Yeast RNA Polymerase II Subunit RPB9
MAPPING OF DOMAINS REQUIRED FOR TRANSCRIPTION ELONGATION*

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The RPB9 subunit of RNA polymerase II regulates transcription elongation activity and is required for the action of the transcription elongation factor, TFIIIS. RPB9 comprises two zinc ribbon domains joined by a conserved linker region. The C-terminal zinc ribbon is similar in sequence to that found in TFIIIS. To elucidate the relationship between the structure and transcription elongation function of RPB9, we initiated a mutagenesis study on the Saccharomyces cerevisiae homologue. The individual zinc ribbon domains, in isolation or in combination, could not stimulate transcription by a polymerase lacking RPB9, pol IIΔ9. Mutations in the N-terminal zinc ribbon had little effect on transcription activity. By contrast, mutations in the acidic loop that connects the second and third β-strands of the C-terminal zinc ribbon were completely inactive for transcription. Interestingly, the analogous residues in TFIIIS are also critical for elongation activity. A conserved charged stretch in the linker region (residues 89–95, DPTLPR) mediated the interaction with RNA polymerase II.

The eukaryotic transcription elongation factor TFIIIS stimulates RNA polymerase II to cleave the 3′ end of nascent transcripts, which in turn promotes transcription through blocks to elongation. TFIIIS homologues have been identified in species ranging from yeast to man. The protein is composed of three structurally independent domains (I, II, and III) (1), which comprise a four-helix bundle, a three-helix bundle, and a zinc ribbon domain, respectively (1–4). The central three-helix bundle mediates binding to RNA polymerase II (5), whereas residues within the zinc ribbon region are important for transcript cleavage (6).

The zinc ribbon is defined by a conserved zinc-chelating motif, CX6CX42CX6C, that forms a three-stranded anti-parallel β-sheet with the four cysteines coordinating the zinc ion (2, 4). Although first identified in TFIIIS, sequence and structural analysis studies have suggested that zinc ribbons are present in many eukaryotic transcription-associated proteins, including transcription factors TFIIA and TFIIE, as well as RNA polymerase II subunits RPB1, RPB2, RPB12, and RPB9 (7, 8). RPB9 is composed primarily of two zinc-binding regions, which are defined by the CX6CX42CX6C motif. The C-terminal zinc domain is very similar (30% identity) to the zinc ribbon domain from TFIIIS (9). RPB9 is highly conserved between yeast (10), archaea (9, 11), Drosophila (12), plants (13), and humans (14). In addition, analogous subunits have been identified as components of the eukaryotic RNA polymerases I and III (15). 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 (17). However, the functional conservation is not universal; Drosophila RPB9 is not functional in the yeast transcription system (13).

The conservation of the zinc ribbon structural motif between TFIIIS and RPB9 extends to function. RPB9 is required for TFIIIS to stimulate elongation readthrough and transcript cleavage (18). RNA polymerase II lacking RPB9 (pol IIΔ9) pauses at intrinsic blocks to elongation at much lower frequency than wild type RNA polymerase II in vitro elongation assays. Pol IIΔ9 is also unable to respond to stimulation by TFIIIS in assays measuring either transcript cleavage or reactivation of elongation at arrest sites. In addition, yeast strains that are deficient for either TFIIIS (19) or RPB9 are both sensitive to the drug 6-azauracil. This drug lowers the cellular pool of GTP and is thought to hamper the process of transcription elongation (21). Strains lacking RPB9 also exhibit slow growth at optimal temperature, as well as cold- and temperature-sensitive phenotypes (10).

RPB9 is also involved in the initiation of transcription. Specifically, RPB9 helps to select the transcript start sites (13, 22, 23). Deletion or disruption of RPB9 in yeast leads to an upstream shift in the start sites at the majority of promoters both in vivo and in vitro (13, 22). In some instances, upstream sites that are normally used infrequently become more commonly used and in others new upstream initiation sites are used.

We initiated this study to explore the relationship between the structure and function of RPB9. We identified the RPB9 residues that mediate the interaction with the Δ9 enzyme, as well as those that are involved in the transcriptional response of pol II to TFIIIS during elongation arrest.

MATERIALS AND METHODS

Purification of Yeast TFIIIS, pol II, and pol IIΔ9—Recombinant wild type yeast TFIIIS used in this study was concentrated to between 1 and 3 mg/ml and stored at −70 °C in 20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM DTT, 10% (v/v) glycerol, 10 μM ZnCl2, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Wild type and RPB9-deficient RNA polymerase II were purified from bakers' yeast and the WY9 yeast strain, respectively, as described (9, 24). The final purified protein preparations had a protein concentration of between 0.4 and 1.2 mg/ml. The proteins were stored at −70 °C in 10 mM Tris-HCl, pH 7.9, 40 mM ammonium sulfate, 10% (v/v) glycerol, 2.5 mM DTT, 100 μM EDTA, and 10 μM ZnCl2.

Cloning of RPB9 Mutants—The open reading frames for the various

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1 C. Carles, personal communication.
2 The abbreviations used are: pol II, polymerase II; DTT, dithiothreitol; HMK, heart myosine kinase; GST, glutathione S-transferase.
3 D. Janama, personal communication.
RPB9 mutants were generated using polymerase chain reaction amplification from a Hin dllIII fragment of yeast genomic DNA cloned into pBluescript (10). Each coding region, including wild type, was appended with a 5′ BamHI site immediately upstream of the start codon and 3′ EcoRI site immediately downstream of the stop codon. Mutants were constructed by polymerase chain reaction using unamplified chromosomal DNAs as previously described (18): the required mutation was generated in one fragment through the use of the appropriate mutagenic oligonucleotide primers. A second polymerase chain reaction fragment was generated containing the remaining wild type sequence. The final open reading frame fragment for each wild type or mutant RPB9 was digested with the BamHI and EcoRI restriction endonucleases. Next, the individual PCR fragments were ligated into the pGEX-2TK vector (Amersham Pharmacia Biotech), which encodes the heart myosine kinase (HMK) recognition sequence directly upstream of the BamHI restriction site. Plasmid DNA was sequenced to confirm the incorporation of each particular mutation.

Expression and Purification of GST-RPB9 Fusion Proteins—The Escherichia 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 (9). The final purified proteins were stored at −70 °C in 5 mM HEPES, pH 7.5, 100 mM NaCl, 10% (v/v) glycerol, 10 mM DTT, and 10 μM ZnCl2. The final preparation for each fusion protein had a protein concentration of between 0.15 and 1.5 mg/ml.

Radiolabeling of GST-RPB9 Fusion Proteins—The wild type and mutant RPB9 proteins were expressed as GST-RPB9 fusion proteins containing the HMK recognition sequence located between the GST and RPB9 regions. GST-RPB9 fusion proteins were phosphorylated using 100 units of HMK (Sigma)/nanomol of fusion protein. Prior to addition to the phosphorylation reaction, the appropriate amount of HMK was activated by preincubation with 40 mM DTT for 10 min at room temperature. The pretreated HMK was incubated with GST-RPB9, 50 mM ATP, and 0.15 μM [γ-32P]ATP in 20 mM HEPES, 50 mM NaCl, and 10 mM MgCl2, for 1 h 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. To quantify the radioactivity, the radiolabeled-RPB9 was resolved on a SDS-polyacrylamide gel, and the total counts/lane were quantified by PhosphorImager (Molecular Dynamics). The percentage of total counts attached to the GST-RPB9 fusion protein was used to determine the specific activity of each radiolabeled GST-RPB9 construct.

Binding Assay between GST-RPB9 and pol II ΔX9—Each of the 32P-labeled GST-RPB9 constructs was incubated with pol II ΔX9. Complex formation was monitored by an electrophoretic mobility shift assay. 1 picomol of 32P-radiolabeled GST-RPB9 (or the various mutants) was incubated with 0.03–5.0 picomol of pol II ΔX9 in 60 mM Tris, pH 8.0, 10 mM ammonium sulfate, 5 mM magnesium acetate, 4 mM spermidine, 1.5 mM DTT, 10% (v/v) glycerol, and 300 μg/ml bovine serum albumin. The reactions were mixed on ice in a total volume of 15 μl and then incubated for 15 min 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. To quantify the radioactivity, the radiolabeled-RPB9 was resolved on a SDS-polyacrylamide gel, and the total counts/lane were quantified by PhosphorImager (Molecular Dynamics). The percentage of total counts attached to the GST-RPB9 fusion protein was used to determine the specific activity of each radiolabeled GST-RPB9 construct.

Transcript Elongation Readthrough Assay—1.3 picomol of the appropriate polymerase was assembled on the tailed H3.3 template as for the readthrough assay described above. After a 90-s chase, the reactions were passed through two successive pre-equilibrated Bio-gel 30 spin columns (Bio-Rad). In the case of pol II ΔX9, 0.21 picomol of the purified pol II ΔX9 was radiolabeled against 5 mM HEPES, 50 mM NaCl, 10% (v/v) glycerol, 10 mM DTT, and 10 μM ZnCl2. The final preparation for each fusion protein had a protein concentration of between 0.15 and 1.5 mg/ml.

Transcript Cleavage Assay—1.3 picomol of the appropriate polymerase was assembled on the tailed H3.3 template as for the readthrough assay described above. After a 90-s chase, the reactions were passed through two successive pre-equilibrated Bio-gel 30 spin columns (Bio-Rad). In the case of pol II ΔX9, 0.21 picomol of the purified pol II ΔX9 was radiolabeled against 5 mM HEPES, 50 mM NaCl, 10% (v/v) glycerol, 10 mM DTT, and 10 μM ZnCl2. The final preparation for each fusion protein had a protein concentration of between 0.15 and 1.5 mg/ml.

RESULTS

RPB9 Mutants

The goal of this study was to understand the relationship between the structure and function of RPB9. In particular, we were interested in uncovering the regions of RPB9 involved in binding of RPB9 to pol II and in mediating the elongation response of pol II to TFIIS.

Sequence analysis revealed that RPB9 is composed of three distinct regions (Fig. 1). The N- and C-terminal regions of RPB9 each contain a zinc-binding domain characterized by the CX5,CX3,CX2,C motif (termed Zn1 and Zn2, respectively). These two domains are connected by a stretch of six amino acids that is highly conserved among eukaryotic RPB9 homologues (termed the linker region) (9, 13).

The Zn2 region of RPB9 has considerable sequence identity (30%) and structural similarity with the zinc ribbon domain of TFIIS (Fig. 1B). The TFIIS zinc ribbon domain forms a three-stranded antiparallel β-sheet with the conserved cysteines coordinating a Zn2 ion. The first and second strands of the sheet are connected by an extended flexible loop. The arrangement of hydrophobic and charged residues in the N-terminal zinc-binding domain of RPB9 was found to be similar to that of TFIIS and the C-terminal domain of 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 (8). Based on these homologies, we postulated that the zinc-binding N-terminal domain of RPB9 also forms a zinc ribbon fold.

The linker region connecting the two zinc-binding domains has no structural homologue. The assignment of structural residues within this region was based on similarity between RPB9 family homologues. As mentioned above, a stretch of six amino acids with the sequence DPTLPF is conserved among eukaryotic homologues. The charged residues within and adjacent to this sequence were targeted for mutagenesis.

We set out to allocate specific functions to the three defined regions of RPB9 using site-directed mutagenesis. We also constructed six deletion mutants, two in each of the three regions of the subunit. Two mutants, which deleted either most of the region between the pairs of cysteines in Zn1 or Zn2, were designed to keep the zinc coordination centers intact but to remove the remainder of the domain. A deletion mutant in the linker region retained the two zinc-binding regions but eliminated most of the connector region. We also engineered three smaller deletions that were designed to be less disruptive to the overall structure of the protein. In these mutants, we removed only the unstructured flexible loop regions in Zn1 or Zn2, or the conserved stretch in the linker region (Fig. 1A). In most cases, the deleted amino acids were replaced with either three or four Ala residues to prevent disruption of the overall domain structure.

We used site-directed mutagenesis in an attempt to ascribe functions to individual residues within each of the three re-
The lower complex is RNA polymerase is also known to dimerize in free pol II radiolabeled GST-RPB9 (Fig. 2). The free radiolabeled wild type GST-RPB9, and complex formation was monitored by nondenaturing electrophoresis (Fig. 2). The free radiolabeled GST-RPB9 migrated as a diffuse band toward the anode. As the concentration of pol II increased, two complexes appeared (Fig. 2A). To understand the nature of the two complexes 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 their constituents (Fig. 2, C and D). Each complex contained both pol IIΔ9 (Fig. 2C) and radiolabeled GST-RPB9 (Fig. 2D) and was distinct from either free pol IIΔ9 or free GST-RPB9. GST-RPB9 is a dimer in solution, and RNA polymerase is also known to dimerize in solution at low salt concentrations (26). The lower complex is shifted only slightly above the free pol IIΔ9; it is likely that it contains a pol IIΔ9 molecule bound to a single GST-RPB9 dimer. The upper complex may contain a dimer of polymerase with one or both polymerase molecules bound to a GST-RPB9 dimer. Alternatively, it may contain two single polymerase molecules, each of which is bound to an RPB9 molecule within the same GST-RPB9 dimer.

Because the two complexes were distinct from the unbound protein and contained both binding constituents, the signals from the upper and lower complexes were combined to quantify the total amount of pol II-GST-RPB9 complex (Fig. 2B). The binding of GST-RPB9 to pol IIΔ9 was stoichiometric; the interaction saturated at a molar ratio of approximately 1:1 for GST-RPB9:pol IIΔ9. An equilibrium dissociation constant was calculated to be 22 nM.

To determine whether 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 IIΔ9 (Fig. 2, E and F). At higher levels of unlabeled GST-RPB9, the amount of radiolabeled GST-RPB9 in the shifted complexes was significantly reduced, indicating that the radiolabeled and unlabeled GST-RPB9 were competing for the same binding site on the polymerase.

To further investigate the reversibility of the interaction between RPB9 and RNA polymerase II, a binding reaction was performed using wild type pol II. Radiolabeled GST-RPB9 was incubated with an equimolar amount of wild type pol II that contained endogenous RPB9. The interaction of GST-RPB9 with pol II was dramatically reduced compared with pol IIΔ9, although some binding was observed (compare lanes 1 and 7 in Fig. 2E). This binding likely reflected exchange between the endogenous RPB9 and the GST-RPB9 added to the reaction. Finally, the relatively weak binding of GST-RPB9 to pol II that we observed supports the contention that the gel shift assay was measuring the interaction of RPB9 with its relevant binding site on the polymerase.

**Binding of RPB9 to pol II Is Not Mediated through Zn1 or**
Zn2—GST-RPB9 assembles with pol IIΔ9 to form a stable complex. We used the above binding assay to identify regions of RPB9 that are involved in mediating the interaction with pol IIΔ9 (Table I). To determine the role of the zinc-binding domains in binding to pol IIΔ9, we analyzed two truncated mutants, GST-RPB91–47 and GST-RPB955–112, as well as several deletion mutants and a series of site-directed mutants in these domains. The zinc-binding domains did not appear to be involved in binding with pol IIΔ9. Neither of the individual zinc domains was sufficient for binding to pol II, nor did any site-directed mutations within these domains grossly affect the binding equilibrium. Several of the internal deletions within Zn1 and Zn2 abrogated binding activity. However, based on the predicted structure of the zinc-binding domains, these mutations probably disrupted the global structure of the protein rather than specifically perturbed the polymerase-binding interface.

The Linker Region Mediates Binding of RPB9 to Pol II—Mutations in the linker region were then assessed for their effect on binding to pol IIΔ9. The charged residues in the conserved DPTLPR motif appeared to be critical for binding of the subunit to the polymerase. The point mutants GST-RPB9K77A, GST-RPB9D72A, and GST-RPB9E54A were unable to assemble with the polymerase (Fig. 3). As well, the deletion mutants GST-RPB916–23 and GST-RPB955–112 were unable to assemble with pol IIΔ9. These constructs contained mutations of conserved charges either immediately N-terminal to, or within, the conserved stretch of amino acids in the linker region. Thus, it appears that certain conserved charges within the linker, especially those within the conserved stretch of residues 65–70, mediate binding of GST-RPB9 to pol IIΔ9.

Zn1 and the Linker Region Are Not Directly Involved in Elongation Activity—With a range of RPB9 mutants in hand, we set out to identify the regions required for transcription elongation activity. We had shown previously that the RPB9-deficient polymerase is compromised for readthrough of blocks to elongation (18). pol IIΔ9 that arrests at the T1a block to elongation in the H3.3 gene is not stimulated to transcribe through pause sites by the addition of a 5-fold molar excess of TFIIS (Fig. 4A); in contrast, the wild type pol II significantly responds to the addition of TFIIS. The elongation activity of arrested pol IIΔ9 can be reconstituted by the addition an excess of GST-RPB9; in the presence of purified RPB9 and TFIIS, the

**Table I**

| Fusion protein | Binding¹ | Readthrough | Cleavage |
|---------------|----------|-------------|----------|
| GST-RPB9      | 22       | ++          | ++       |
| GST-RPB9Δ1–47 | 21       | –           | –        |
| GST-RPB9Δ12–27| –        | –           | –        |
| GST-RPB9Δ16–23| –        | –           | –        |
| GST-RPB9K93A  | 21       | ++          | –        |
| GST-RPB9R92A  | 21       | ++          | –        |
| GST-RPB9R91A  | 26       | ++          | –        |
| GST-RPB9K77A  | 8        | ++          | –        |
| GST-RPB9D72A  | 12       | ++          | –        |
| GST-RPB9E54A  | 15       | ++          | –        |
| GST-RPB9D65A  | 10       | ++          | –        |
| GST-RPB9D65–112| 32      | ++          | –        |
| GST-RPB9R30A  | 13       | ++          | –        |
| GST-RPB9V34A  | 5        | ++          | –        |
| GST-RPB9Δ36–70| –        | –           | –        |
| GST-RPB9Δ55–70| –        | –           | –        |
| GST-RPB9Δ65–70| –        | –           | –        |
| GST-RPB9Δ65A  | 15       | ++          | –        |
| GST-RPB9Δ65–112| 12     | ++          | –        |
| GST-RPB9Δ72A  | 10       | ++          | –        |
| GST-RPB9Δ72–113| 8       | –           | –        |
| GST-RPB9Δ72–113| 8       | –           | –        |
| GST-RPB9Δ80–101| 5       | –           | –        |
| GST-RPB9Δ80–95| 30       | –           | –        |
| GST-RPB9Δ85–101| 20      | –           | –        |
| GST-RPB9Δ85–95| 105      | –           | –        |
| GST-RPB9Δ94A  | 12       | –           | –        |

¹ An estimate of the equilibrium dissociation constant is given in nM.
majority of the pol IIΔ9 complexes paused at the T1a were able to transcribe through the pause site to produce full-length transcript.

Each of the GST-RPB9 mutants was tested for the ability to restore polymerase response to stimulation by TFIIIS. The mutants were assayed at a 5-, 100-, and 300-fold molar excess over pol IIΔ9 (Table I). The purified Zn1 and Zn2 domains, alone or in combination, were not able to reconstitute elongation activity, even at a 300:1 ratio over pol IIΔ9.

None of the mutations in the Zn1 region affected the ability of GST-RPB9 to restore response to TFIIIS in the readthrough assay. All of the Zn1 substitution mutants were able to restore wild type activity at a 5-fold molar excess (Fig. 4C), with the exception of the two deletions in this region (mutants GST-RPB9Δ12–27 and GST-RPB9Δ16–23) that abolished activity but that also would be predicted to destabilize the protein. Thus, residues within Zn1 are likely not responsible for mediating the response of pol IIΔ9 to TFIIIS.

The linker region is important for binding of RPB9 to pol IIΔ9. Mutations within this region also affected elongation activity. However, these mutations only affected elongation to the extent that they affected binding. For example, GST-RPB9Δ35A and GST-RPB9Δ72A, which were able to bind to pol IIΔ9, were also able to restore response to TFIIIS at a 5-fold molar excess (Table I). By contrast, mutants from this region that only weakly bound pol IIΔ9 (GST-RPB9Δ61A, GST-RPB9Δ65A, and GST-RPB9Δ70A) were unable to restore response to TFIIIS at a 5:1 ratio of fusion protein:pol IIΔ9 (Fig. 4C) unless high levels of fusion protein were added (100:1 ratio). For these mutants, this amount would permit interaction between the GST-RPB9 mutants and pol IIΔ9. The two deletion mutants from the linker region, which are severely crippled for binding, could not restore readthrough response to TFIIIS, even at a 300:1 ratio of GST-RPB9 fusion:pol IIΔ9. Thus, the effect on elongation activity of mutations in the linker region is correlated exactly with the effect of the mutations on the RPB9/pol IIΔ9 interaction. We conclude that the linker region does not play a direct role in mediating transcription elongation.

The Putative Flexible Loop in Zn2 Is Required for Readthrough Response—The Zn2 region of RPB9, which has homology to the zinc ribbon domain of TFIIIS, possesses a series of charged residues (residues 89–95 in RPB9) that are analogous to those in TFIIIS that are essential for elongation activity (6). These charged residues are located in the flexible loop that connects the second and third β-strand. Point mutations in the RPB9 charged residues, Arg81, Arg82, Lys83, and Asp84, as well as the Δ989–95 deletion mutation were completely inactive for elongation activity (Table I). These mutations had no effect on binding to pol IIΔ9, even at a 300:1 ratio over pol IIΔ9 (Fig. 4C). These data suggest that the flexible loop of Zn2 from RPB9, like the flexible loop from TFIIIS, is responsible for mediating the activity of RPB9 in transcript elongation.

Transcript Cleavage Assay—TFIIS reactivates arrested RNA polymerase II complexes in part by inducing the polymerase to cleave the nascent transcript at the 3’ end (27). This process appears to be at least a two-step procedure, resulting in the
Fig. 5. Transcript cleavage assay. A, cleavage assays performed with pol II, pol IIΔ9, or pol IIΔ9 and a 5-fold molar excess of GST-RPB9. Samples were taken at 0, 1, 5, or 10 min after the time of addition of TFIIS, as indicated. After 10 min, nucleotides were added back to reactions, and the reaction was incubated for an additional 10 min (lanes labeled nt). C1 indicates the position of the first cleavage product, and C2 indicates that for the second cleavage product. B, cleavage assays performed with pol IIΔ9 and representative GST-RPB9 mutants. The assays were performed with a 5- or 100-fold molar excess of fusion protein over polymerase and were done in the presence of 5-fold molar excess of TFIIS. Details for each mutant are summarized in Table I.

DISCUSSION

Pol II Binding Region—We had previously demonstrated that recombinant RPB9 or GST-RPB9 could restore pol II activity in elongation assays (18). Here, we demonstrate that GST-RPB9 forms a stable interaction with pol IIΔ9 and that this interaction appears to be required for transcription activity. In our analysis, each mutant that lost binding activity also lost transcription activity. We localized the polymerase binding region to the conserved sequence DPTLPR in the linker region of RPB9. The two charged residues in this sequence (D61A and D65A), and a third charged residue N-terminal to this sequence (R70A), were found to be essential.

The residues that we have identified as important for the interaction between RPB9 and pol IIΔ9 are conserved among eukaryotic RPB9 homologues (9, 13) but not in the analogous subunits in archaeabacteria and eukaryotic RNA polymerase I. Interestingly, the archaeabacterial subunits do contain a conserved sequence in the linker region that is composed of conserved aliphatic and charged residues (TVIK--K-K) (9). 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 archaeabacterial polymerases. In accordance with this idea, human RPB9 could partially complement growth defects in Δ9 yeast strains and could fully restore wild type start site preferences in a yeast in vitro assay (17).

Role of RPB9 in Elongation—The Δ9 polymerase is defective for certain transcription elongation activities (9). Without RPB9, RNA polymerase II is less efficient at recognizing pause sites within the template, and the Δ9 pol II molecules that do recognize and arrest at intrinsic pause sites are unable to read through and resume transcribing in response to stimulation by TFIIS. The arrested Δ9 enzyme is able to cleave the nascent release of two short RNA oligomers when performed in the absence of nucleotide substrates (16, 18). Only the first cleavage event is required for resumption of transcription (20), but TFIIS mutants that can only stimulate the first cleavage event cannot activate polymerase readthrough activity (5).

The transcript cleavage assay has been used previously to demonstrate that pol IIΔ9, 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 unable to produce the second cleavage product (C2). These data, along with other results, led to the hypothesis that reactivation of arrested complexes requires cleavage of the nascent transcript to C1 as well as a conformational change in the arrested complex. Wild type transcript cleavage activity as assessed by the appearance of the C2 transcript can be restored to arrested pol IIΔ9 complexes by the addition of purified RPB9 and TFIIS (Fig. 5A).

Accordingly, we tested each of the GST-RPB9 constructs for the ability to restore complete cleavage activity to the Δ9 polymerase. Because pol IIΔ9 can respond to TFIIS stimulation by producing C1 in the absence of GST-RPB9, we looked specifically for the ability of the fusion proteins to stimulate pol IIΔ9 to produce C2 in the presence of TFIIS.

Mutants in the Zn1 and Linker Regions Restore Transcript Cleavage—In general, the cleavage activity of each of the GST-RPB9 mutants in the Zn1 and linker regions mirrored the activity measured in the readthrough assay (Fig. 5B). Polymerase cleavage activity was restored at the same levels of fusion protein that restored polymerase readthrough activity (Table I). For the most part, this relationship also held true for mutants in Zn2. However, in a few mutants, weak transcript cleavage activity could be detected without corresponding readthrough activity (Table I and Fig. 5B). For example, no stimulation of readthrough was seen for mutants within the flexible loop of Zn2, but at high concentrations of fusion protein, some cleavage activity was restored. The small deletion mutant and the substitution mutants from the Zn2 region of RPB9 for which we were unable to detect readthrough did not restore cleavage activity at a 100-fold molar excess.
Transcript to produce a primary cleavage product (C1) when incubated with TFIIS. However, the α9 enzyme cannot perform the subsequent cleavage event to produce a second cleavage product (C2). GST-RPB9 can restore each of these activities in vitro when added to arrested ternary complexes.

The Zn2 domain of RPB9 is responsible for activating pol II to respond to TFIIS stimulation in transcript readthrough and cleavage assays. Specifically, the putative flexible loop of the zinc ribbon is required to restore these elongation activities to pol IIα9. Mutations that deleted the charged loop of the Zn2 region (QQRRKDT) or removed each of the charges within the loop (R, K, or D) abrogated the ability of RPB9 to respond to TFIIS. These mutants did not restore the readthrough activity of pol IIα9 in response to TFIIS, even at a 300-fold molar excess.

Mutants that were able to induce a readthrough response always were able to induce polymerase to cleave back to C2 at the same level of fusion protein. By contrast, the ability to cleave the transcript did 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 (5). These results suggest that transcript cleavage is absolutely required for readthrough but is not sufficient.

Thus, in addition to sequence and structural 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 (QQRSAD). 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 (6). Thus, this domain is functionally analogous in RPB9 and TFIIS and represents a conserved elongation stimulatory motif.

Proposed Model for RPB9 Mode of Action—We suggest that RPB9 employs the zinc ribbon structural motif to exert its influence on the active site of polymerase and specifically that Zn2 is required for the stimulation of arrested ternary complexes. The Zn2 domain of RPB9 does not act alone to stimulate transcription elongation. The reactivation of polymerase after elongation arrest is dependent on TFIIS (27) and most importantly the TFIIS zinc ribbon domain (ZnIIS) (5, 6). Apparently, Zn2 of RPB9 acts in concert with ZnIIS to affect transcript cleavage and readthrough. The first cleavage event that occurs in reactivating arrested elongation complexes requires TFIIS but not RPB9, because pol IIα9 and TFIIS can perform this reaction (18). The second cleavage event and the essential "post-cleavage" step require the concerted effort of RPB9 and TFIIS. We suggest that the first cleavage event brings the 3′ end of the transcript and the active site on the template back into register. Pol II is unable to resume transcription at this stage. We then suggest that Zn2 and ZnIIS act together to induce a rearrangement of the enzyme that switches 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.

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