Yeast RNA Polymerase II Lacking the Rpb9 Subunit
Is Impaired For Interaction with Transcription Factor IIF

Lynn M. Ziegler, Denys A. Khaperskyy, Michelle L. Ammerman and Alfred S. Ponticelli‡

Department of Biochemistry
School of Medicine and Biomedical Sciences, State University of New York,
Buffalo, New York 14214-3000

(716) 829-2473, FAX (716) 829-2725

E-mail: asp@buffalo.edu

Copyright 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Running Title: Impaired interaction between yeast TFIIF and ΔRpb9 RNAPII

\[\text{Corresponding author}\]
Previous studies have shown that transcription factors IIB (TFIIB), IIF (TFIIF), and RNA polymerase II (RNAPII) play important roles in determining the position of mRNA 5’-ends in the yeast *Saccharomyces cerevisiae*. Yeast strains containing a deletion of the small, non-essential Rpb9 subunit of RNAPII exhibit an upstream shift in the positions of mRNA 5’-ends, whereas mutation of the large subunit of yeast TFIIF (Tfg1) can suppress downstream shifts that are conferred by mutations in TFIIB. In this study, we report an approach for the production of functional recombinant yeast holoTFIIF (Tfg1/Tfg2 complex) and use of the recombinant protein in both reconstituted transcription assays and gel mobility-shifts in order to investigate the biochemical alterations associated with the δRpb9 polymerase. The results demonstrated that upstream shifts in the positions of mRNA 5’-ends could be conferred by the δRpb9 RNAPII in transcription reactions reconstituted with highly-purified yeast general transcription factors, and importantly, that these shifts are associated with an impaired interaction between the δRpb9 polymerase and TFIIF. Potential mechanisms by which an altered interaction between the δRpb9 RNAPII and TFIIF confers an upstream shift in the positions of mRNA 5’-ends are discussed.
INTRODUCTION

The initiation of messenger RNA (mRNA) synthesis by eukaryotic RNA polymerase II (RNAPII) is a critical step in the regulation of eukaryotic gene expression. Accurate and efficient transcription of eukaryotic protein-coding (class II) genes involves the concerted action of RNAPII and a host of accessory proteins. A subset of these proteins are known as the general transcription factors (GTFs) that includes TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (reviewed in 1). The GTFs and additional accessory factors are being intensively studied with the objective of determining their respective functions during the different stages of RNAPII transcription which include 1) formation of a preinitiation complex (PIC) on the promoter; 2) melting of the promoter DNA; 3) transcription initiation; 4) clearance of RNAPII from the promoter; 5) elongation of the nascent transcript; and 6) transcription termination.

Transcription of class II genes is governed by both regulatory DNA sequence elements and core promoter elements. Regulatory elements contain binding sites for protein factors that modulate the overall activity of the promoter and can be located within a few hundred base pairs upstream of the mRNA start sites or at much greater distances either upstream or downstream of the start sites. Core promoter elements serve to direct the location of PIC assembly and comprise a TATA element and/or an Initiator element. TATA elements, containing the consensus sequence TATAa/tAa/t, are located upstream of the mRNA start sites and are specific binding sites for the TATA-binding protein (TBP) subunit of TFIID (2,3). Initiator elements, containing the consensus motif PyPyANt/aPyPy, were identified in class II promoters.
from higher eukaryotic cells and encompass the transcription start site (4). In higher eukaryotes, transcription initiation usually occurs at a discrete site located approximately 25-30 base pairs downstream of the TATA element. In marked contrast, the positions of mRNA 5'-ends in the yeast *Saccharomyces cerevisiae* frequently map to multiple sites ranging from 45-120 base pairs or greater downstream of the TATA element (reviewed in 5,6). The ability of *S. cerevisiae* RNAPII to generate transcripts with 5'-ends mapping far downstream of a TATA element reflects an interesting and fundamental difference in the initiation mechanism relative to that in mammalian cells. Although the mechanistic basis for this difference remains unknown, it is clear from both genetic and biochemical studies that TFIIB and RNAPII play critical roles in determining the positions of mRNA 5'-ends in *S. cerevisiae*. Genetic studies have identified mutations in both TFIIB and the largest subunit of RNAPII (Rpb1) that confer downstream shifts in the positions of mRNA 5'-ends (7-11). In contrast, deletion of the small non-essential Rpb9 subunit of RNAPII, or mutation disrupting a C-terminal zinc-ribbon in Rpb9, confers an upstream shift in the position of mRNA 5'-ends (12,13). Moreover, biochemical studies have shown that the pairwise exchange of *S. cerevisiae* TFIIB and RNAPII with their homologs from *Schizosaccharomyces pombe* in an *in vitro* reconstituted system is sufficient to convert the transcription initiation pattern to that of *S. pombe* (14).

In addition to the roles played by TFIIB and RNAPII, the possibility that TFIIF may also play a role in determining the positions of yeast mRNA 5'-ends was suggested by the identification of a mutation in the large subunit of *S. cerevisiae* TFIIF (Tfg1) as a suppressor of a TFIIB mutation conferring downstream shifts in the positions of mRNA 5'-ends (15). Native *S.
*cerevisiae* TFIIF comprises 3 protein subunits designated Tfg1, Tfg2 and Tfg3. Tfg1 and Tfg2 are homologous to the human TFIIF RAP74 and RAP30 subunits, respectively, and are essential for yeast cell viability (16). The Tfg3 subunit, also known as yeast TAF14, has no known counterpart in mammalian TFIIF and yeast strains containing deletions of Tfg3 are viable.

To date, biochemical analyses of yeast TFIIF have been hampered by the inability to produce recombinant yeast TFIIF due to toxicity of the Tfg1 subunit in *E. coli* (16). In this paper, we describe an approach for the production of functional recombinant yeast holoTFIIF (Tfg1/Tfg2 complex) and report on a comparison of the activities of the recombinant protein *in vitro* to those of TFIIF purified from *S. cerevisiae* extracts (Factor g, Tfg1/Tfg2/Tfg3 complex). Importantly, use of the recombinant TFIIF in reconstituted transcription assays and in gel mobility-shift assays revealed that the alterations in the positions of mRNA 5'-ends conferred by yeast RNAPII lacking the Rpb9 subunit both *in vitro* and *in vivo* is associated with an impaired interaction between the ∆Rpb9 mutant polymerase and TFIIF. We discuss two models for the mechanism by which an altered interaction between the ∆Rpb9 RNAPII and TFIIF confers an upstream shift in the positions of mRNA 5'-ends in *S. cerevisiae*.

**EXPERIMENTAL PROCEDURES**

*Plasmids* - The dual-expression plasmid pRK/Tfg1,2 was constructed using the low-copy broad host range plasmid pRK415 as a starting vector (17) and contains the β-lactamase gene
substituted for the tetracycline-resistance marker and the coding regions for the yeast TFIIF Tfg1 and Tfg2 subunits (Tfg1p: hexahistidine-tag at the C-terminus, myc-epitope tag at the N-terminus; Tfg2p: hexahistidine-tag at the N-terminus) under the control of T7 promoters in adjacent expression cassettes (see Fig. 1A; complete lineage available upon request). Plasmid p316/A has been described previously (9) and contains the yeast ADH1 promoter (positions -500 to +12, where +1 is the initiating ATG) fused to the HIS3 coding region (positions +1 to +900) in vector pRS316. Plasmid pCR-HIS4 contains the yeast HIS4 gene (positions -145 to +744) in vector pCR-Blunt (Invitrogen Inc.).

Production and purification of recombinant yeast holoTFIIF - Overnight cultures of E. coli strain BLR (Novagen) harboring pRK/Tfg1,2 were grown at 37°C in LB media containing 200 µg/ml carbenicillin and then diluted to an initial OD600 of 0.1 in 500 ml of LB media containing 200 µg/ml carbenicillin, 0.2% maltose, 10 mM MgSO4. The culture was grown at 37°C to an OD600 of 1.0 and expression of the recombinant Tfg1 and Tfg2 proteins was induced with the addition of 20 ml bacteriophage CE6 (carrying T7 RNA polymerase, Novagen) in LB media containing 200 µg/ml carbenicillin, 0.2% maltose, 10 mM MgSO4 at a titer of 1.0 x 10^10 pfu/ml.

The culture was incubated at 37°C without shaking for 15 min., grown with shaking at 37°C for 3 hrs., and the cells were harvested by centrifugation and frozen at -80°C. Frozen cell pellets were thawed on ice, resuspended with 10 ml of ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 1 mM PMSF) containing 2 mg/ml lysozyme, and the suspension
was incubated on ice for 30 min. with occasional mixing. Cells were lysed by the addition of 1 ml of 10% Triton X-100 (in lysis buffer), and MgCl$_2$, RNAse A and DNAse I were added to final concentrations of 10 mM, 100 $\mu$g/ml and 40 $\mu$g/ml, respectively. The suspension was mixed by inversion, incubated on ice for 45 min. with occasional mixing, and then centrifuged at 15,000 x g for 20 min. at 4°C. The cleared supernatant was adjusted to a final concentration of 5 mM imidazole, batch loaded onto 1 ml Ni-NTA resin (Qiagen) for 60 min. at 4°C, and the resin was subsequently packed into a column. The column was washed sequentially with 30 ml of lysis buffer containing 20 mM imidazole, 10 ml of lysis buffer containing 30 mM imidazole, and the protein was eluted with 10 ml of lysis buffer containing 250 mM imidazole, collecting 1 ml fractions. Peak fractions of Tfg1,2 protein were pooled (2-3 ml final), diluted with an equal volume of ice-cold TE$_{500}$ (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 500 mM NaCl, 1 mM PMSF), and batch loaded onto a 1 ml myc-antibody column with gentle mixing overnight at 4°C (column prepared using cyanogen-bromide activated Sepharose and DEAE-purified 9E10 antibody from ascites fluid, Covance). The resin was packed into a column and washed sequentially with 8 ml cold TE$_{500}$ and 8 ml cold TE$_{500}$ containing 30% glycerol. Protein elution was initiated by the addition of 0.75 ml of 0.5 mg/ml myc peptide (Roche) in TE$_{500}$ containing 15% glycerol and incubation for 20 min. at 30°C with occasional mixing. The eluant was collected and the resin was washed twice by incubating with 1 ml TE (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM PMSF) containing 20% glycerol for 10 min. at 30°C and collecting both wash fractions. Peak fractions of holoTFIIF (Tfg1/Tfg2 complex) that were eluted from the antibody column from two
preps (500 ml cultures each) were pooled (about 5 ml total), dialyzed against 500 ml Buffer F (20 mM HEPES-KOH pH 7.6, 250 mM potassium acetate, 20% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 mM Benzamidine) at 4°C for 3 hrs, and then concentrated to approximately 0.5 ml using an Ultrafree-4-Biomax 5KNMWL spin column (Millipore). Typical yield was 100-150 µg of holoTFIIF protein per 1 liter of starting culture.

**Purification of yeast TBP, TFIIB, RNAPII, TFIIE and TFIIH** - Recombinant hexahistidine-tagged yeast TBP was purified essentially as described previously (18) and stored in Buffer D (25 mM HEPES-KOH pH 7.6, 200 mM potassium acetate, 20% glycerol, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.01% NP40). Recombinant hexahistidine-tagged yeast TFIIB was purified as described previously (19) and stored in Buffer B (20 mM HEPES-KOH pH 7.6, 100 mM potassium acetate, 20% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 µM zinc acetate). Recombinant yeast holoTFIIE (6HisTfa1/Tfa2 complex) was purified through the DEAE column step as described previously (20) and stored in Buffer E (20 mM HEPES-KOH pH 7.6, 100 mM potassium acetate, 15% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). Yeast holoTFIIH was purified from yeast strain YPH/TFB1 6His through the Mono-Q HPLC column step as described previously (20,21) and stored in Buffer H (20 mM HEPES-KOH pH 7.6, 600 mM potassium acetate, 20% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). Yeast RNA polymerase II was purified from strain BJ2168 and from a strain containing a deletion of the Rpb9 subunit (WY9, reference (12) essentially as described previously (22) but with the following modifications: Cells were harvested and washed with 0.2 volumes of 5X Buffer A (250 mM Tris-HCl pH 7.9, 50% glycerol, 50 mM DTT, 5 mM EDTA, 5% DMSO, 50 µM ZnCl₂, protease inhibitors). After cell disruption,
the supernatant was decanted from the glass beads and the beads were washed with 1X Buffer A until the wash was clear. The supernatant and washes were pooled and adjusted to a final concentration of 150 mM KCl and centrifuged at 15,000 rpm for 45 min. in a JLA-17 rotor at 4°C. The cleared supernatant was fractionated by heparin-Sepharose chromatography and protein was precipitated by the addition of solid ammonium sulfate to 50% of saturation as described. The pellet was resuspended in Buffer B’ (50 mM Tris-HCl pH 7.5, 10 mM DTT, 1 mM EDTA, 10µM ZnCl₂, protease inhibitors) to a conductivity corresponding to an ammonium sulfate concentration of 500 mM and passed over a DEAE-Sephacel column. Protein in the flow-through was again concentrated by ammonium sulfate precipitation and the pellet was resuspended in Buffer B’ to a conductivity corresponding to an ammonium sulfate concentration of 500 mM. The solution was bound to monoclonal antibody 8WG16-Sepharose for 2 hours at 4°C, and the resin was washed and protein was eluted as described. The eluant was dialyzed against Buffer P (50 mM Tris-HCl pH 7.5, 10% glycerol, 100 mM ammonium sulfate, 10 mM DTT, 1mM EDTA, 10µM ZnCl₂) and concentrated using an Ultrafree-4-Biomax 5KMWL spin column.

**Reconstituted transcription assays** - Transcription reactions (30 µl final volume) contained 50 mM HEPES-KOH pH 7.6, 5-8% glycerol, 80 mM potassium acetate, 10 mM magnesium acetate, 5 mM EGTA, 2.5 mM DTT, 40 units RNAsin (Promega Inc.), 30 mM creatine phosphate, 1.5 units/ml creatine kinase, 250 ng plasmid template, 50 ng TBP, 150 ng TFIIB, 300 ng RNAPII, 250 ng TFIIF, 30 ng TFIIE and 60 ng TFIIH. Reaction components were initially...
mixed at 4°C and then incubated for 30 min. at room temperature. Transcription was then initiated by the addition of NTPs and the reactions were incubated for an additional 20 min. at room temperature. When using the CYC1/G-less cassette template pG5CG-, reactions also contained 330 µM 3'-O-methyl GTP, 10 µCi of α-32P-UTP, and transcription was initiated by the addition of 1 µl 30X G-NTPs (12 mM ATP, 12 mM CTP, 45 µM UTP). G-less cassette reactions were terminated, treated with RNAse T1, and the products subjected to electrophoresis on 7% denaturing polyacrylamide gels as described previously (10). For reactions using templates containing the ADH1 promoter (p316/A) or HIS4 promoter (pCR-HIS4), transcription was initiated by the addition of 1 µl 12 mM NTPs, terminated after 20 min. with the addition of 300 µl of stop buffer (100 mM sodium acetate pH 5.2, 10 mM EDTA, 1% SDS, 75 µg/ml carrier tRNA), and then treated with Proteinase K for 20 min. at 37°C (170 µg/ml final). The products were extracted twice with phenol-chloroform, ethanol precipitated, and the transcripts were analyzed by primer extension as described below.

Primer extension mapping of mRNA 5'-ends - Total in vivo RNA (30 µg) or the products of reconstituted in vitro transcription reactions were analyzed by primer extension as described previously (10). In vivo RNA was analyzed using either an ADH1-specific primer (5'-dGTATTCCAACCTACGGATCG-3', corresponding to positions +63 to +39, where +1 is the initiating ATG) or a HIS4-specific primer (5'-dCAGATCATACATTAACCGGTAG-3', corresponding to positions +36 to +16, where +1 is the initiating ATG). The products of reconstituted in vitro transcription reactions with plasmid pCR-HIS4 as template were analyzed using the HIS4-specific primer; reactions with plasmid p316/A as template were analyzed using
a HIS3-specific primer (5’-dGGTTTACATTTGTAATACGCTTTAC-3’, corresponding to positions +45 to +22, where +1 is the initiating ATG)

*Electrophoretic mobility-shift assays* - The TATA-containing DNA probe was a 45 base pair internally labeled fragment containing the Adenovirus early 1B TATA box (23). Reactions (20 µl) contained 20 mM HEPES-KOH pH 7.6, 5% glycerol, 80 mM potassium acetate, 50 mM potassium chloride, 10 mM magnesium acetate, 2.5 mM DTT, 50 µg/ml BSA, 200 ng poly (dG-dC), 20 fmoles labeled probe, and the amount of purified transcription factors as indicated in the figure legends. Reaction components were mixed at 4°C and the reactions were incubated at room temperature for 30 min. Ten µl of the reaction products was then immediately loaded onto a native gel polyacrylamide gel (5% polyacrylamide, 30:1) and the gels run at 4°C in 25 mM Tris, 190 mM glycine (pH 8.3) at 200 V for 2 hrs.

**RESULTS**

*Production and characterization of recombinant yeast TFIIF* - As noted earlier, biochemical studies of yeast TFIIF have been hampered by the inability to produce recombinant protein due to toxicity of the Tfg1 subunit in *E. coli* (16). Previous attempts to produce recombinant Tfg1 revealed that the vast majority of successfully expressed Tfg1 protein underwent proteolytic cleavage in *E. coli* (Ziegler and Ponticelli, unpublished results). We speculated that proteolytic cleavage of Tfg1, and perhaps the associated toxicity of the Tfg1 plasmid, might be reduced by co-expression of the yeast TFIIF Tfg2 subunit. Presented in Figure 1A is an outline of the
scheme used for the expression and purification of recombinant holoTFIIF (Tfg1/Tfg2 complex). The main features of the approach included 1) the use of a low-copy broad host range vector for construction of the plasmid with Tfg1 and Tfg2 expression under the control of adjacent T7 promoters; 2) the addition of a hexahistidine-tag at the C-terminus of Tfg1, a myc-epitope tag at the N-terminus of Tfg1, and a hexahistidine-tag at the N-terminus of Tfg2; 3) the propagation of the expression plasmid in a recombination-deficient E. coli strain lacking T7 RNA polymerase; 4) the induction of Tfg1 and Tfg2 expression by infection with bacteriophage lambda carrying T7 RNA polymerase; and 5) the purification of the Tfg1/Tfg2 complex containing full-length Tfg1 protein by sequential chromatography on nickel agarose and myc-antibody columns (see Experimental Procedures for details). The combined features of this scheme resulted in decreased proteolytic cleavage of the Tfg1 subunit, decreased toxicity/instability of the expression plasmid, and recovery of the Tfg1 and Tfg2 recombinant proteins in apparently stoichiometric amounts at greater than 95% purity (Fig. 1B, data not shown).

To assess the functionality of the recombinant Tfg1/Tfg2 complex, the activity of the recombinant protein was compared to that of purified native yeast TFIIF ("Factor g", Tfg1/Tfg2/Tfg3 complex) using both gel electrophoretic mobility-shift assays and reconstituted transcription assays in vitro. The recombinant Tfg1/Tfg2 complex exhibited a slightly higher activity than purified Factor g for both the formation of TBP-TFIIB-TFIIF-RNAPII-DNA complexes in the electrophoretic mobility-shift assays (DBPolF, Fig. 2A) and for supporting transcription in the reconstituted assays (Fig. 2B). These results indicated that the recombinant
Tfg1/Tfg2 complex was apparently fully active for both the assembly of higher-order transcription complexes and for supporting transcription in vitro.

Yeast RNAPII lacking the Rpb9 subunit confers upstream shifts in the positions of mRNA 5’-ends in vivo and in reconstituted transcription assays in vitro - Previous studies demonstrated that yeast RNAPII lacking the Rpb9 subunit confers upstream shifts in the positions of mRNA 5’-ends both in vivo and in transcription assays in vitro using nuclear extracts (12). To begin an investigation of the altered protein-protein interactions responsible for the shifts in mRNA 5’-ends conferred by the $\Delta$Rpb9 RNAPII, we tested whether upstream shifts in the positions of mRNA 5’-ends are generated in vitro using a well-defined reconstituted transcription system containing highly purified yeast general transcription factors. Transcription assays were performed using plasmid templates containing either the S. cerevisiae ADH1 or HIS4 promoters, purified recombinant factors (TBP, TFIIB, TFIIE and TFIIF), and wild-type RNAPII, the $\Delta$Rpb9 RNAPII, and TFIIH purified from yeast extracts (see Experimental Procedures for details). Primer extension analyses of the transcription reaction products revealed that, compared to the reactions containing wild-type RNAPII, the reactions containing the $\Delta$Rpb9 RNAPII yielded diminished amounts of more downstream transcripts and/or enhanced levels of more upstream transcripts from both the ADH1 and HIS4 promoters (Fig. 3A, lanes 2, 3; Fig. 3B, lanes 2, 3). These results demonstrated that upstream shifts in mRNA 5’-ends conferred by the $\Delta$Rpb9 RNAPII were generated in the well-defined reconstituted transcription system and suggested that the altered interaction(s) responsible for the shifts involve the $\Delta$Rpb9 RNAPII and one or more of the purified general transcription factors.
Yeast RNAPII lacking the Rpb9 subunit is impaired for interaction with TFIIF - To further investigate the altered protein-protein interactions involving the ΔRpb9 RNAPII, electrophoretic mobility-shift assays were utilized to compare the relative activities of wild-type RNAPII and the ΔRpb9 RNAPII in the formation of stable intermediates of PICs. Initial control reactions containing only individual transcription factors demonstrated the ability of TBP, wild-type RNAPII, ΔRpb9 RNAPII, and TFIIH to form stable complexes with the DNA probe, whereas no binding was detected with either TFIIF or TFIIE (Fig. 4A). In reactions containing combinations of the factors leading to PIC formation, the results demonstrated that the wild-type and ΔRpb9 polymerases were equally proficient for the formation of stable TBP-TFIIB-RNAPII-DNA (DBPol) complexes (Fig. 4B, lanes 2, 3). In sharp contrast, reactions containing the ΔRpb9 RNAPII yielded dramatically reduced levels of the next higher-order complex containing TFIIF (TBP-TFIIB-RNAPII-TFIIF-DNA (DBPolF) complexes) (Fig. 4B, lanes 4, 5), as well as the subsequent higher-order complexes that also contain TFIIE and TFIIH (Fig. 4B, lanes 6-9).

In light of the observed proficiency of the ΔRpb9 polymerase in the formation of DBPol complexes, the dramatically reduced activity of the ΔRpb9 polymerase in the formation of the next higher-order complexes containing TFIIF (DBPolF) suggested an impairment in the interaction between the ΔRpb9 RNAPII and TFIIF. However, an alternative possibility is that the ΔRpb9 RNAPII is proficient for the formation of DBPol complexes, but the resulting complexes have an altered structure that impairs the subsequent association of TFIIF to TFIIB and/or RNAPII. To discriminate between these possibilities, electrophoretic mobility-shift assays were
performed with the omission of TBP and TFIIB from the binding reactions in order to assess the direct association of TFIIF to either the wild-type or ΔRpb9 RNAPII. The results demonstrated that the wild-type and ΔRpb9 polymerases were equally active in the formation of DNA-RNAPII (Pol) complexes (Fig. 5B, lanes 2, 4), but that the ΔRpb9 polymerase was dramatically impaired for the association of TFIIF (PolF complexes, Fig. 5B, lanes 3, 5).

DISCUSSION

In this study, we have investigated the molecular basis underlying the alterations in the positions of mRNA 5′-ends that are conferred both in vivo and in vitro by an S. cerevisiae RNA polymerase II mutant lacking the Rpb9 subunit. To facilitate these studies, we developed an approach for the production of functional recombinant yeast holoTFIIF (Tfg1/Tfg2 complex) and demonstrated that the recombinant protein was as active as native TFIIF (Tfg1/Tfg2/Tfg3 complex) purified from yeast extracts for both the formation of TBP-TFIIB-TFIIF-RNAPII-DNA complexes and for supporting transcription in reconstituted in vitro assays. Incorporation of purified ΔRpb9 RNAPII into the reconstituted transcription assays, which included RNAPII and TFIIH purified from yeast extracts and purified recombinant yeast factors TBP, TFIIB, TFIIF and TFIIIE, demonstrated that alterations in the positions of mRNA 5′-ends conferred by the ΔRpb9 polymerase could be generated in a well-defined system of purified yeast general transcription factors. Electrophoretic mobility-shift assays utilizing the purified factors subsequently revealed that the alterations in the positions of mRNA 5′-ends were associated with an impaired interaction between the ΔRpb9 polymerase and TFIIF.
The purification of native *S. cerevisiae* TFIIF, also known as yeast Factor g, led to the identification of three co-purifying polypeptides that were designated Tfg1, Tfg2 and Tfg3 (16,24). The Tfg3 polypeptide was found to be identical to yeast TAF14/ANC1, which is also a component of the TFIID complex and the SWI/SNF chromatin remodeling complex (25-27). Since yeast strains containing deletions of Tfg3 are viable, further studies are needed in order to elucidate the potential roles of the Tfg3 polypeptide in the activities of the TFIID, TFIIF and SWI/SNF complexes (16). The Tfg1 and Tfg2 polypeptides were found to be the *S. cerevisiae* homologs of the human TFIIF RAP74 and RAP30 subunits, respectively, and their presence is required for the maintenance of yeast cell viability (16). In contrast to the situation with the Tfg2 polypeptide, attempts to produce recombinant Tfg1 protein were previously unsuccessful, apparently due to toxicity of the Tfg1 expression plasmid in *E. coli* (16). To facilitate biochemical studies of yeast TFIIF, we developed an approach for the co-expression of the recombinant Tfg1 and Tfg2 polypeptides and purification of a Tfg1/Tfg2 complex. Biochemical assays comparing the activities of the recombinant Tfg1/Tfg2 complex to those of native TFIIF purified from yeast extracts (Factor g, Tfg1/Tfg2/Tfg3 complex) demonstrated that the recombinant Tfg1/Tfg2 complex was slightly more active than purified Factor g for both the formation of TBP-TFIIB-TFIIF-RNAPII-DNA (DBFPol) complexes in electrophoretic mobility-shift assays and for supporting transcription from the yeast CYC1 promoter in reconstituted reactions (Figs. 2A, B). These results indicated that the recombinant Tfg1/Tfg2 complex was apparently fully active for both higher-order transcription complex assembly and for transcription *in vitro* and they suggest that the presence of the Tfg3 polypeptide has no significant effect on the activity of the purified native TFIIF in the assays employed. Further studies will be required in order to determine
whether the Tfg3 polypeptide affects the activity of yeast TFIIF in the transcription from other yeast promoters and/or from chromatin templates.

As discussed earlier, previous work demonstrated that a yeast RNAPII mutant lacking the small non-essential Rpb9 subunit confers upstream shifts in the positions of mRNA 5'-ends both in vivo and in transcription assays in vitro using nuclear extracts (12). In the studies reported here, we confirmed that yeast strains containing a deletion of Rpb9 manifest upstream shifts at the ADH1 and HIS4 promoters in vivo and demonstrated that alterations in the positions of mRNA 5'-ends could also be conferred by the ∆Rpb9 polymerase in vitro in a well-defined reconstituted transcription system containing highly purified yeast general transcription factors (Fig. 3). These results suggested that the underlying biochemical basis for the alterations in the positions of mRNA 5'-ends conferred by the ∆Rpb9 RNAPII involves an altered interaction involving the mutant polymerase and one or more of the purified general transcription factors in the reconstituted reactions. In this regard, it was recently reported that a GAL4-Rpb9 fusion protein containing the GAL4 DNA binding domain fused to a truncated Rpb9 lacking the N-terminal 32 amino acids interacted with the large subunit of yeast TFIIE (Tfa1) in a yeast two-hybrid assay (28). Although this result suggests that Rpb9 might physically interact with TFIIE, the detected interaction in the two-hybrid assay was reported to be very weak with a GAL4-Rpb9 fusion protein containing the entire Rpb9 coding sequence. In addition, the proficiency of RNAPII lacking the Rpb9 subunit in the assembly of stable higher-order intermediate complexes leading to PIC formation was not directly tested. The use of highly-purified general transcription factors and electrophoretic gel-mobility assays in our work reported here revealed
that the ΔRpb9 RNAPII is impaired for direct interaction with yeast TFIIF (Fig. 5B). Moreover, association of TFIIE to TBP-TFIIB-TFIIF-RNAPII-DNA (DBFPol) complexes containing the ΔRpb9 polymerase was detected in the mobility-shift assays (Fig. 4B, lanes 5 vs. 7). Thus, we suggest that the primary biochemical alteration resulting from deletion of the Rpb9 subunit is an impaired TFIIF-RNAPII interaction. Our results do not exclude the possibility that an Rpb9-TFIIE interaction is manifest subsequent to PIC formation / transcription initiation and plays a role in the activity of RNAPII during transcript elongation.

How might an altered interaction between the ΔRpb9 RNAPII and TFIIF confer an upstream shift in the position of mRNA 5'-ends in S. cerevisiae? To address this issue, one must consider the potential mechanism of RNAPII transcription initiation in S. cerevisiae. As noted earlier, S. cerevisiae exhibits a unique transcription initiation pattern compared to other eukaryotes, with mRNA 5'-ends frequently mapping to multiple sites ranging from 45-120 base pairs or greater downstream of the TATA element (reviewed in 5,6). Although the mechanism by which this is achieved remains to be determined, it has been proposed that yeast RNAPII may be released from the preinitiation complex and scan the downstream sequence for preferred initiation sites (29). The fact that TFIIF has been shown to directly interact with RNAPII to affect transient pausing and processivity strongly suggests that a TFIIF-RNAPII interaction plays a role in the DNA recognition/interaction properties of RNAPII (30,31). Thus, within the context of a scanning polymerase model for S. cerevisiae transcription initiation, an impaired interaction between the ΔRpb9 RNAPII and TFIIF could result in an increased number of the mutant polymerases escaping the promoter without the accompaniment of TFIIF. In the
absence of bound TFIIF, the ΔRpb9 polymerase could exhibit enhanced pausing/recognition of sub-optimal start site sequences encountered early in the scanning process, resulting in the observed upstream shifts in the positions of mRNA 5'-ends.

An alternative explanation for the ability of *S. cerevisiae* RNAPII to initiate transcription far downstream of a TATA element is that the architecture of yeast PICs is significantly different than that of higher eukaryotic PICs. Although the overall structure of *S. cerevisiae* PICs has yet to be reported, published work from numerous laboratories strongly support a model for the architecture of mammalian PICs that involves the wrapping of approximately 90 base pairs of promoter DNA around the PIC (32-36). The wrapping of promoter DNA around a mammalian PIC is reportedly induced by TFIIF and involves DNA sequences ranging from about 30 base pairs upstream to 55 base pairs downstream of the TATA element (32). Since mammalian TFIIF functions as a heterotetramer of the RAP30 and RAP74 subunits and the yeast TFIIF Tfg1 and Tfg2 subunits are both significantly larger than their mammalian counterparts (16), it is conceivable that yeast PICs have the potential to wrap significantly more promoter DNA than mammalian PICs in order to contact transcription start sites further downstream of the TATA element. Within the context of a DNA-wrapping model for the mechanism of *S. cerevisiae* transcription initiation, an altered interaction between the ΔRpb9 polymerase and TFIIF could affect the extent and/or tightness of DNA wrapping that is induced by TFIIF, thereby resulting in a subpopulation of PICs that initiate transcription closer to the TATA element than normal. Continued biochemical analyses regarding both the architecture and post-assembly activities of PICs containing wild-type or mutant RNAPII should provide additional insight into the
mechanism by which the positions of mRNA 5'-ends are determined in *S. cerevisiae*.

**Acknowledgements** - The authors thank Yuichiro Takagi and Roger Kornberg for providing purified yeast Factor g, plasmid Dabp28 for the production of recombinant yeast TFIIIE, and yeast strain YPH/TFB1 6His for the purification of yeast TFIIH. Members of the Ponticelli laboratory are also thanked for helpful discussions and comments on the manuscript. This work was supported by a Public Health Service grant (GM51124) from the National Institutes of Health to A.S.P.

**REFERENCES**

1. Orphanides, G., Lagrange, T., and Reinberg, D. (1996) *Genes Dev* **10**, 2657-2683.
2. Nakajima, N., Horikoshi, M., and Roeder, R. G. (1988) *Mol Cell Biol* **8**, 4028-4040.
3. Parker, C. S., and Topol, J. (1984) *Cell* **36**, 357-369.
4. Smale, S. T., and Kadonaga, J. T. (2003) *Annu Rev Biochem* **19**, 19
5. Guarente, L. (1987) *Annu. Rev. Genet.*** **21**, 425-452.
6. Struhl, K. (1987) *Cell* **49**, 295-297.
7. Bangur, C. S., Pardee, T. S., and Ponticelli, A. S. (1997) *Mol Cell Biol* **17**, 6784-6793.
8. Berroteran, R. W., Ware, D. E., and Hampsey, M. (1994) *Mol Cell Biol* **14**, 226-237.
9. Faitar, S. L., Brodie, S. A., and Ponticelli, A. S. (2001) *Mol Cell Biol* **21**, 4427-4440.
10. Pardee, T. S., Bangur, C. S., and Ponticelli, A. S. (1998) *J Biol Chem* **273**, 17859-17864.
11. Pinto, I., Wu, W. H., Na, J. G., and Hampsey, M. (1994) *Journal of Biological Chemistry* **269**, 30569-30573

12. Hull, M. W., McKune, K., and Woychik, N. A. (1995) *Genes Dev* **9**, 481-490.

13. Sun, Z. W., Tessmer, A., and Hampsey, M. (1996) *Nucleic Acids Res* **24**, 2560-2566.

14. Li, Y., Flanagan, P. M., Tschochner, H., and Kornberg, R. D. (1994) *Science* **263**, 805-807.

15. Sun, Z. W., and Hampsey, M. (1995) *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3127-3131

16. Henry, N. L., Campbell, A. M., Feaver, W. J., Poon, D., Weil, P. A., and Kornberg, R. D. (1994) *Genes Dev* **8**, 2868-2878.

17. Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988) *Gene* **70**, 191-197.

18. Lee, M., and Struhl, K. (1997) *Mol Cell Biol* **17**, 1336-1345.

19. Bangur, C. S., Faitar, S. L., Folster, J. P., and Ponticelli, A. S. (1999) *J Biol Chem* **274**, 23203-23209.

20. Myers, L. C., Leuther, K., Bushnell, D. A., Gustafsson, C. M., and Kornberg, R. D. (1997) *Methods* **12**, 212-216.

21. Svejstrup, J. Q., Feaver, W. J., LaPointe, J., and Kornberg, R. D. (1994) *J Biol Chem* **269**, 28044-28048.

22. Edwards, A. M., Darst, S. A., Feaver, W. J., Thompson, N. E., Burgess, R. R., and Kornberg, R. D. (1990) *Proc Natl Acad Sci U S A* **87**, 2122-2126.

23. Kao, C. C., Lieberman, P. M., Schmidt, M. C., Zhou, Q., Pei, R., and Berk, A. J. (1990) *Science* **248**, 1646-1650

24. Henry, N. L., Sayre, M. H., and Kornberg, R. D. (1992) *J Biol Chem* **267**, 23388-23392.
25. Burns, L. G., and Peterson, C. L. (1997) Biochim Biophys Acta 1350, 159-168.

26. Cairns, B. R., Henry, N. L., and Kornberg, R. D. (1996) Mol Cell Biol 16, 3308-3316.

27. Welch, M. D., Vinh, D. B., Okamura, H. H., and Drubin, D. G. (1993) Genetics 135, 265-274.

28. Van Mullem, V., Wery, M., Werner, M., Vandenhaute, J., and Thuriaux, P. (2002) J Biol Chem 277, 10220-10225.

29. Giardina, C., and Lis, J. T. (1993) Science 261, 759-762.

30. Tan, S., Aso, T., Conaway, R. C., and Conaway, J. W. (1994) J Biol Chem 269, 25684-25691.

31. Kephart, D. D., Wang, B. Q., Burton, Z. F., and Price, D. H. (1994) J Biol Chem 269, 13536-13543.

32. Robert, F., Douziech, M., Forget, D., Egly, J. M., Greenblatt, J., Burton, Z. F., and Coulombe, B. (1998) Mol Cell 2, 341-351.

33. Forget, D., Robert, F., Grondin, G., Burton, Z. F., Greenblatt, J., and Coulombe, B. (1997) Proc Natl Acad Sci U S A 94, 7150-7155.

34. Kim, T. K., Lagrange, T., Wang, Y. H., Griffith, J. D., Reinberg, D., and Ebright, R. H. (1997) Proc Natl Acad Sci U S A 94, 12268-12273.

35. Darst, S. A., Edwards, A. M., Kubalek, E. W., and Kornberg, R. D. (1991) Cell 66, 121-128.

36. Buratowski, S., Hahn, S., Guarente, L., and Sharp, P. A. (1989) Cell 56, 549-561.

FIGURE LEGENDS
FIG. 1. **Production and purification of recombinant yeast TFIIF.**  
A, Scheme used for the expression and purification of recombinant yeast TFIIF.  
B, Myc-antibody column purification of recombinant yeast TFIIF. Shown is a SDS 8% polyacrylamide gel stained with Coomassie Blue R-250 containing molecular mass standards (lanes 1, 6); the Ni-NTA column eluant that was loaded onto the myc-antibody column (L, lane 2); the myc-antibody column flow-through (FT lane 3); the pooled myc-antibody column wash fractions (W, lane 4); and the eluant from the myc-antibody column (E, lane 5). The mobilities of the Tfg1p and Tfg2p TFIIF subunits are designated by the arrows.

FIG. 2. **Invitro functional comparison of recombinant yeast TFIIF vs. native TFIIF purified from yeast extract.**  
A, Electrophoretic mobility shift assay. Reactions contained a $^{32}$P-labeled 45 base pair DNA fragment containing the adenovirus early 1B TATA element, 10 ng recombinant yeast TBP, 50 ng recombinant yeast TFIIB, 100 ng yeast RNAPII, and either 50 ng TFIIF purified from yeast extract or 25 ng, 50 ng, or 100 ng of recombinant yeast TFIIF. The positions of the TBP-DNA (D), TBP-TFIIB-DNA (DB), TBP-TFIIB-RNAPII-DNA (DBPol), TBP-TFIIB-RNAPII-recombinant TFIIF-DNA (DBPolF') and TBP-TFIIB-RNAPII-native TFIIF-DNA (DBPolF) complexes are indicated by the arrows.  
B, Transcriptional activity. Transcription reactions contained 250 ng of plasmid DNA template (pG5CG-), 50 ng TBP, 150 ng TFIIB, 300 ng RNAPII, 30 ng recombinant yeast TFIIE, 60 ng yeast TFIIH and the indicated amount of either recombinant or native TFIIF.
FIG. 3. **RNAPII lacking the Rpb9 subunit confers an upstream shift in the position of mRNA 5'-ends both *in vivo* and in reactions reconstituted with purified GTFs *in vitro***. Transcript 5'-ends were mapped by primer extension using an ADH1-specific (A) or HIS4-specific (B) oligonucleotide primer and either RNA synthesized *in vitro* from reconstituted reactions with wild-type or ΔRpb9 RNAPII (lanes 1-3) or total RNA isolated from yeast strains N220 or WY9, which contain either wild-type or ΔRpb9 RNAPII, respectively (lanes 4-5).

FIG. 4. **RNAPII lacking the Rpb9 subunit is impaired for formation of stable TBP-TFIIB-TFIIF-RNAPII-DNA complexes**. A, Electrophoretic mobility-shift assays with individual transcription factors. Reactions contained the 45 base pair 32P-labeled E1B TATA probe and either 10 ng TBP, 100 ng TFIIB, 100 ng recombinant TFIIF, 100 ng wild-type RNAPII, 100 ng ΔRpb9 RNAPII, 90 ng TFIIE, or 60 ng TFIIH. B, Electrophoretic mobility-shift assays for higher-order complex formation. Reactions were performed as above but contained the indicated combinations of TBP, TFIIB, wild-type RNAPII, ΔRpb9 RNAPII, TFIIF, TFIIE and TFIIH. The mobilities of the TBP-TFIIB-RNAPII-DNA (DBPol) and TBP-TFIIB-RNAPII-TFIIF-DNA (DBPolF) are indicated.

FIG. 5. **RNAPII lacking the Rpb9 subunit is impaired for interaction with TFIIF**. A, SDS-16% polyacrylamide gel (stained with silver) containing 2 µg of either wild-type or ΔRpb9 RNAPII used in these experiments. Shown on the right is a portion of the polyacrylamide gel stained for a longer time with silver to demonstrate the presence of the Rpb6 subunit. B, electrophoretic mobility shift assay. Reactions contained the labeled E1B TATA probe and 100 ng of wild-type
RNAPII, 100 ng of ΔRpb9 RNAPII or 100 ng recombinant TFIIF as indicated. The mobilities of the RNAPII-DNA (Pol) and RNAPII-TFIIF-DNA (PolF) complexes are indicated.
**A**

- In E. coli strain BLR
- pRK/Tfg1,2
- TFG1
- TFG2
- T7
- Myc
- 6His
- Amp

- Infect with λphage carrying T7 RNAP
- 37°C, 3 hrs.
- Lysate to Ni-NTA column
- Ni-NTA eluant to Myc antibody column

**B**

- L
- FT
- W
- E

- Tfg1p
- Tfg2p
Yeast RNA polymerase II lacking the Rpb9 subunit is impaired for interaction with transcription factor IIIF
Lynn M. Ziegler, Denys A. Khaperskyy, Michelle L. Ammerman and Alfred S. Ponticelli

J. Biol. Chem. published online September 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309656200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts