{R5A, F6A}	38	++	++
GST-RPB9 _{R8A, D9A}	12	++	++
GST-RPB9 _{R17A}	33	++	++
GST-RPB9 _{E18A}	25	++	++
GST-RPB9 _{D19A}	18	++	++
GST-RPB9 _{K20A}	15	++	++
GST-RPB9 _{E21A}	10	++	++
GST-RPB9 _{R30A}	13	++	++
GST-RPB9 _{Y34A}	5	++	++
GST-RPB9 _{Δ36-70}	—	—	—
GST-RPB9 _{Δ65-70}	—	—	—
GST-RPB9 _{E54A}	15	++	++
GST-RPB9 _{D61A}	—	+	+
GST-RPB9 _{D65A}	—	+	+
GST-RPB9 _{R70A}	—	+	+
GST-RPB9 _{D72A}	10	++	++
GST-RPB9 ₅₅₋₁₁₂	—	—	—
GST-RPB9 _{K77A}	8	++	++
GST-RPB9 _{Δ80-101}	—	—	(+)
GST-RPB9 _{Δ89-95}	5	—	+
GST-RPB9 _{R91A}	26	—	+
GST-RPB9 _{R92A}	20	—	+
GST-RPB9 _{K93A}	105	—	+
GST-RPB9 _{D94A}	12	—	+

++ Wildtype pol II activity was restored at a 5:1 ratio of fusion: pol II Δ9.

+ Wildtype pol II activity was restored at a 100:1 ratio of fusion: pol II Δ9.

(+) Wildtype pol II activity was restored at a 300:1 ratio of fusion: pol II Δ9.

— Wildtype pol II activity was not restored, even at a 300:1 ratio of fusion: pol II Δ9.

^aAn estimate of the equilibrium dissociation constant is given in units of nM.

no restoration of cleavage activity was seen at a five-fold molar excess. However, restoration of cleavage ability in response to TFIIS was observed at a hundred-fold molar excess. The large deletion mutant from Zn2, GST-RPB9_{Δ80-101}, did not demonstrate binding to pol II Δ9, nor any ability to restore readthrough response to TFIIS. Yet pol II Δ9 was able to cleave the transcript to the second cleavage product at a 300:1 molar excess of this mutant.

These results imply that the readthrough response occurs via transcript cleavage to produce the second cleavage product. In this study, mutants that are able to induce a readthrough response are always associated with an ability to induce polymerase to cleave back to C2 at the same level of fusion protein. The reverse is not true. Stimulation of polymerase to cleave at high levels of fusion protein does not always correlate with a stimulation of readthrough ability even at higher levels of fusion protein than could induce cleavage. Similar results were seen in a mutational study of TFIIS (Awrey *et al*, 1998). Certain mutants within the Zn ribbon domain of TFIIS could stimulate pol II to cleave the transcript to produce C2 but could not induce stimulation of readthrough. However, no mutants in this region could stimulate readthrough without a stimulation of cleavage at the same or lower levels of TFIIS.

3.6 Complementation of Growth Phenotype *in vivo*

3.6.1 RPB9 Corrects Sensitivity of the $\Delta 9$ Strain to High and Low Temperatures and 6-azauracil

In addition to the functions of RPB9 that could be measured with *in vitro* assays, certain *in vivo* roles for RPB9 were known. The $\Delta 9$ yeast strain is sensitive to both low and high temperature extremes, and grows more slowly than the wildtype strain, even at the optimal growth temperature (Woychik *et al*, 1991). The genotype of the strain used here is different from that of previously characterized $\Delta 9$ strains. Thus, we confirmed the growth phenotypes for the $\Delta 9$ strain used in this study (Figure 15). Transformation of the $\Delta 9$ yeast strain with a low copy plasmid carrying the *RPB9* gene under the control of its endogenous promoter fully restored normal growth characteristics.

Yeast strains deficient for TFIIIS have been shown to be sensitive to the drug 6-azauracil (Hubert *et al*, 1983). This phenotype is thought to reflect a defect in transcription elongation (Exinger and Lacroute, 1992). Since RPB9 is required for the functional interaction between RNA polymerase II and TFIIIS, we tested the $\Delta 9$ strain used in this study for its ability to use 6-azauracil as its only uracil source. The $\Delta 9$ strain grew more slowly on medium containing 6-azauracil than the wildtype strain. Transformation of the $\Delta 9$ yeast strain with the *RPB9* gene on a low copy plasmid fully complemented the 6-azauracil sensitivity (Figure 15).

3.6.2 The Majority of RPB9 Mutant Alleles are Able to Complement Growth Defects

We used these growth characteristics to assay each of the *rpb9* alleles for their ability to restore normal growth. The $\Delta 9$ strain was transformed with the low copy

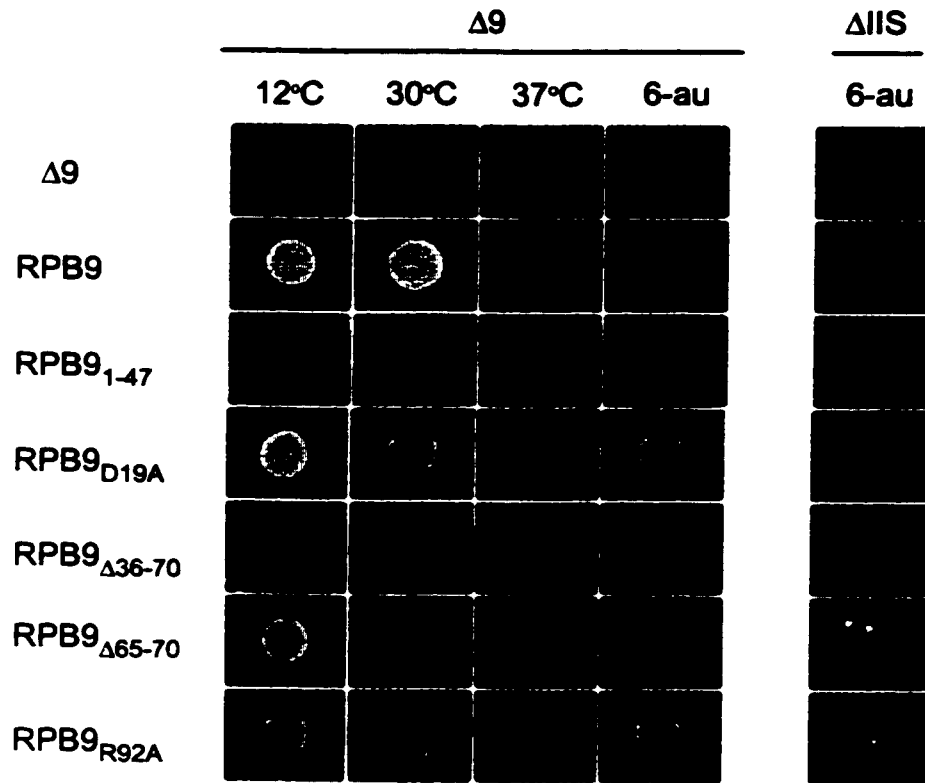


Figure 15. Complementation of Phenotype by Various RPB9 Mutants. The $\Delta 9$ and ΔIIS yeast strains were transformed with either the parent plasmid (pRS314) or a derivative plasmid containing one of the various RPB9 mutant alleles under the RPB9 endogenous promoter. Details pertaining to plasmid construction and growth conditions are described in Materials and Methods. Transformed cells were spotted on either selective medium and grown at the indicated temperatures (12°C, 30°C and 37°C) or selective medium containing 6-aza-uracil and grown at 30°C (6-au). Phenotypic details for each RPB9 mutant are shown in Table 4.

plasmid pRS314 carrying each of the *rpb9* mutant alleles. The majority of the *rpb9* alleles were able to restore wildtype growth characteristics (Table 4), with the following exceptions.

The Zn1 domain was not able to act independently in phenotype complementation, since the truncation mutant RPB9₁₋₄₇ did not correct the growth defects (Figure 15). This allele contains the N-terminal zinc-binding domain and part of the linker region. As well, the two deletion mutations within the Zn1 region (RPB9_{Δ12-27} and RPB9_{Δ16-23}) did not restore wildtype characteristics. It seems that RPB9 requires both Zn binding regions to restore growth phenotype, and that minor mutations that were sufficient to abolish activity in the *in vitro* assays, including the binding assay, had no effect on growth phenotype complementation.

The large deletion mutation in the linker region, RPB9_{Δ36-70}, was able to partially, but not fully, complement the growth defects of the knockout strain. This mutant has both Zn regions, but the intervening sequence is almost completely removed. Thus, although this construct has two intact Zn regions, their spatial arrangement may be disrupted.

It should also be noted that the three growth defects of temperature sensitivity, cold sensitivity and 6-azauracil sensitivity appear to be linked. Every mutant that complemented one of the growth defects complemented all three to the same extent; no mutant was able to complement only one or two of the growth defects. Therefore, the same effect of deletion of RPB9 seems to be influencing all three phenotypic characteristics.

The growth complementation assay was replicated in a RPB9/TFIIS double knockout strain (data not shown), and the trends were the same as those obtained for the $\Delta 9$ strain. Wildtype RPB9 could partially correct the slow growth phenotype of the double knockout. Mutants that did not restore growth phenotype in the $\Delta 9$ strain also did not partially correct growth defects in the $\Delta 9/\Delta IIS$ strain. The remaining mutants were able to correct growth defects to the same extent as RPB9.

The TFIIS deletion strain (ΔIIS) is also sensitive to 6-azauracil. This strain was transformed with each of the RPB9 alleles to determine if any of the RPB9 mutations could suppress the drug sensitivity caused by the deletion of TFIIS. No effect was seen with any of the RPB9 alleles in the ΔIIS strain (Figure 15).

3.7 Correction of Initiation Start Site Selection *in vivo*

3.7.1 Expression of RPB9 Corrects Altered Start Site Preferences in $\Delta 9$ and $\Delta 9/\Delta TFIIS$ Yeast Strains

Yeast strains deficient for RPB9 have been shown to exhibit altered preferences for start sites of transcription initiation (Furter-Graves *et al*, 1994); (Hull *et al*, 1995); (Sun *et al*, 1996). The effects of deleting RPB9 have been measured on a variety of different transcripts; the majority of those analyzed demonstrate an upstream shift of the 5' end of the transcript. In this study the *ADHI* gene, which shows a distinctive difference in transcript start site between the wildtype and $\Delta 9$ strains, was used to analyze the effect of the various RPB9 alleles on initiation start site preferences. In addition, a TFIIS knockout strain, and a RPB9/TFIIS double knockout strain were analyzed to

determine the effect of TFIIIS on initiation start site. Primer extension analysis was performed on RNA isolated from the different yeast strains using a primer directed against the 3' end of the *ADH1* gene.

The start site selection of the wildtype strain was compared to three deletion strains: the $\Delta 9$, Δ IIIS, and $\Delta 9/\Delta$ IIIS strains. The RNA for this primer extension analysis was prepared from these strains after they were grown in a rich medium. The pattern of reverse transcripts between the wildtype and the $\Delta 9$ strains is easily discernible; the reverse transcripts prepared from the $\Delta 9$ strain are longer and indicate an increase in *ADH1* transcripts that start upstream of the -37 position (Figure 16). The deletion of transcription factor TFIIIS appears to have no influence on start site selection, even in conjunction with the deletion of RPB9. The Δ IIIS strain has a reverse transcript pattern identical to that of the wildtype strain, and the result for the $\Delta 9/\Delta$ IIIS double knockout is identical to that of the $\Delta 9$ strain.

3.7.2 The Zn1 Region is Sufficient for Correction of Altered Start Site Preferences

In analyzing the effect of the various RPB9 mutations on selection of transcript start site, RNA was prepared from strains grown in a selective medium. The results from this primer extension analysis are not as distinctive as the ones from the previous analysis (Figure 17). The pattern of reverse transcripts from the wildtype strain includes some of the longer species, indicating an increased usage of the start sites upstream of -37 in the *ADH1* gene under these growth conditions. As with phenotype complementation, the



Figure 16. Primer Extensions of the ADH1 Transcript from Wildtype and Deletion Strains of *S. cerevisiae*. Total RNA was isolated from wildtype, $\Delta 9$, ΔIIS , and $\Delta 9/\Delta IIS$ yeast strains. In order to map the transcript start site primer extensions were performed using a primer directed against the ADH1 transcript. An autoradiogram of the reverse transcripts is shown. The leftmost lane contains DNA standards, with the sizes of the standards indicated (in bases) to the left of the figure. The size references indicated on the right of the figure refer to the number of bases upstream from the ATG codon in the transcript. Refer to the Materials and Methods section for experimental details.

majority of *rpb9* alleles were able to restore wildtype characteristics in this assay (Table 4).

Two striking results are those obtained with the Zn1 truncation and deletion mutants. Although the RPB9₁₋₄₇ allele did not restore wildtype activity in any other assay including phenotype complementation, it was able to correct the alteration of start site selection (Figure 17). Similarly, the small deletion from the Zn1 region corrected start site preferences, but not any other activity of RPB9. Together these results indicate that the Zn1 region is sufficient for restoration of start site preferences, but the putative flexible loop within Zn1 is not required for this restoration.

In contrast, the RPB9_{Δ36-70} mutation, which was able to partially complement growth defects, could not correct the altered start site selection. This mutant has an unaltered Zn1 domain, but has a deleted linker region. This suggests that it is not only the presence of the Zn1 region, but that the orientation or accessibility of Zn1 in the context of the subunit is important.

These *in vivo* assays have been able to detect mutations that act independently in growth phenotype and initiation start site selection. We conclude that these two defects of the Δ9 yeast strain are due to different effects of loss of RPB9 and are not directly linked.

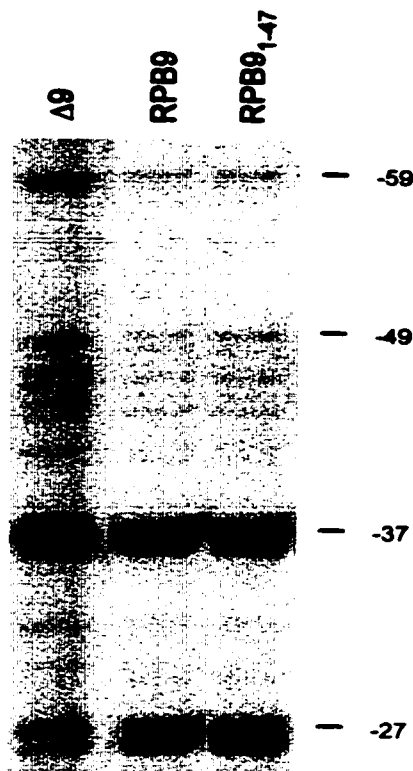


Figure 17. Primer Extension of the ADH1 Transcript from a Yeast Strain Carrying the Truncated Zn1 Allele of RPB9. Primer extensions directed against the ADH1 transcript were performed using total RNA prepared from the $\Delta 9$ yeast strain carrying either pRS314 or a derivative plasmid containing one of the various RPB9 mutant alleles. Shown here is an autoradiogram displaying the reverse transcripts, separated on a denaturing gel, from the $\Delta 9$ strains transformed with the plasmid alone ($\Delta 9$), the plasmid encoding wildtype RPB9, or the Zn1 truncated RPB9 allele (RPB9₁₋₄₇). Primer extension results for each of the RPB9 mutants are presented in Table 4.

Table 4. Summary of *in vivo* Analyses of RPB9 Mutant Alleles

RPB9 Mutant	Phenotype Complementation	Start Site Selection via Primer Extension
RPB9	++	++
RPB9 ₁₋₄₇	—	++
RPB9 _{Δ12-27}	—	—
RPB9 _{Δ16-23}	—	++
RPB9 _{R5A, F6A}	++	++
RPB9 _{R8A, D9A}	++	++
RPB9 _{R17A}	++	++
RPB9 _{E18A}	++	++
RPB9 _{D19A}	++	++
RPB9 _{K20A}	++	++
RPB9 _{E21A}	++	++
RPB9 _{R30A}	++	++
RPB9 _{Y34A}	ND	ND
RPB9 _{Δ36-70}	+	—
RPB9 _{Δ65-70}	++	++
RPB9 _{E54A}	++	++
RPB9 _{D61A}	++	++
RPB9 _{D65A}	++	++
RPB9 _{R70A}	++	++
RPB9 _{D72A}	++	++
RPB9 ₅₅₋₁₁₂	ND	ND
RPB9 _{K77A}	++	++
RPB9 _{Δ80-101}	++	++
RPB9 _{Δ89-95}	++	++
RPB9 _{R91A}	++	++
RPB9 _{R92A}	++	++
RPB9 _{K93A}	++	++
RPB9 _{D94A}	++	++

++ Allele completely restores wildtype characteristics.

+ Allele partially restores wildtype characteristics.

— Allele does not restore wildtype characteristics.

ND Experiment was not performed.

3.8 Summary

The results presented here represent a mutational analysis characterizing RPB9, a subunit of yeast RNA polymerase II. Twenty-seven mutants were designed, subcloned and analyzed in five different assays for their ability to restore RPB9 activity to pol II $\Delta 9$.

The first assay performed was a reconstitution assay measuring the reassembly of RPB9 with pol II $\Delta 9$. As no assay existed, we developed a gel mobility shift assay to measure the affinity of GST-RPB9 for pol II $\Delta 9$. The interaction was shown to be saturatable, stoichiometric, and reversible. A conserved stretch of amino acids in the linker region of RPB9 was shown to be necessary for binding between RPB9 and pol II. However, these assays were done with GST-RPB9 fusion proteins, and this should be considered when interpreting the results.

The next two assays performed measured the ability of RPB9 to mediate a response between pol II and TFIIS during elongation. The flexible loop in the Zn2 region was found to be required for both readthrough and full cleavage activity of RPB9. Zn2 is the region of RPB9 that is homologous to the Zn ribbon of TFIIS. The analogous loop in TFIIS is known to be required for elongation stimulation of pol II. The results for these assays indicate that readthrough activity is dependent on the ability of polymerase to completely cleave the nascent transcript back to the C2 product. This finding agrees with studies done on TFIIS.

The mutant alleles of *rpb9* were transformed into appropriate yeast strains for the final two assays. Complementation of growth at temperature extremes and the ability to

restore growth on 6-azauracil was measured. The Zn1 truncation mutant could not correct growth phenotypes, implying that perhaps the linker or Zn2 regions of RPB9 are also required. The large deletion mutant from the linker region contains both Zn domains, but with very little intervening sequence. This mutant was able to partially correct growth phenotype.

Correction of start site preference was also measured. The truncation mutant containing Zn1 was able to correct start site selection. The implication is that only Zn1 is involved in start site selection. However, the two deletion mutants in the linker region both contain an intact Zn1 domain. The large deletion did not restore start site preference, although the small deletion was able to restore wildtype preferences. This suggests that perhaps it is the orientation or accessibility of Zn1 in the context of the subunit that is important.

Altogether, we have performed an extensive characterization of the RNA polymerase II subunit RPB9. It has provided insight into how RPB9 functions within the RNA polymerase II enzyme. We have demonstrated that recombinant RPB9 reassembles with RNA polymerase II $\Delta 9$ to form an intact enzyme. In addition, we have identified residues required both for reassembly with RNA polymerase II and for response to elongation stimulation by TFIIS. With this study we have also provided evidence that the three growth defects of the $\Delta 9$ yeast strain are linked, but the altered start site selection is independent of the growth defects. The Zn1 domain can correct the start site preference, but the putative loop within this domain is not required. In all, this study has furthered our understanding of how RPB9 functions in transcription.

CHAPTER 4 DISCUSSION

4.1 General Discussion

We initiated this study in order to define the transcriptional role of the RNA polymerase II subunit RPB9. We specifically wished to put the various functions of RPB9 into a structural context. To this end, we developed a set of mutants of RPB9 to test which regions of RPB9 were required in certain functional assays. The design of mutants was based, in part, on the assumption that the two zinc-binding regions of RPB9 were likely to adopt the zinc ribbon fold. NMR studies on archaebacterial RPB9 have subsequently demonstrated that this is the case for the second zinc-binding domain (Wang *et al.*, 1998). In particular, we targeted conserved regions within the two zinc regions that we predicted to be analogous to the charged flexible loop of TFIIS (Qian *et al.*, 1993a). As well, we targeted the conserved DPTLPR sequence located in the region that connects the two zinc regions of RPB9.

Through characterization of the mutants in our study, we have identified and isolated regions of RPB9 that are responsible for particular functions of the RNA polymerase II subunit. We have been able to assign the functions of polymerase binding, elongation response to TFIIS, and initiation start site selection to unique regions within RPB9. These activities have been attributed to the linker region, domain Zn2, and domain Zn1, respectively.

We have identified the residues involved in protein-protein interactions with RNA polymerase II. The conserved linker sequence DPTPLR was found to be essential for assembly of RPB9 with the polymerase in our binding assay. Specifically, the two charged residues and a third charge located N-terminally to this sequence were found to be important. However, in order to maintain the solubility of RPB9, the binding assays were performed with GST fusion proteins. The fact that the fusion proteins contain an extra 28 kDa region attached to the N-terminus of RPB9 must be taken into account when interpreting the results.

The second zinc-binding region was found to be involved in mediating the elongation response of RNA polymerase II to TFIIS. The residues that compose the flexible loop analogous to that of TFIIS were required to restore response to TFIIS *in vitro*. We targeted the charged residues in this sequence and demonstrated that these residues were necessary for arrested polymerase molecules to resume elongating in response to stimulation by TFIIS. This result is similar to those obtained for TFIIS; charged residues within the flexible loop were essential for TFIIS stimulation of polymerase (Jeon *et al*, 1994). Thus, the zinc ribbon domain shared by these two proteins appears to be an elongation stimulation domain, and both are required to influence arrested polymerase molecules to continue transcribing.

The first zinc-binding domain was able to restore correct start site preferences as an independent domain. In contrast to the elongation activity of Zn2, correction of start site selection by Zn1 was found not to be dependent on the flexible loop. Despite the ability to restore wildtype start site selection, Zn1 could not independently correct growth

phenotype. These results demonstrate that growth phenotypes induced by deletion of RPB9 are not directly due to an upstream shift of transcript start site.

These results and their significance to RPB9 function will be analyzed in detail in the following discussion sections. In addition, I will present a model of the mechanism by which RPB9 mediates RNA polymerase II activity during transcription.

4.2 RNA Polymerase II Binding Region

We had previously demonstrated that recombinant RPB9 or GST-RPB9 could restore wildtype RNA polymerase activity in elongation assays (Awrey *et al*, 1997). *In vitro* experiments measuring start site selection also demonstrated that GST-RPB9 could restore wildtype preferences to the $\Delta 9$ RNA polymerase (Hull *et al*, 1995). We developed a gel mobility shift assay to determine how the recombinant subunit restores these transcriptional activities. We wished to determine if GST-RPB9 assembles with pol II $\Delta 9$ in a stable manner to form a functional, intact enzyme, as opposed to acting on pol II $\Delta 9$ in a transient manner. Using radiolabeled GST-RPB9, we demonstrated that GST-RPB9 does form a stable interaction with pol II $\Delta 9$. We detected a stable binary complex of GST-RPB9 and RNA polymerase II $\Delta 9$; the complex forms a high affinity interaction, with an estimated equilibrium dissociation constant of 22 nM.

Our mutational study has identified the region of RPB9 involved in binding to the $\Delta 9$ RNA polymerase. The conserved sequence DPTLPR in the linker region is required for binding to pol II $\Delta 9$. The two charged residues in this sequence, and a third charged residue N-terminal to this sequence, were found to be essential. The deletion mutant

$\Delta 65-70$ and the point mutants D61A, D65A and R70A did not exhibit binding in our gel mobility shift assay. Thus it appears, at least in the context of this assay, that this region is responsible for the interaction between RPB9 and pol II $\Delta 9$.

The residues that we have identified as important for the interaction between RPB9 and pol II $\Delta 9$ are conserved only among eukaryotic RPB9 homologues (Kaine *et al*, 1994); (Hull *et al*, 1995). The homologous subunits that have been identified from archaebacteria and eukaryotic RNA polymerase I do not contain the D---DPTLPR sequence. However, the archaebacterial subunits do contain a conserved sequence in the linker region that is composed of conserved aliphatic and charged residues (TVIK---K-K) (Kaine *et al*, 1994). This sequence may represent the RNA polymerase binding region within the archae subunits. These patterns of sequence conservation suggest that the region of RNA polymerases involved in binding to their respective RPB9 homologous subunits is conserved within a class of RNA polymerase, but not between polymerase classes. Yeast RPB9 likely interacts with a region of the polymerase that is highly conserved between eukaryotic RNA polymerase II homologues, but is not contained within RNA polymerase I or archaebacterial polymerases. In accordance with this idea, human RPB9 could partially complement growth defects in $\Delta 9$ yeast strains, and could fully restore wildtype start site preferences in a yeast *in vitro* assay (McKune *et al*, 1995).

Although the conserved charge D61 and the conserved sequence DPTLPR are essential for the binding of GST-RPB9 to pol II $\Delta 9$ *in vitro*, they may form the central part of a larger binding face on RPB9 rather than defining the entire interface. It is important to note that the binding assay was performed with GST-RPB9 fusion proteins,

rather than RPB9 alone. GST is a 28 kDa protein that forms a dimer in solution. The fusion of this larger globular protein to the 15 kDa RPB9 may have some steric effect on the assembly of the subunit with pol II $\Delta 9$. The GST-RPB9 fusion is still able to restore activity *in vitro* (Hull *et al*, 1995); (Awrey *et al*, 1997) as well as bind to the polymerase with high affinity ($K_d = 22$ nM). Thus, the addition of GST does not significantly affect the activity of wildtype RPB9. However, if GST were partially blocking a larger binding surface, the effects of mutations within the remaining binding face may have greater influence on a GST-RPB9 construct than they would on RPB9 alone. Consistent with this idea, mutations that disrupted binding of GST-RPB9 fusions *in vitro* did not affect activity of RPB9 *in vivo*. The binding mutants were able to fully restore wildtype growth characteristics and start site preferences when transformed into a $\Delta 9$ yeast strain.

Another factor that may contribute to the differences between the *in vitro* and *in vivo* effects of the binding mutants is the enzyme assembly process. The RPB9 constructs were expressed in yeast under the endogenous *RPB9* promoter and would therefore have been expressed and incorporated into the polymerase as it was assembled from the various subunits. In contrast, binding assays with GST-RPB9 involved the association of the subunit with an already formed pol II $\Delta 9$ complex. It is conceivable that RPB9 may be involved in extensive protein-protein contacts with the other subunits. The residues identified in our binding assay may be central to, but not entirely responsible for, the incorporation of RPB9 into RNA polymerase II. Therefore, mutations that significantly destabilize the interaction between GST-RPB9 and the pre-

formed enzyme do not have as extreme an effect on RPB9 that is incorporated into pol II as it assembles.

The ability of RPB9 to restore activity to the $\Delta 9$ polymerase appears to depend on its ability to assemble with the polymerase. Results obtained with the *in vitro* elongation assays demonstrate that mutants that could not bind to pol II $\Delta 9$ were not able to restore readthrough and cleavage responses to TFIIS at wildtype levels. However, these mutants did restore elongation activities at 100-fold molar excess. The ability of the binding mutants to restore activity at extremely high levels may be due to a limited ability to bind pol II $\Delta 9$ at very high concentrations. It is possible that these mutants have a very low affinity for the polymerase that we could not detect in our binding assay. Alternatively, RPB9 may have a limited ability to influence polymerase in a transient manner. This type of transient interaction may be relevant only at high concentrations of RPB9. In either event, these data suggest that prior assembly with the polymerase is important for RPB9 to restore activities at wildtype levels of the subunit.

4.3 Role of RPB9 in Elongation

The $\Delta 9$ polymerase is defective for certain transcription elongation activities (Awrey *et al.*, 1997). Without RPB9, RNA polymerase II is less efficient at recognizing pause sites within the template. The $\Delta 9$ pol II molecules that do recognize and arrest at intrinsic pause sites are unable to readthrough and resume transcribing in response to stimulation by TFIIS. The arrested $\Delta 9$ enzyme demonstrates an ability to cleave the nascent transcript to produce a primary cleavage product (C1) when incubated with

TFIIS. However, the $\Delta 9$ enzyme cannot perform the subsequent cleavage event to produce a second cleavage product (C2). GST-RPB9 can restore these activities when added to *in vitro* elongation assays. We used these characteristics of $\Delta 9$ RNA polymerase II to identify the region of RPB9 that is involved in mediating the elongation response of RNA polymerase II to TFIIS.

RPB9 contains two zinc-binding domains that we predict adopt the zinc ribbon conformation. The second of these domains (Zn2) possesses 30% identity with the zinc ribbon domain of TFIIS. We found that this domain of RPB9 is responsible for activating pol II to respond to TFIIS stimulation in transcript readthrough and cleavage assays. Specifically, we found that the putative flexible loop of the zinc ribbon is required to restore these elongation activities to pol II $\Delta 9$. Mutations that deleted the charged loop of the Zn2 region (QRRKDT), or removed each of the charges within the loop (R, R, K, or D), resulted in RPB9 constructs that were unable to induce pol II $\Delta 9$ to respond to TFIIS stimulation at wildtype levels. At very high concentrations (one hundred-fold molar excess) these mutants induced pol II $\Delta 9$ to cleave the transcript to produce C2 in the presence of TFIIS. However, these mutants did not restore the readthrough activity of pol II $\Delta 9$ in response to TFIIS, even with a three hundred-fold molar excess.

In addition to sequence similarity, the Zn2 domain of RPB9 and the zinc ribbon domain of TFIIS share a functional similarity. The elongation stimulation activity of TFIIS is also dependent on its zinc ribbon domain, and particularly the charges within the flexible loop (QTRSADE). Mutation of the aspartic acid and glutamic acid residues

within the loop completely abolished the ability of TFIIS to stimulate the readthrough and cleavage activities of RNA polymerase II. As well, only positively charged residues could substitute for the arginine residue (Jeon *et al*, 1994). Thus, this domain is functionally analogous in RPB9 and TFIIS, and represents an elongation stimulatory domain in the two proteins.

The conserved zinc ribbon domains of RPB9 and TFIIS are both necessary to stimulate the readthrough and cleavage activities of arrested RNA polymerase II. Both domains are required for the readthrough activity of the polymerase; mutation of the zinc ribbon of either protein eliminated elongation stimulation of arrested polymerase molecules (Jeon *et al*, 1994); (Awrey *et al*, 1998); (this study). However, they differ in their effects on transcript cleavage. The transcript cleavage activity is contained within the polymerase, but is dependent on TFIIS for stimulation (Reines *et al*, 1992). Mutation of critical residues in the zinc ribbon of TFIIS completely abrogated stimulation of cleavage (Jeon *et al*, 1994); (Awrey *et al*, 1998). In contrast, deletion of RPB9 does not completely abolish polymerase cleavage activity. Pol II $\Delta 9$ can cleave the transcript to produce C1 in response to TFIIS stimulation but is unable to produce C2 (Awrey *et al*, 1997). Furthermore, experiments in our laboratory designed to interchange the zinc regions of the two proteins were not successful, indicating that each zinc ribbon domain possesses unique functional characteristics (Chris Koth, personal communication). Therefore, the conserved zinc ribbon domains in RPB9 and TFIIS are both required to fully stimulate RNA polymerase II to overcome transcript arrest. The two domains act in a concerted manner to influence the activity of arrested RNA polymerase II complexes.

We found that the readthrough activity of RNA polymerase II correlates with the ability of the polymerase to produce the second cleavage product, C2. The $\Delta 9$ polymerase can produce C1, but not C2, in response to stimulation by TFIIS. Furthermore, arrested pol II $\Delta 9$ cannot read through intrinsic pause sites in response to TFIIS. Addition of GST-RPB9 restores both of these activities to the polymerase (Awrey *et al*, 1997). We observed that the mutants in the Zn2 region of RPB9 were defective for restoring both readthrough and cleavage activities. However, at high levels these mutants could restore the ability of the polymerase to produce the C2 product. This partial restoration of cleavage activity did not lead to restoration of readthrough activity in the course of this experiment. In addition, we did not observe any RPB9 mutants that were able to restore the readthrough activity of polymerase without also restoring the ability to cleave to C2. Thus, cleavage to C2 can occur in the absence of readthrough activity, but readthrough activity always correlates with the capability to cleave to C2. These results imply that the ability to cleave to produce the C2 product is a prerequisite for readthrough activity. However, it should be noted that these cleavage experiments were performed in the absence of nucleotides. In the presence of nucleotides, the polymerase may retain the ability to produce C2, but the actual cleavage event may not occur.

Mutational studies performed on yeast TFIIS confirm our finding that a stalled ternary complex must be capable of producing C2 if it is to read through the pause site and resume transcribing (Awrey *et al*, 1998). Mutations in TFIIS that disrupted stimulation of cleavage to C2, but left the ability to stimulate cleavage to C1, also disrupted stimulation of polymerase readthrough activity. Moreover, the stimulation of

readthrough activity correlated highly with the stimulation of the polymerase to produce the C2 product.

Our cleavage results seem to conflict with transcriptional studies performed with mammalian TFIIS. RNA and DNA mapping experiments demonstrated definitively that when arrested ternary complexes were stimulated by TFIIS, only one cleavage event was required prior to activation of the polymerase to resume transcription (Gu *et al*, 1993). However, these experiments were performed in the presence of nucleotides, allowing for polymerase to resume transcribing as soon as it became translocation competent. As with our results, two cleavage products were observed in this study when TFIIS was added to arrested ternary complexes in the absence of nucleotides. Therefore, our results are consistent with the results of this study, as we measured the ability to produce C2 in the absence of nucleotides.

A two-step model has been proposed to reconcile these two lines of apparently contradictory evidence (Awrey *et al*, 1998). The model states that TFIIS acts on the translocation-incompetent (arrested) ternary complex to induce cleavage to C1. Subsequent to C1 cleavage, TFIIS induces the ternary complex to switch to a translocation-competent conformation. In the presence of nucleotides, the polymerase can proceed through the pause site, and continue transcribing. In the absence of nucleotides, the polymerase is now paused, but not arrested. It responds to the lack of nucleotides by further cleaving the transcript to produce C2.

This model is consistent with the observation that in the absence of nucleotides, TFIIS activates both arrested and paused ternary complexes to cleave the 3' end of the

transcript. Oligonucleotides of either 7-14 or 2 bases in length are released, respectively (Reines *et al*, 1992); (Izban and Luse, 1993). In the mammalian TFIIS study described above, the first cleavage product observed was shortened by 6 to 9 nucleotides; the second cleavage product was shortened by an additional two nucleotides (Gu *et al*, 1993). Thus, these results conform to the mechanism proposed by the model, that TFIIS induces polymerase to switch out of the arrested state after the initial cleavage event, and that the second cleavage event is a reaction to the lack of nucleotide substrates.

This model also agrees with our transcript cleavage and readthrough data. We propose that the second step involving the switching of RNA polymerase from a translocation-incompetent to a translocation-competent form is dependent on RPB9. This transformation is represented by the ability of the polymerase to cleave the transcript to C2 in the absence of nucleotides. We found that this activity requires the charged residues of the Zn2 loop of RPB9. We contend that readthrough activity is dependent on the ability to produce C2, although not necessarily dependent on the actual occurrence of the second cleavage event. After TFIIS has activated a re-positioning of the polymerase active site with respect to the 3' end of the transcript, RPB9 acts in concert with TFIIS to effect a second rearrangement that results in the ability to resume transcribing. Only in the absence of nucleotides does cleavage to C2 occur. This reaction is dependent on the conserved zinc ribbon domains of both TFIIS and RPB9.

4.4 Role of RPB9 in Initiation

RPB9 plays a role in the selection of start site usage during transcription initiation. Deletion of RPB9 results in an upstream shift in the preference of RNA polymerase II for particular start sites at most promoters (Furter-Graves *et al*, 1994); (Hull *et al*, 1995); (Sun *et al*, 1996). We transformed the $\Delta 9$ yeast strain with the mutant *rpb9* constructs in order to assess the effects of the various mutations on the selection of transcript start sites. We found that most of the mutations in RPB9 did not affect the ability of the subunit to influence start site preferences; the majority of mutants tested in our study restored wildtype start site patterns.

Interestingly, our study indicates that the Zn1 domain of RPB9 is sufficient to restore wildtype start site preferences. The truncation mutant that comprises the Zn1 region of RPB9 was able to correct start site preferences as an independent domain. We predict that this region of RPB9 adopts a zinc ribbon fold, as it contains the conserved cysteine pattern that typifies the zinc ribbon motif. The large deletion within Zn1, $\Delta 12-27$, removed the majority of the region between the two pairs of cysteines residues without affecting expression levels of the subunit (Stephen Orlicky, personal communication). This mutation constitutes deletion of two of the β -strands and the flexible loop region within the zinc ribbon domain. The large Zn1 deletion within the context of full-length RPB9 disrupted the ability of Zn1 to restore correct start site usage. Together, these two results imply that the Zn1 domain is responsible for the contribution of RPB9 to initiation start site selection.

We also found that this activity does not involve the charged residues within the predicted flexible loop. Each of the single amino acid substitution mutants within the within the loop did restore wildtype start site usage. As well, the $\Delta 16-23$ mutant, which has the entire predicted flexible loop removed, restored wildtype preferences. These results imply that the ability to influence start site selection does not require the flexible loop of Zn1. This is in direct contrast with the elongation domain of RPB9, Zn2. The activity of Zn2, and the analogous domain of TFIIS, is dependent on the charges within the flexible loop of the zinc ribbon (Jeon *et al*, 1994); (this study).

The results obtained with our *in vitro* elongation assays demonstrated that assembly with the polymerase was an important factor for the ability of RPB9 constructs to restore activities to RNA polymerase II. We have demonstrated that the Zn1 truncation mutant did not assemble with pol II $\Delta 9$ in our *in vitro* binding assay, but was able to restore correct start site selection *in vivo*. This result is not unique; many of the mutants that did not bind to pol II $\Delta 9$ *in vitro* did fully restore both start site preferences and growth characteristics *in vivo*. As mentioned previously, it is conceivable that the polymerase-binding surface of RPB9 consists of more than just the conserved D---DPTLPR sequence. If the Zn1 mutant is in fact assembling with the polymerase, then part of this surface must be contained within the first zinc-binding domain of RPB9. Alternatively, the Zn1 domain could be influencing transcript start site usage through a transient interaction with the pre-initiation complex.

In contrast with the above results, the large deletion mutant within the linker region, $\Delta 36-70$, did not restore wildtype start site preferences. This mutant contains

intact Zn1 and Zn2 regions, but 34 amino acids from the linker region have been removed, resulting in the juxtaposition of the two zinc-binding domains. Although this RPB9 construct has a functional Zn1 region, having an immediately adjacent Zn2 domain must inhibit the function of Zn1 in start site selection. Steric interference by Zn2 would affect the activity of this mutant, whether Zn1 was acting via assembly with polymerase, or via a transient means.

Our results regarding the requirement for Zn1 in start site selection conflict with the analysis of experiments involving the *ssu73-1* mutant of RPB9 (Sun *et al*, 1996). The *ssu73-1* mutation is a point mutation at codon 107, resulting in an early stop codon and loss of the final 16 residues of RPB9. This mutant suppresses the cold-sensitive phenotype and the altered start site selection defect imparted by the *sua7-1* mutation of TFIIB. As this mutation occurs at the residue immediately following the final cysteine of Zn2, it is extremely likely that Zn2 is not folded. The authors interpret the results to indicate that Zn2 is necessary for start site selection, but is not required for correction of growth phenotype. However, the growth assays are presented in the context of the triple *sua7-1 ssu72-1 ssu73-1* triple mutant. It is possible that the unfolding of Zn2 destabilizes RPB9, and the *ssu73-1* mutant is not stably expressed. In this case, the results would be the same as those expected for the *rpb9* null mutant. We interpret the *ssu73-1* results to imply that the deletion of RPB9 suppresses the *sua7-1* cold growth phenotype and start site selection defect in the presence of the *ssu72-1* mutation. Our results suggest that stable expression of Zn1 would relieve the suppression of the start site defect but not the cold growth phenotype.

4.5 Growth Phenotype Correction

In addition to altered transcript start site patterns, the $\Delta 9$ yeast strains exhibit slow growth at optimal temperature, with an increased sensitivity to high and low temperatures, and a lower tolerance for the drug 6-azauracil (Woychik *et al*, 1991); (David Jansma, personal communication). We used the $\Delta 9$ yeast strain to assess the ability of each of the mutant *rpb9* alleles to restore wildtype growth characteristics under each of the various growth conditions. As with start site selection, we found that the majority of the *rpb9* alleles in our study were able to correct growth defects.

We observed that the growth characteristics appear to be linked. None of the mutants tested in this study were able to separate the various phenotypes. Any mutant that complemented one of the growth characteristics was able to complement all of them to the same extent. Consequently, it appears that a single effect of the deletion of RPB9 causes yeast cells to grow more slowly, become more sensitive to temperature extremes, and be less resistant to the effects of 6-azauracil.

In contrast with start site selection, expression of the Zn1 domain alone was not sufficient to correct growth defects. Neither the Zn1 truncation mutant, nor the $\Delta 16-23$ deletion mutant restored wildtype growth characteristics despite their ability to correct start site usage. Together these results indicate that the residues in the flexible loop of Zn1 are required for complementation of growth phenotype, but an intact Zn1 domain is not sufficient for this complementation. As well, the effect of RPB9 deletion that causes the growth defects is not the same one that is responsible for the alteration in start site

preferences. We have demonstrated that the temperature and drug sensitivities are separable from the start site selection defects of the $\Delta 9$ yeast strains.

Deletion of the linker region of RPB9 resulted in partial restoration of wildtype growth characteristics. The deletion mutant, $\Delta 36-70$, possesses complete but juxtaposed Zn1 and Zn2 regions. The partial complementation by this mutant implies that it may contain some, but not all of the regions of RPB9 required for correction of growth defects. Part of the deleted linker region may be required in addition to the two zinc regions. A second interpretation is that only the two zinc regions are required, but deletion of the linker region has resulted in an alteration of their spatial arrangement with respect to each other or the polymerase. The repositioning of the zinc regions may result in their partially blocking each other, or otherwise being unable to make full contact with other proteins in the required manner.

One result that is not consistent with our *in vitro* findings is the effect of mutations within Zn2 on the resistance to 6-azauracil. None of the deletion or single amino acid mutations within Zn2 had any effect on the ability of RPB9 to complement growth defects, including the sensitivity to 6-azauracil. This result is surprising, as we have identified Zn2 to be involved in elongation, and particularly to interact with TFIIS in stimulating arrested ternary complexes. Deletion of TFIIS imparts sensitivity to 6-azauracil to yeast strains (Hubert *et al*, 1983). The $\Delta 9$ yeast strains are less sensitive to this drug than the Δ IIS strains, and the extent of 6-azauracil sensitivity is dependent on the genetic background of the parental yeast strain (Rod Weilbaecher and David Jansma, personal communications). The double deletion mutant $\Delta 9/\Delta$ IIS exhibits a synthetically

lethal phenotype when grown on 6-azauracil (David Jansma, personal communication). This drug depletes cellular GTP levels, and sensitivity to it is thought to indicate a defect in elongation (Exinger and Lacroute, 1992). However, the exact cause for the drug sensitivities in the $\Delta 9$ and Δ IIS strains is not known. The sensitivities of these two strains may not even be due to a single cause. Rather than being due to the interruption of the interaction between TFIIS and RPB9, they may be due to defects in other elongation-related phenomena.

4.6 RPB9 Zn Ribbon Structure

The structure of the C-terminal zinc-binding region of the RPB9 homologue from the archaeobacterium *Thermococcus celer* has been solved by NMR techniques (Wang *et al*, 1998). This structure confirms our predictions that the Zn₂ domain of RPB9 adopts the zinc ribbon conformation. The overall fold is conserved with respect to the human TFIIS (Qian *et al*, 1993b) and archaeal TFIIB (Zhu *et al*, 1996) zinc ribbons. The RPB9 domain has one additional short β -strand, making the domain a four-stranded anti-parallel β -sheet with an apical zinc ion coordinated by four cysteines. As well, this structure demonstrates that the charged residues we found to be essential for the elongation activities of RPB9 do form part of a flexible loop analogous to the loop from the TFIIS domain.

Additional features that are conserved between the RPB9 and TFIIS zinc ribbons are the hydrophobic patches that are located on the front and back faces of the zinc ribbon domain (Qian *et al*, 1993b); (Olmstead *et al*, 1998). These patches may represent

surfaces that are involved in protein-protein interactions. Alternatively, it has been suggested that these patches function as nucleic acid binding sites (Wang *et al*, 1998); (Awrey *et al*, 1998). Mutation of three of the conserved aromatic residues in yeast TFIIS severely reduced the ability of TFIIS to stimulate the cleavage and readthrough activities of RNA polymerase II. A molecular model for the mechanism of TFIIS proposes that one of these residues forms contacts with the 3' end of the RNA during the first cleavage event (Awrey *et al*, 1998). We do not currently have evidence as to whether these hydrophobic patches in RPB9 are involved in interactions with protein or nucleic acid moieties.

This structure represents the third zinc ribbon domain to be identified in transcription-related proteins. The structures of zinc ribbons from TFIIS (Qian *et al*, 1993a); (Qian *et al*, 1993b); (Olmstead *et al*, 1998), TFIIB (Zhu *et al*, 1996), and now RPB9, have been confirmed by NMR methods. As well, experiments using protein threading algorithms predict that the zinc-binding regions of TFIIE (Jeon *et al*, 1993b), RPB2 and RPB12 (Wang *et al*, 1998) adopt the zinc ribbon fold. Thus, this fold depicts a conserved structural motif among initiation and elongation proteins. We have demonstrated that the function of the zinc ribbon domain is also conserved with respect to RPB9 and TFIIS.

4.7 Model of RPB9 Mode of Action

RPB9 influences both the initiation and the elongation stages of transcription. We have demonstrated that it achieves these effects through interactions involving its Zn1

and Zn²⁺ zinc ribbon domains, respectively. The transcriptional events that require RPB9 involve the rearrangement of the active site of RNA polymerase II. In addition, these events require the participation of TFIIB and TFIIS, proteins that also contain zinc ribbon domains. We cannot provide a detailed molecular mechanism of RPB9 action, but we can provide insight into the transcriptional events that involve RPB9. Here we present a model to explain how RPB9 may influence start site selection during initiation and reactivation of RNA polymerase II during elongation arrest.

RPB9 appears to influence the placement of the active site of polymerase with respect to either the template or the transcript. RPB9 is involved in defining the upstream boundary of the window in which initiation occurs (Furter-Graves *et al*, 1994); (Hull *et al*, 1995); (Sun *et al*, 1996). Deletion of RPB9 causes RNA polymerase II to select upstream start sites for transcription initiation. RPB9 also influences RNA polymerase during elongation arrest (Awrey *et al*, 1997). Deletion of RPB9 prevents RNA polymerase II from becoming translocation-competent in response to stimulation by TFIIS. Both of these events presumably involve movement or conformational change of the active site.

We suggest that RPB9 employs the zinc ribbon structural motif to exert its influence on the active site of polymerase. Our study has demonstrated that it is the Zn1 region of RPB9 that influences start site selection. We propose that this domain adopts the zinc ribbon conformation, but that the charged loop of this domain is not necessary for start site selection. We have shown that Zn²⁺ is required for the stimulation of arrested ternary complexes. An archaeal homologue of this domain has been shown to form a zinc

ribbon (Wang *et al*, 1998). The residues that define the charged loop of this domain are required for the elongation activity of RPB9.

The zinc ribbon domains of RPB9 do not act independently to influence the active site. Both activities also require an additional transcription factor, each of which contains a zinc ribbon domain. Accurate start site selection involves TFIIB; TFIIB defines the downstream boundary of the start site selection window (Pinto *et al*, 1992). The N-terminal region of TFIIB that interacts with polymerase (Malik *et al*, 1993) contains a zinc ribbon domain (ZnIIB) (Zhu *et al*, 1996). Disruption of the zinc ribbon abolished the interaction with polymerase (Buratowski and Zhou, 1993). Furthermore, the reactivation of polymerase after elongation arrest is dependent on TFIIS (Reines, 1992), and particularly the zinc ribbon domain (ZnIIS) (Jeon *et al*, 1994); (Awrey *et al*, 1998). Both TFIIB and TFIIS demonstrate a functional interaction with RPB9 during these processes (Sun *et al*, 1996); (Awrey *et al*, 1997). Thus, we propose that the zinc ribbon domains of RPB9 act in concert with ZnIIB and ZnIIS to orient the active site of polymerase during start site selection and the reactivation of arrested ternary complexes, respectively.

We propose that ZnI and ZnIIB influence the orientation of the polymerase active site with respect to the promoter via opposing interactions. Yeast RNA polymerase II initiates transcription from specific sites within an 80 basepair window (Struhl, 1987). This wide range of start site positioning indicates there is substantial flexibility within the pre-initiation complex. Whether this wide range of positioning is due to an adjustment of the polymerase active site, or due to a shift in the location of polymerase at the promoter,

is not known. In either event, ZnI must interact with pol II to “aim” the active site downstream, while ZnIIB interacts to “aim” the active site upstream.

We suggest that these domains act in a concerted manner to define the location of the start site window. Mutation of RPB9 suppresses a start site mutant of TFIIB, resulting in no change in the boundaries of the start site window (Sun and Hampsey, 1996). However, mutation of one or the other of the two proteins does significantly alter the location of the start site window with respect to the promoter (Pinto *et al*, 1992); (Sun *et al*, 1996). The fact that the double mutation results in no change in the start site preferences compared to wildtype indicates that the two domains are influencing the same property of the polymerase active site, although in an opposing manner. If these domains acted independently, a widening of the start site window would be expected with the double mutant.

Our model also includes the role of RPB9 in elongation. We propose that Zn2 and ZnIIS act together during elongation arrest to effect a conformational change of the polymerase active site. The first cleavage event that occurs with TFIIS stimulation of arrested ternary complexes does not involve RPB9, as pol II $\Delta 9$ can perform this reaction (Awrey *et al*, 1997). The occurrence of the second cleavage event is not essential for the polymerase to read through a pause site (Gu *et al*, 1993), but the ability to produce C2 is required for readthrough (Awrey *et al*, 1998); (this study). It is this reaction that requires the concerted effort of RPB9 and TFIIS. It is likely that the first cleavage event brings the 3' end of the transcript and the active site on the template back into register. However, the active is unable to transcribe at this stage. Zn2 and ZnIIS then act together

to induce a rearrangement of the active site, switching the polymerase into a translocation-competent mode. RNA polymerase II then resumes transcribing if nucleotide substrates are available, or performs the second cleavage event if nucleotides are absent.

A recent model proposes that ZnIIS binds the 3' end of the RNA by positioning the transcript across the face of the zinc ribbon, distorting the transcript and exposing the C1 cleavage site. The acidic residues within the ZnIIS loop are suggested to deprotonate key residues of the polymerase active site, priming them for nucleophilic attack of the C1 phosphodiester bond of the transcript (Awrey *et al*, 1998).

We would like to extend this model to include the activity of RPB9. The charges within the loop of Zn2 are predominantly basic, and may perform the complementary function to ZnIIS. We suggest that the basic residues of the Zn2 loop may reprotonate the polymerase residues involved in the cleavage event, thereby resetting the active site. This rebalancing of the charges of the active site may not be the only event required to allow the second cleavage event. However, we propose that it is a critical step in the active site rearrangement prior to reactivation of transcription. The catalytic residues would then be ready for a second interaction with TFIIS, inducing either a resumption of chain elongation or, in the absence of nucleotides, a second cleavage event.

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