RNA Polymerase II Trigger Loop Mobility

INDIRECT EFFECTS OF Rpb9*

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Rpb9 is a conserved RNA polymerase II (pol II) subunit, the absence of which confers alterations to pol II enzymatic properties and transcription fidelity. It has been suggested previously that Rpb9 affects mobility of the trigger loop (TL), a structural element of Rpb1 that moves in and out of the active site with each elongation cycle. However, a biochemical mechanism for this effect has not been defined. We find that the mushroom toxin α-amanitin, which inhibits TL mobility, suppresses the effect of Rpb9 on NTP misincorporation, consistent with a role for Rpb9 in this process. Furthermore, we have identified missense alleles of RPB9 in yeast that suppress the severe growth defect caused by rpb1-G730D, a substitution within Rpb1 α-helix 21 (α21). These alleles suggest a model in which Rpb9 indirectly affects TL mobility by anchoring the position of α21, with which the TL directly interacts during opening and closing. Amino acid substitutions in Rpb9 or Rpb1 that disrupt proposed anchoring interactions resulted in phenotypes shared by rpb9Δ strains, including increased elongation rate in vitro. Combinations of rpb9Δ with the fast rpb1 alleles that we identified did not result in significantly faster in vitro misincorporation rates than those resulting from rpb9Δ alone, and this epistasis is consistent with the idea that defects caused by the rpb1 alleles are related mechanistically to the defects caused by rpb9Δ. We conclude that Rpb9 supports intra-pol II interactions that modulate TL function and thus pol II enzymatic properties.

Rpb9 is a small (122 amino acids in Saccharomyces cerevisiae) subunit of RNA polymerase II (pol II)2 that is highly conserved among eukaryotes (1, 2). It is composed of two zinc ribbon domains characterized by the zinc-chelating motif (CCHILD). Although Rpb9 is not essential for growth in yeast, rpb9 null mutants have a number of phenotypes, including slow growth (2), hypersensitivity to several drugs (2–5), upstream shifts in transcription start site (2, 6), defects in transcription-coupled repair that result in enhanced sensitivity to UV light (7), and decreased transcriptional fidelity in vivo (4, 8) and in vitro (9, 10).

The fidelity defect of pol II that is lacking Rpb9 (pol IIΔ9) has been examined in several studies. Decreased selectivity for correct (complementary to the DNA template) versus incorrect NTP substrates has been observed (10). However, our prior experiments have not provided evidence for a selectivity defect, and, in our hands, the decreased fidelity of pol IIΔ9 appears to be the result of inefficient proofreading caused by an increased rate of RNA extension from a mismatched 3′-end combined with a decreased efficiency of TFIIS-mediated mismatch removal (9). A role for Rpb9 in fidelity is consistent with reported roles for the homologous subunits in RNA polymerases I and III (A12.2 and C11, respectively) (11, 12) as well as the related archaean protein TTS (13, 14). However, the role that Rpb9 plays in pol II fidelity is probably distinct from these homologs, all of which function as hybrid proteins combining functions of both Rpb9 and TFIIS.

Rpb9 is located on the leading edge of pol II at a position that is not in immediate proximity to the active site (15), and the absence of Rpb9 has little or no effect on the binding affinity of TFIIS with pol II (16). This leaves open the question of how Rpb9 exerts its effects on elongation and cleavage. Using rapid quench-flow kinetic techniques, it has been demonstrated that the absence of Rpb9 promotes the sequestration of NTP substrates in the active site before formation of a new phosphodiester bond (10). This behavior was similar to that observed with pol II containing Rpb1-E1103G (pol II-(Rpb1-E1103G)) (17), which contains a substitution within the trigger loop (TL), a mobile structural element that closes on the pol II active site and interacts with the substrate NTP (reviewed in Refs. 18–21). Due to the proximity of Rpb9 to the TL, it was proposed that the C-terminal domain of Rpb9 interacts directly with the open pol II TL and thereby stabilizes its open conformation (10). However, in this same study, direct mutational analysis of an amino acid proposed to interact with the open TL (Rpb9-K93) showed no fidelity (or any other) defect.

Although deletion of RPB9 and particular substitutions within the TL can confer similar phenotypes and biochemical defects, no mechanism through which Rpb9 and the TL could interact has been defined. In this study, we provide additional genetic and biochemical evidence that Rpb9 affects pol II activity through the TL but does so indirectly through stabilization of Rpb1 α-helices 20 and 21 (α20 and α21) and a small loop between them that we term the Rpb1 anchor loop (AL). We describe a plausible biochemical model for how this could

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2 The abbreviations used are: pol II, RNA polymerase II; TL, trigger loop; pol IIΔ9, RNA polymerase II that is lacking Rpb9; TFIIS, transcription factor IIS; EC, elongation complex; α21, Rpb1 α-helix 21; pol II-(Rpb1-E1103G), RNA polymerase II in which amino acid X at position m of subunit Rpb1 is replaced by amino acid Z; α20, Rpb1 α-helix 20; WCE, whole cell extract; MPA, myco- phenolic acid; AL, anchor loop; nt, nucleotide(s); NTA, nitrilotriacetic acid.
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occur and confirm predictions suggested by this model related to phenotypes of specific alleles of RPB9 and RPB1.

Results

Effect of Rpb9 on Pol II Activity in the Presence of α-Amanitin—Although previous work had shown that the absence of Rpb9 promotes NTP sequestration in the pol II active site, the proposed mechanism, in which sequestration is increased through altered mobility of the TL, was without direct experimental support (10). We decided to test the general idea that Rpb9 affects TL mobility using an experimental approach that exploits the mushroom toxin α-amatin, which has been shown to inhibit pol II by locking the TL in a position where it cannot participate directly in active site chemistry (22, 23). Elongation by pol II is strongly inhibited in the presence of saturating concentrations of α-amatin, but it is not completely prevented (24, 25). We reasoned that if Rpb9 affects TL mobility, then, by immobilizing the TL with α-amatin, the effects of Rpb9 on pol II activity would be markedly altered.

We have previously reported that the absence of Rpb9 increases the rate of pol II-catalyzed RNA synthesis in a single-nucleotide misincorporation assay (9). We chose to examine the effects of α-amatin with this assay rather than alternatives that would assess the faster rate of incorporation of correct, template-matched NTPs (23) (see below) because of the relatively low affinity of yeast pol II for α-amatin (26). We feared that the rapid rate of elongation with template-matched NTPs might compete with the “on” rate of α-amatin binding such that, even at saturation, a small amount of free pol II in equilibrium with α-amatin-bound pol II could, during its brief period of freedom from the inhibitor, sometimes elongate, and this would be more likely to occur in a variant with a faster elongation rate. Indeed, observations consistent with this effect for yeast TL mutations that increase elongation rate have been reported (23).

To assess the effect of α-amatin on misincorporation, purified pol II or pol IIΔ9 was immobilized on Ni2+–NTA beads via a His6 tag on Rpb3, and elongation complexes (ECs) were assembled with DNA and RNA oligonucleotides in which the 10-nt RNA was completely complementary to the template DNA and was 5′-end-labeled with 32P (Fig. 1A). Rather than supplying CTP, the nucleotide specified by the template for extension of the labeled RNA oligonucleotide, a nearly saturating (9) amount of ATP (1 mM) was added, and the time course of A-for-C misincorporation was followed in the presence or absence of α-amatin (Fig. 1, B and C). The fraction extended to 11 nt was plotted against time, and the data were fit to a single exponential equation (Fig. 1, D and E). The faint band at position 12 at some of the later time points in Fig. 1C probably reflects extension of the mismatched 3′-end by ATP, which is the template-specified nucleotide at this position. We have previously shown that such mismatch extension can occur at a rate consistent with its appearance here and that it is 2–3-fold faster for pol IIΔ9 than for pol II (9). Although small in size, we combined signal in this band with the band at position 11 because both reflect misincorporation. We confirmed that the extension to 11 nt was the result of misincorporation and not the result of contaminating CTP because the 11-nt product was not rapidly extended by the addition of CTP, GTP, and UTP (data not shown), consistent with a mismatched 3′-end (9, 27). In the absence of α-amatin, the misincorporation rate for pol II

FIGURE 1. Effect of α-amatin on A-for-C misincorporation. A, schematic representation of assembled EC. Template and non-template DNA oligonucleotides depict the transcription bubble. A RNA oligonucleotide labeled at its 5′-end with 32P is between the DNA strands. The small box indicates the template position for extension by the next correct NTP, and the shaded area represents the approximate region associated with pol II, B and C, time course of A-for-C misincorporation. The EC depicted in A was assembled with purified pol II (B) or pol IIΔ9 (C) and incubated with ATP (1 mM). Aliquots were removed at the indicated times, and the products were separated by denaturing polyacrylamide gel electrophoresis and visualized on a phosphor imager. Samples in lanes 8–13 contained α-amatin (160 μg/ml), which was added 15 min before the addition of ATP. D, E, quantitation of extension time course. The fraction of ECs extended to 11 nt was plotted versus time and fit to a single exponential equation (open circles, pol II; closed circles, pol IIΔ9). Y axis was normalized to the individual reaction end points to emphasize relative rates. Error bars on each data point indicate the S.D. for three independent experiments. The values of kext are based on a fit to a single exponential equation performed as described under “Experimental Procedures.” Error for kext indicates the 95% confidence interval for the non-linear regression fit of the experimental data to the first order exponential curve.
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TABLE 1
Yeast strains

| Strain           | Genotype                        | Source                  |
|------------------|---------------------------------|-------------------------|
| BJ5464 His-Bio   | ura3-52 trp1 leu2Δ1 his3Δ200 pep4Δ: HIS3 prb1Δ1.6R can1 GAL MATα* | Mikhail Kashlev (22)   |
| dpoY127          |                                 | Peterson laboratory (19) |
| CKY283           |                                 | Kaplan laboratory (23)  |
|                  |                                 | This study              |
|                  |                                 |                         |
|                  |                                 |                         |
|                  |                                 |                         |

(k_{obs} = 0.069 ± 0.006 min⁻¹) was a little more than half that observed for pol IIΔ9 (k_{obs} = 0.122 ± 0.017 min⁻¹), consistent with our previously reported observations (9), but in the presence of α-amanitin (160 μg/ml), the rates for pol II (k_{obs} = 0.029 ± 0.004 min⁻¹) and pol IIΔ9 (k_{obs} = 0.025 ± 0.002 min⁻¹) were essentially identical. This concentration of α-amanitin was saturating, because concentrations as high as 640 μg/ml had no additional effect on the rate. Overall, the misincorporation reaction was less sensitive to α-amanitin than the incorporation of correct NTPs (data not shown), consistent with published observations (23), but immobilization of the TL by α-amanitin completely eliminated the effect of Rpb9 on the rate. This observation is consistent with the idea that the effect of Rpb9 on the rate of misincorporation is mediated, at least in part, by affecting the TL in some manner.

**rpB9 Alleles That Suppress rpb1-G730D (sit1-7)—Our interest in the molecular mechanism(s) by which Rpb9 could alter TL activity fortuitously merged with a more general approach that we had pursued to identify mutations that affect Rpb9 function. An allele of RPB1 (rpb1-G730D) was originally isolated as sit1-7 (suppressor of initiation of transcription) based on its ability to support low level transcription from the HIS4 gene in the absence of normally required activating factors (28). Subsequently, it was independently isolated as a suppressor of the menadione hypersensitivity of rpB9A strains (29). A severe growth defect is conferred by rpb1-G730D in the presence of Rpb9, but nearly wild-type growth can be restored in rpb1-G730D strains if they also contain certain alleles of Rpb9, including null alleles or alleles encoding proteins in which Rpb9 residues 48–122, 65–70, or 89–95 are deleted (29). Previous experiments had shown that these deletions do not prevent binding of Rpb9 to pol II (7, 30); thus, it is likely that some kind of negative interaction between Rpb9 and rpb1-G730D is mediated through the C-terminal half of the 122-residue Rpb9.

Pol II (rpb1-G730D) has been shown to be catalytically slow, with an elongation rate only about 10% that of wild-type pol II (31). To examine the interaction of Rpb9 and rpb1-G730D more closely, we took advantage of yeast strain CKY283 (23), in which the only copy of RPB1 is provided on a centromeric plasmid (Table 1). We introduced a RPB9 ORF knock-out into this strain (32, 33) to create dpoY249 (Table 1) and then replaced RPB1 with rpb1-G730D via a plasmid shuffle (34). ECs were assembled with pol II that had been immobilized from whole cell extract (WCE) of these strains via a TAP tag on Rpb3, and A-for-C misincorporation assays were performed. Rates of misincorporation for pol II derived from WCE from wild-type and rpb9ΔA strains (Fig. 2) were very similar to those obtained with highly purified pol II and pol IIΔ9 (Fig. 1), with the absence of Rpb9 resulting in an increase in misincorporation rate of about 2-fold. The more convenient analysis of pol II variants from WCE thus provides a reliable measure of relative enzyme activity, as we have reported previously (9). When the rpb1-G730D allele was shuffled into the RPB9 strain, the misincorporation rate decreased, consistent with the previously reported decrease in the elongation rate with template-matched NTPs (31). However, in the rpB9A background, we observed mutual suppression of the fast rate characteristic of pol IIΔ9 and the slow rate associated with pol II (Rpb1-G730D), and the result was a rate near that of wild-type polymerase (Fig. 2). This suggested that specific rpB9 alleles that relieve the negative genetic interaction with rpb1-G730D, such as those that delete Rpb9 residues 48–122, 65–70, or 89–95, might also affect the pol II elongation rate, and we therefore decided to screen additional rpb9 alleles for their ability to relieve the negative interaction between RPB9 and rpb1-G730D.

Our initial results were consistent with the results of Koyama et al. (29) (Fig. 3A). The severe growth defect in rpb1-G730D RPB9 strains was partially relieved when RPB9 was deleted, and the rpb1-G730D rpb9Δ strain retained only the growth defect caused by rpb9Δ. Providing RPB9 on a single-copy plasmid restored the negative interaction with rpb1-G730D, as did an allele that changed Rpb9 amino acids Arg-91, Arg-92, Lys-93, and Asp-94 to alanine (rpb9-(91–94)A). (The Rpb9 amino acid numbering system used throughout this paper does not take into account the 8-amino acid N-terminal FLAG tag present in all of our constructs.) In contrast, an allele in which Rpb9 amino acids 88–97 are deleted (rpb9-(88–97)Δ) or an allele encoding only the N-terminal 59 amino acids of Rpb9 (rpb9-(1–59)) did not restore this negative interaction. Rpb9 encoded by all of the tested alleles appeared to be expressed and capable of associating with pol II because all of them restored nearly wild-type growth to an Rpb1 rpb9Δ strain, and in the rpb1-G730D rpb9Δ background, both rpb9-(1–59) and rpb9-(88–97)Δ grew significantly better than when the vector alone was present. These results strongly suggest that Rpb9 residues 88–90 and/or 95–97 play a major role in the negative interaction between RPB9 and rpb1-G730D.

Additional rpb9 alleles were constructed to further dissect this region of the protein. rpb9-Q87A, rpb9-S96A, rpb9-(88–90)A, and rpb9-(95–97)A all restored wild-type growth to a rpb9Δ strain, but only rpb9-(95–97)A also completely avoided the negative interaction with rpb1-G730D (Fig. 3B). The absence of the negative interaction does not appear to be the result of an inability of Rpb9-(95–97)A to bind the polymerase, because growth of the rpb1-G730D rpb9-(95–97)A strain was much more robust than that of the rpb1-G730D rpb9Δ strain. This is consistent with published work in which amino acid substitutions near this region of Rpb9 do not affect pol II bind-
at NTP (Fig. 4B), and transcription was monitored over time (Fig. 4, B and C). After gel electrophoresis of the RNA products, the half-time for the synthesis of products 61 nt in length was estimated using the empirical observation that the time course of the synthesis of these products closely followed a single exponential equation (Fig. 4C), and the average rate of elongation was determined by dividing the actual number of nt added to the 10-nt primer at completion (51 nt) by this calculated half-time. Pausing of the polymerase clearly plays a role in the time course, but this assay has proven to be a useful method for comparing catalytic activity of pol II variants (23).

that observed when Rpb9 was present (5.8 ± 2.4 nt/s). As described in greater detail below, the A-for-C misincorporation rate of pol II-Rpb9-(95–97)A is also fast, yet, like that of pol IIΔ9, the misincorporation rate is essentially identical to that of wild-type pol II in the presence of α-amanitin (see Fig. 8), which suggests that TL function may be affected by the amino acid changes in Rpb9-(95–97)A.

Although the in vivo phenotypes of both RPB1 rpb9-(95–97)A and rpb1-G730D rpb9-(95–97)A strains appeared to preclude a major defect in association of Rpb9-(95–97)A with the rest of the polymerase, we directly addressed this possibility using a pull-down assay. dopY127, a rpb9Δ strain with a His$_6$ tag on Rpb3, was transformed with a centromeric plasmid containing either RPB9 or rpb9-(95–97)A, each of which was FLAG epitope-tagged at its N terminus. Pol II was immobilized from WCEs derived from these strains using Ni$_2$+NTA beads, and the beads were washed just as they would be for in vitro transcription assays, such as those described in Fig. 4. Bead-bound proteins were dissolved in SDS, fractionated by SDS-PAGE, and probed with antibodies against the N-terminal FLAG tag of Rpb9 as described under “Experimental Procedures.” A single protein band of ~15 kDa, consistent with the expected size of Rpb9, was detected in lanes derived from extracts from cells with the RPB9 or rpb9-(95–97)A plasmid but not the control extract made from rpb9Δ cells carrying only the empty vector. The membrane was then gently washed and probed with an antibody against the Rpb1 C-terminal domain, which revealed a large protein, consistent with the size of Rpb1 (191 kDa), in each sample while retaining the signal from the FLAG-tagged Rpb9 (Fig. 5). Quantitation yielded a ratio of Rpb9 to Rpb1 that is very similar for wild-type Rpb9 and Rpb9-(95–97)A, indicating that there is no major defect for binding with the Rpb9 variant. This is not surprising because the area critical for Rpb9 binding appears to reside in residues nearer to the linker between the Zn1 and Zn2 zinc ribbon domains of Rpb9 (30). These results were not compromised by the presence of the 8-amino acid FLAG tag because all of the RPB9 alleles shown in Figs. 2 and 3 contain it.

A Model for Interaction between Rpb9 and the TL—Our initial results with α-amanitin were consistent with the idea that Rpb9 could affect TL mobility. However, although a nearly atomic resolution crystal structure of pol II with the TL in the closed position is available (PDB entry 2E2H (35)), the study of TL mobility has been complicated by the lack of a comparable structure in the open conformation. Portions of the TL are not visible in most crystal structures in which the TL is not locked in its closed position interacting with substrate NTP (e.g. PDB entries 1Y1W (36) and 2E2J (35)), suggesting that the open TL may be composed of multiple conformations of similar stability.

Structures of several open TL conformations have, however, been published (36, 37), and one of these (PDB entry 1Y1V (36)), in which the open TL is stabilized by the presence of TFIIIS, served as a starting point for our analysis. In this structure, Rpb9 amino acids 95–97, as well as nearby residues, are in position to interact with the ends of Rpb1 α20 and α21, which form part of the “jaw” domain of pol II, as well as the small loop that connects them (Fig. 6A), which we have termed the Rpb1
FIGURE 3. Suppression of the growth defect in rpb1-1G730D strains by alleles of RPB9. Strains based on CKY283 or dopY249 with the genotype shown were transformed with plasmids derived from pPS413 carrying various alleles of RPB9. Strains were grown to saturation in SC-His medium, and serial 10-fold dilutions were spotted onto SC-His plates. The pictures show growth after incubation for 2 days at 30 °C. A, suppression of rpb1-1G730D by rpb9 alleles (1–59), (88–97)A, and (91–94)A. B, suppression of rpb1-1G730D by rpb9 alleles Q87A, S96A, (88–90)A, and (95–97)A.

AL. Major features include interactions between Rpb1-Arg711 and Rpb9 residues Gln87 and Met97, as well as insertion of the side chain of Rpb9-Met97 into a hydrophobic region between α20 and α21, created in part by Rpb1-Phe714. In addition, Rpb9-Thr95 has potential hydrogen bonding with the Rpb9 backbone at amino acids Arg92 and Gln90, which may be important in maintaining the correct orientation of Rpb9-Gln90 and Rpb9-Met97, and Rpb9-Ser112 forms a hydrogen bond with the backbone carbonyl of AL residue Gln696. Importantly, the open TL in 1Y1V is positioned to interact with the N-terminal tip of α21 and the AL in a manner that could stabilize the open conformation (Fig. 6B). Notable features of this interaction include a series of hydrogen bonds between Rpb1-Thr709, Rpb1-Glu712, and the backbone amide nitrogen of TL residue Ala1090, as well as van der Waals interactions of AL residue Met780 within a hydrophobic region between TL residues Val1089 and Ala1090.

Two additional open TL conformations have been reported (37), both of which showed elongation complexes with a full transcription bubble that was stabilized by the presence of TFIIIF, although TFIIIF was not clearly visible in the crystal structures. One of these structures (PDB entry 5C4X) was obtained with a complete, 12-subunit pol II, whereas the other (PDB entry 5C4J) was obtained with a 10-subunit polymerase missing Rpb4 and Rpb7. In both of these structures, the interactions between Rpb9 and Rpb1 α20-AL-α21 are very similar to those depicted in Fig. 6A. The only notable difference is that in one of them (5C4X), the orientation of Rpb9-Thr95 is such that Rpb1-Arg711 makes a hydrogen bond with this residue as well as with Rpb9-Gln87. The TL in both of these structures is less open than that in 1Y1V (Figs. 6, C and D), but all three of the open positions share a common feature in which the TL has direct interaction with α21.

The model that emerges from this analysis is that Rpb9 may anchor the ends of α20, α21, and the AL in positions that are favorable for interaction with the open TL. Stabilization of the open conformation provides time for binding of NTP substrate or proofreading of errors without TL interference. This is similar to a model proposed by Walmacq et al. (10), but it does not involve a previously proposed direct interaction between Rpb9 and the TL. Instead, our model requires two interaction interfaces, one between Rpb9 and Rpb1 α20-AL-α21 and a second between the AL or α21 and the TL. It should be noted that there are additional interactions that could also contribute to stabilization of the TL in all three of the stable open positions, but Fig. 6 shows only those to which Rpb9 probably contributes. We note that Rpb1 residue Gly730 (shown in red in Fig. 6) is also in α21, and our model provides a plausible explanation for the negative genetic interaction between RPB9 and rpb1-1G730D, which is described under “Discussion.”

If the molecular interactions are as suggested in Fig. 6, mutations that alter them should result in in vivo phenotypes that mimic at least some of the phenotypes of rpb9Δ strains, and some previously published observations are consistent with this idea. For example, rpb1-S713P, in which the proline would disrupt the N terminus of α21 and potentially alter interactions with both Rpb9 and the TL, causes an upstream shift in the transcription start site of ADH1 (38), a trait shared by rpb9Δ strains (2) and one that is common to mutations that increase the pol II rate of elongation (39). In addition, a screen for suppressors of the downstream shift in the transcription initiation site caused by sua7–1, an allele of the gene that encodes TFIIH, identified an allele of RPB9 that encodes only amino acids 1–106 (6), disrupting potential Rpb9 interactions, such as the hydrogen bond between Rpb9-Ser112 and the AL (Fig. 6A). Although the TL does not appear to make any direct contact with α20, the two helices are maintained in a parallel orientation through a series of hydrophobic interactions along their helical interface, and the positioning of α20 probably affects α21 as well.

Phenotypes of Mutations That Alter Potential Interaction Interfaces—To test this model, we introduced mutations that are likely to disrupt potential interactions between Rpb9, α20-
AL-α21, and the TL. In addition to the previously described rpb9-(95–97)A, we constructed rpb1 alleles (707–709)A and R711A, and rpb1-S713P (38) was also available. In addition, an ongoing genetic screen for mutations within the TL identified alleles rpb1-S1091C, rpb1-S1091W, and rpb1-K1092E,3 and they were also chosen for analysis.

Mutations were characterized using three phenotypes of rpb9/H9004 strains, decreased growth, hypersensitivity to mycophenolic acid (MPA), and suppression of galactose sensitivity conferred by the gal10Δ56 background (Gal5). MPA is a potent inhibitor of IMP dehydrogenase, an enzyme important in purine biosynthesis. It has been widely assumed that MPA hypersensitivity in yeast is indicative of a defect in transcription elongation that is exacerbated when nucleotide concentrations are low. However, it has been shown that hypersensitivity is almost always a much more specific defect that results from the failure of low concentrations of GTP to cause induction of the yeast IMD2 gene (40), which encodes a relatively MPA-resistant form of IMP dehydrogenase (41). Induction is caused by a shift to a downstream transcription initiation site that is required to generate the long, stable transcript that includes the IMD2 ORF (42). Thus, strains with mutations that prevent the efficient shift from the upstream start site (which begins with GTP) to the downstream site (which begins with ATP) when GTP concentrations are low become MPA-hypersensitive. Recent evidence suggests that one (but not the only) determin-
interaction interfaces should result in phenotypes shared by rpb9Δ.

To assess possible biochemical phenotypes of these pol II alleles, we examined in vitro elongation and misincorporation rates. Pol II immobilized from WCE of rpb1-(707–709)A, rpb1-R711, and rpb1-S1091C strains all share the fast overall elongation rate (Fig. 8A) and fast misincorporation rate (Fig. 8B) of pol IIΔ9 and pol II-(Rpb9-(95–97)A). In addition, just as with pol IIΔ9 and pol II-(Rpb9-(95–97)A), the presence of saturating amounts of α-amanitin decreased the misincorporation rate of all of the polymerases with mutations of the TL near that observed with wild-type pol II (Fig. 8B), consistent with the idea that all of these mutations may be affecting a TL-related function.

Epistasis of Anchor Loop and a21 Mutations with rpb9Δ—TL mutations that affect the rate of elongation tend to be additive when combined (39). Fast, gain-of-function alleles combined with slow, loss-of-function alleles usually have an elongation rate between the two extremes, similar to what we observed with the combination of rpb9Δ and rpb1-G730D (Fig. 2), whereas combinations of two fast or two slow TL alleles are often lethal. In contrast, when we combined rpb9Δ with the fast rpb1 alleles that we identified, the combinations did not result in polymerases that were significantly faster than that caused by rpb9Δ alone in the A-for-C misincorporation assay (Fig. 9). This epistasis supports a model where the defects imposed by rpb1 alleles R711A, (707–709)A, and S1091C are related mechanistically to the defects caused by rpb9Δ and rpb9-(95–97)A. This epistasis is not observed with all fast TL variants, because the combination of rpb9Δ and rpb1-E1103G, a gain-of-function mutation (23) located near one of the hinges of the TL, is lethal (10), an observation that we have independently confirmed (data not shown).

Discussion

The rate of transcription elongation in eukaryotes is fine tuned to the rates of other processes, such as mRNA capping, transcriptional proofreading, splicing, and termination (38), all of which must operate together to generate a functional RNA product. Our experiments, as well as previously published studies (9, 10), indicate that the small pol II subunit Rpb9 plays a role in maintaining elongation at a speed that keeps all of these processes in synchrony. The α-amanitin sensitivity of the effect of Rpb9 on misincorporation (Fig. 1) as well as the observed increase in NTP sequestration in the pol II active site in the absence of Rpb9 (10) are consistent with the idea that Rpb9 affects pol II activity, at least in part, by delaying TL closure and thereby slowing elongation. However, a plausible biochemical mechanism by which Rpb9 could affect TL mobility has not been described. Our experiments provide genetic and biochemical evidence for an indirect mechanism by which Rpb9 affects TL mobility by positioning Rpb1 elements α20 and α21 as well as the AL that connects them into positions that allow the open TL to interact directly with α21 or the AL.

In addition to the crystal structures shown in Fig. 6, a molecular dynamics study of TL mobility (49) has identified numerous potential interactions between the TL and α21 that may be important. In this study, the open TL conformation was
assigned starting with a crystal structure in which the TL conformation was not completely defined (PDB entry 2E2J (35)). Various TL positions were sampled, and a preferred open conformation was chosen based on an internal free energy calculation. The molecular dynamics simulation identified a stable intermediate state between the completely open and closed conformations, suggesting that this intermediate state may provide sufficient TL opening for elongation to occur and that the completely open state may be attained only when the polymerase pauses or backtracks. The presence of this stable intermediate state defined two kinetic barriers for complete opening of the TL and, if correct, suggests that the effects of Rpb9 on catalytic activity could be mediated not only through stabilization of the open TL conformation but also through effects on the stability of the intermediate state or either of the two transition states that define the kinetic barriers between the relatively stable open, intermediate, and closed TL conformations. Among many amino acid interactions observed in the molecular dynamics simulations, of particular interest here are salt bridges between TL amino acids Lys1092 or Lys1093 and Rpb1 α21 amino acids Asp716 (in the open TL), Glu712 (in the transition state between the open TL and the stable intermediate), and Asp716 (in the intermediate state) as well as contacts between TL residues Phe1086 and Ala1087 and α21 residue Lys726 in the transition and intermediate states, respectively. Our results, which suggest that Rpb9 has an important role in orienting α21, can be interpreted in the context of the open TL positions identified in crystal structures as well as in the context of interactions between the TL and α21 that are predicted by molecular dynamics simulation of TL movement.

A role for Rpb9 in establishing a correct orientation for Rpb1 α21 might at least partially explain some other phenotypes of rpb9Δ yeast strains. For example, the α21 mutation rpb1-G730D has a strong negative interaction with RPB9 (29) (see Fig. 2). Gly730 is in a small hydrophobic pocket that can accommodate neither the size nor the charge of aspartate, and it is likely that the G730D substitution alters the position of the C-terminal end of α21 and perhaps the orientation of the entire helix. It is easy to imagine that such a reorientation could restrict TL movement, resulting in the slow rate of elongation characteristic of pol II-(Rpb1-G730D). It is also possible that removal of the anchoring interactions with Rpb9 (as in strains containing rpb9Δ or rpb9-(95–97)A) could allow α21 to adopt a position that is less restrictive to TL movement, consistent with suppression of rpb1-G730D by rpb9Δ and rpb9-(95–97)A (Figs. 2 and 3).

Rpb1 α21 also plays a role in TFIIH-mediated RNA cleavage of stalled or backtracked elongation complexes. In order to
facilitate cleavage, a long arm of TFIIIS is inserted into the active site (36). This arm contains two acidic residues (Asp290 and Glu291 in *S. cerevisiae*) that chelate a Mg\(^{2+}\)/H\(^{+}\) ion and reprogram the pol II active site for cleavage (50). The pathway of the TFIIIS arm into the active site is immediately adjacent and parallel to Rpb1, with salt bridge or hydrogen bond interactions between TFIIIS and Rpb1 at amino acids Arg720, Asp727, Arg731, and Glu734 (36) (PDB entry 1Y1V). For the *Thermus aquaticus* RNA polymerase, it appears that the TL and Gre, a protein that stimulates cleavage of backtracked ECs in a manner very similar to that of TFIIIS (51), compete for position in the active site (52). Thus, it seems plausible that the effects of Rpb9 on TFIIIS-mediated cleavage (4, 9, 30, 53, 54), which occur without affecting the affinity of TFIIIS for pol II (16), may be due, at least in part, to positioning of \(\alpha_21\), which is important for both the TL and TFIIIS to access the active site.

Our work strongly supports the idea that Rpb9 indirectly affects TL mobility. Our results also hint at the multifunctionality of Rpb9, with C-terminal functions relating to the TL and N-terminal functions supporting additional roles that may be

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**FIGURE 7.** *Growth phenotypes of mutations at potential interaction interfaces.* Strains based on CKY283 or dopY249 with the genotype shown were transformed with plasmids derived from pRS415 (SC-Leu) or pRS413 (SC-His) carrying the indicated alleles of *RPB9* or *RPB1*. MPA hypersensitivity. Strains were grown to saturation in appropriate media, and serial 10-fold dilutions were spotted onto solid media containing the indicated concentrations of MPA. Growth after incubation for 2 days at 30 °C is shown. A, Gal\(^{+}\) phenotype. Strains were grown to saturation in media selective for plasmid retention, and serial 10-fold dilutions were spotted onto solid media containing the indicated concentrations of MPA. Growth after incubation for 2 days (YP Raf) or 4 days (YP Raf/Gal) at 30 °C is shown.
more directly related to the effect of Rpb9 on cell growth or the genetic interactions between RPB9 and genes encoding components of certain chromatin-modifying complexes (48, 54). Future structural and functional work along the lines of our approaches here will illuminate the molecular nature of these possible Rpb9 functions.

Experimental Procedures

Yeast Strains and Media—Yeast strains used in this study are described in Table 1. Strain BJ5464 His-Bio (generously provided by Mikhail Kashlev (55)) contains a His6 tag on Rpb3, which allows pol II to be immobilized from WCE with Ni2+–NTA beads. CKY283 (23) contains a TAP tag on Rpb3, which allows pol II to be immobilized from WCE with IgG-agarose beads. The RPB9 ORF was deleted from BJ5464 His-Bio and CKY283 (rpb9/H9004::KANMX4) by PCR-mediated one-step gene disruption (32, 33) to generate strains dopY127 and dopY249, respectively. Yeast media and propagation used standard procedures (56).

Construction of rpb9 and rpb1 Alleles—Missense alleles of RPB9 were constructed in the context of various pRS4XX plasmids (33, 57) by PCR-mediated site-directed mutagenesis (58). The rpb9(1–59) allele, which encodes only the N-terminal 59 amino acids of Rpb9, was constructed by PCR-mediated site-directed deletion of DNA encoding amino acids 60–122. The RPB9 sequences in each plasmid included DNA 285 bp upstream and 635 bp downstream of the RPB9 ORF. All of the alleles of RPB9 included an N-terminal FLAG epitope tag (DYKDDDDK) inserted between amino acids 1 and 2. All constructs were confirmed by DNA sequencing.

RPB1 alleles R711A and (707–709)A were constructed by site-directed mutagenesis (58), and alleles S1091C, S1091W, and K1092E were isolated as part of an ongoing genetic screen for mutations in the TL. The RPB1 allele S713P has been described previously (38). All constructs were confirmed by DNA sequencing.

Preparation of Whole Cell Extract—WCE was prepared as described by Kireeva et al. (55). Protein concentrations in WCE were 30–45 mg/ml as estimated by absorbance at 280 nm.

Purification of Pol II and Pol IIΔ9—Pol II and pol IIΔ9 were purified essentially as described by Kireeva et al. (55) with modifications described by Sydow et al. (59).

Elongation Complex Assembly and in Vitro Transcription—RNA and DNA oligomers were purchased from Integrated DNA Technologies (Coralville, IA). RNA oligonucleotides...
(16.7 pmol) were labeled with $[\gamma-^{32}P]$ATP (16.7 pmol) (3000 Ci/mmol, PerkinElmer Life Sciences catalog no. BLU002A) using T4 polynucleotide kinase (Promega catalog no. M4101) in transcription buffer TB(40) (20 mM Tris- HCl (pH 7.9), 40 mM KCl, 5 mM MgCl$_2$, 2 mM 2-mercaptoethanol). RNA oligonucleotides were then annealed with the appropriate DNA template strand (16.7 pmol) as described by Kireeva et al. (55). Unlabeled NTPs were obtained from GE Healthcare.

ECs were assembled essentially as described (9, 55). For assays using purified polymerase, pol II or pol IIΔ9 (250 ng) was added to 25 µl of Ni$^{2+}$-NTA beads, which had been equilibrated in transcription buffer. For assays using WCEs, 25–50 µl of extract (~1.5 mg of total protein) were added to 25 µl of Ni$^{2+}$-NTA beads (or human IgG-agarose beads for CKY283-derived strains carrying the Rpb3 TAP tag). Purified pol II or pol II from WCEs was then allowed to bind the beads for 30 min at room temperature in an orbital shaker (1000 rpm). After the initial incubation, beads were washed with 1 ml of buffer identical to TB(40) except with 1 mM KCl (TB(1000)) for 10 min, followed by three 1-ml washes with TB(40). The preannealed RNA/DNA hybrid (1 pmol) was then added to the beads in a total volume of 100–150 µl and incubated for 10 min, followed by the addition of the non-template strand (10 pmol), which was allowed to incubate for an additional 10 min. ECs were then washed with 1 ml of TB(1000) for 10 min, followed by three washes with 1 ml of TB(40). The final suspension of washed ECs was in 100–200 µl of TB(40).

Transcription was initiated by the addition of substrate NTPs at concentrations described in the figure legends, and aliquots (10 µl) of the reaction mixture were removed at various time intervals and added to 10 µl of 2× loading buffer (10 M urea, 50 mM EDTA (pH 7.9), 0.005% bromophenol blue, 0.005% xylene cyanol), which stopped the reaction. The resulting extension products were resolved in 13.5% gel (for longer template as in Figs. 4 and 8A) or 20% polyacrylamide gels as described (9, 23). For assays using α-amanitin, assembled ECs were incubated with α-amanitin (Calbiochem) for 15 min before the addition of NTPs. Quantification of elongation products was performed using a Bio-Rad PhorusFX Plus phosphorimager. In order to account for possible sampling inconsistencies that could be caused by non-uniform distribution of beads in the reaction mixture, signals from bands of interest were normalized to the total signal from all bands in the same lane of the gel.

For experiments with the longer template (Figs. 4 and 8A), the half-time of the reaction was determined based on the observed progress of the reaction, which empirically was found to closely fit that of a simple first-order reaction (see Fig. 4C). Plots, curve fits, and statistics were prepared using KaleidaGraph (version 4.1; Synergy Software) or Prism (version 6.0; GraphPad Software) graphing software. Errors shown for values of $k_{obs}$ (misincorporation assays) represent 95% confidence intervals derived from the curve fits. Errors in elongation rates (longer template) represent 95% confidence intervals calculated from curve fit-derived errors in reaction half-times.

Western Blotting Analysis—WCEs (300 µl) prepared from a dopY127-derived strain carrying an empty vector (pRS413) or the same vector containing DNA encoding either Rpb9 or Rpb9-(95–97)A were incubated with 20 µl of Ni$^{2+}$-NTA beads for 30 min and washed as described for preparation of ECs. After the washes, most of the final wash buffer was removed to reduce the volume to ~20 µl, and 20 µl of 2× Laemmli buffer (120 mM Tris- HCl (pH 6.8), 20% glycerol, 4% SDS, 0.005% bromophenol blue, and 5% 2-mercaptoethanol) was added. Samples were boiled for 2 min and loaded onto a 4–20% SDS-polyacrylamide gradient gel along with fluorescently labeled molecular weight size standards. The resolved proteins were electrophoretically transferred to a PVDF membrane overnight at room temperature. The membrane was then blocked with TBS containing 3% nonfat dry milk for 1 h, followed by an overnight incubation at 4 °C with mouse anti-FLAG antibody (Sigma catalog no. F1804) (diluted 1:1000 in TBS containing 3% nonfat dry milk). The membrane was then rinsed several times in water, followed by five washes with TBS. Anti-mouse IgG (Sigma catalog no. A9044) (diluted 1:30,000 in TBS containing 3% nonfat dry milk) was then added to the blot and incubated for 30 min at room temperature, followed by five washes with TBS. The blot was visualized by incubation with Supersignal West Femto (Thermo Fisher catalog no. 34095) for 5 min, with the signal detected by a chemiluminescent imager (Bio-Rad ChemiDoc XRS). The membrane was then washed with TBS, reblocked, and probed with anti-Rpb1 C-terminal domain mouse antibody 8W4G16 (Covance catalog no. MMS-126R-500) (diluted 1:500 in TBS containing 3% nonfat dry milk), washed, treated with secondary antibody, and imaged as described above.

Growth Phenotypes of Yeast Strains—Strains were grown to saturation at 30 °C in appropriate selection media, and aliquots were transferred to one row of a 96-well plate. Six 10-fold serial dilutions (in sterile water) were prepared for each strain, spotted onto the indicated media using a pin spotter (Dan-Kar), and grown for the indicated number of days.

Author Contributions—B. C. K. conducted most of the experiments, analyzed the results, and wrote the paper with D. O. P. K. C. K. purified pol II and pol IIΔ9 and conducted and analyzed the experiment in Fig. 1. C. D. K. constructed various TL mutant strains, analyzed results, and provided valuable input during the writing of the manuscript. D. O. P. provided the initial concept for the experiments, analyzed the results, and wrote the paper with B. C. K. All authors reviewed the results and approved the final version of the manuscript.

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Rpb9 and Pol II Trigger Loop Mobility

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