Unstructured N Terminus of the RNA Polymerase II Subunit Rpb4 Contributes to the Interaction of Rpb4·Rpb7 Subcomplex with the Core RNA Polymerase II of Saccharomyces cerevisiae

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Two subunits of eukaryotic RNA polymerase II, Rpb7 and Rpb4, form a subcomplex that has counterparts in RNA polymerases I and III. Although a medium resolution structure has been solved for the 12-subunit RNA polymerase II, the relative contributions of the contact regions between the subcomplex and the core polymerase and the consequences of disrupting them have not been studied in detail. We have identified mutations in the N-terminal ribonucleoprotein-like domain of Saccharomyces cerevisiae Rpb7 that affect its role in certain stress responses, such as growth at high temperature and sporulation. These mutations increase the dependence of Rpb7 on Rpb4 for interaction with the rest of the polymerase. Complementation analysis and RNA polymerase pulldown assays reveal that the Rpb4·Rpb7 subcomplex associates with the rest of the core RNA polymerase II through two crucial interaction points: one at the N-terminal ribonucleoprotein-like domain of Rpb7 and the other at the partially ordered N-terminal region of Rpb4. These findings are in agreement with the crystal structure of the 12-subunit polymerase. We show here that the weak interaction predicted for the N-terminal region of Rpb4 with Rpb2 in the crystal structure actually plays a significant role in interaction of the subcomplex with the core in vivo. Our mutant analysis also suggests that Rpb7 plays an essential role in the cell through its ability to interact with the rest of the polymerase.

Studies of transcriptional regulation have focused mainly on the role of DNA-bound regulatory proteins and their contacts with the general transcription factors, mediator, and other accessory proteins in the transcriptional machinery. In most eukaryotes, the 12-subunit RNA polymerase II (Pol II) is thought to have little or no influence on the regulation of transcription. Rpb4 and Rpb7 form a subcomplex within the polymerase that dissociates easily under mild denaturing or non-denaturing conditions and shows variable association with the polymerase at different growth stages, leading to the suggestion that the subcomplex could be analogous to the σ subunit of the bacterial RNA polymerase (1–3). Whether such a regulatory role can be ascribed to the subcomplex has been a matter of some debate, but the phenotypes of the deletion mutants and the interactions mediated by these subunits suggest that they might have some regulatory role in stress response and transcription (4).

Rpb7 is essential for survival of Saccharomyces cerevisiae, whereas Rpb4 is not (5). However, rpb4Δ strains are temperature-sensitive and cold-sensitive, show poor recovery from stationary phase, are defective in sporulation (a response to severe nutritional starvation), and are predisposed to pseudohyphae formation (a response to mild nutritional starvation) (4). Apart from its roles in stress response, Rpb4 is involved in transcription under moderate and extreme temperatures (6). The polymerase lacking Rpb4 and Rpb7 is defective for promoter-dependent initiation of transcription but not for promoter-independent chain elongation (2).

Pol II activity at extreme temperatures is dependent on the presence of Rpb4, leading to a suggestion that the Rpb4·Rpb7 subcomplex controls the stability of Pol II under extreme temperatures (7). However, rpb4Δ strain is also defective for activated transcription from a subset of genes at moderate temperatures (8).

Early studies showed that Pol II purified from rpb4Δ strain lacks Rpb7, suggesting that the Rpb4 stabilizes the interaction of Rpb7 with the rest of the Pol II (9, 10). Consistent with such a hypothesis, overexpression of Rpb7 can partially rescue the temperature sensitivity and sporulation defects of the rpb4Δ strain (10, 11). In addition, overexpression of Rpb7 can rescue activated transcription only from certain stress promoters but not from the nonstress promoters, suggesting that ScRpb7 may

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4 The abbreviations used are: Pol II, RNA polymerase II; RNP, ribonucleoprotein; OB, oligonucleotide/oligosaccharide binding; YPD, yeast extract peptone dextrose medium; TAP, tandem affinity purification; WT, wild type; aa, amino acids.
not have a generalized role in promoter-dependent transcription in vivo (8).

Rpb4 plays a Rpb7-independent role in promoting mRNA export and maintaining transcript stability of a subset of genes (12, 13). On the other hand, overexpression of Rpb7 in the Rpb4 heterodimer in the polymerase based on the low resolution crystal structure of the 12-subunit Pol II (24, 25) confirmed that Rpb7 is in a position to interact with the nascent RNA transcript released from the transcribing complex. This prediction has been recently validated by UV cross-linking studies (26). Although the subcomplex has been shown to bind the nascent transcript, a clearly defined molecular function for the subcomplex has not yet emerged.

The structure of the 12-subunit RNA polymerase from S. cerevisiae determined at higher resolution allowed a more detailed view of the interaction between the Rpb4-Rpb7 complex and the polymerase core (20). The tip loop of the RNP domain of Rpb7 interacts with a region of Pol II made up of the linker region of Rpb1 and Rpb6. Furthermore, the authors propose that the N-terminal residues of Rpb4 may be involved in the interaction with Rpb2. However, the relative contributions of the reported points of interaction between the subcomplex and the core polymerase II and the consequences of disrupting them have not been studied in detail. Given the essential nature of Rpb7, the complexity of its roles in multiple phenotypes and the lack of useful information from deletion-based analyses, we decided to use genetic screens to isolate mutants of Rpb4 and Rpb7. The structure of the 12-subunit RNA polymerase from S. cerevisiae was recently shown to disrupt the Rpb7 heterodimer in the polymerase based on the low resolution crystal structure of the 12-subunit Pol II (24, 25) confirmed that Rpb7 is in a position to interact with the nascent RNA transcript released from the transcribing complex. This prediction has been recently validated by UV cross-linking studies (26). Although the subcomplex has been shown to bind the nascent transcript, a clearly defined molecular function for the subcomplex has not yet emerged.

## Experimental Procedures

**Yeast Strains**—The yeast strains used in this study are listed in Table 1. These strains were transformed with the appropriate plasmids and assayed for various phenotypes. Yeast transformations were performed routinely using the modified lithium acetate protocol that does not involve heat treatment of cells (28). For high efficiency transformation for the genetic screens, the method of Finley and Brent (29) was followed. Yeast strains were grown in rich YPD medium or in synthetic dextrose medium containing 2% glucose or 2% galactose as a carbon source and the required amino acids.

## Plasmids

The gapped plasmid (containing sequences 650 bp upstream and downstream of Rpb7 open reading frame separated by a HindIII site) pVS134 used in the genetic screen to isolate loss of function mutations in Rpb7 was generated by subcloning the 1.5-kb PvuII fragment from pBP86 in between the PvuII sites of pPS7 (YEpIac195). The plasmid pVS154 expressing the rpb7−7 mutant and the plasmid pSP186 expressing wild type RPB7 from pPS7 were used to separate the

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**Table 1**

| Strain | Genotype | Source |
|--------|----------|--------|
| SY10   | MATa, leu2-3, lys2-112, ura3-52, rpb4Δ::HIS3 | Ref. 8 |
| SYD101 | MATa/a, his3Δ200::his3Δ200, ura3-52::ura3-52, leu2-3::leu2-3, lys2-1::lys2-1, rpb4Δ::HIS3/rpb4Δ::HIS3 | Ref. 12 |
| SYD7   | MATa/a, ADE2/ade2-101, his3Δ200::his3Δ200, leu2-3::leu2-3, TRP1/trp1-901, lys2-112/lys2-112, RPB7::RPB7Δ::LEU2 | Ref. 15 |
| BY4741 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Euroscarf, Germany |
| Y7005  | MATa rpb4Δ::kanr his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | This study |
| SY1011 | MATa, ade2Δ ade7Δ ura3-1, his3-115, trp1-1, leu2-3112, can1-100, UBR1::GAL-HA-UBR1(HIS3) | Euroscarf, Germany |
| SY1012 | MATa, ade2Δ ade7Δ ura3-1, his3-115, trp1-1, leu2-3112, can1-100, UBR1::GAL-HA-UBR1(HIS3), N-deg Rpb7(KAN') | This study |

### Strain Genotype Source

| Strain | Source |
|--------|--------|
| MATa, ade2-1, ura3-1, his3-115, trp1-1, leu2-3112, can1-100, UBR1::GAL-HA-UBR1(HIS3) | Euroscarf, Germany |
| N. deg 7 MATa, ade2-1, ura3-1, his3-115, trp1-1, leu2-3112, can1-100, UBR1::GAL-HA-UBR1(HIS3), N-deg Rpb7(KAN') | Euroscarf, Germany |

**List of strains used in this work**

**Interaction of Rpb4-Rpb7 Subcomplex with Core Pol II**
N- and the C-terminal mutations of rpb7−7. The plasmid, pVS357 expressing the rpb7−7 N-terminal mutations was generated by a three-way ligation between a 0.9-kb EcoRI-DraI fragment from pVS154, a 0.5-kb DraI-Sall fragment from pSP186, and the EcoRI-Sall-digested pPS7. The plasmid pVS358 expressing the rpb7−7 C-terminal mutations was generated by a three-way ligation between a 0.95-kb BamHI-DraI fragment from pVS154, a 0.9-kb DraI-Sall fragment from pSP186, and the BamHI-Sall-digested pPS7. Plasmids pBB651 and pBB652 are the WT RPB4 and RPB7 transformed with different alleles from the pVS186 and pVS154 plasmid, respectively, subcloned in the plasmid pPS6 (YEplac112) with TRP1 auxotrophic marker to enable us to accommodate all the plasmids used in this work. Plasmid YKL 187 for amplification of the degron cassette was obtained from Euroscarf (Frankfurt, Germany).

Construction of the TAP-tagged Strains—The N-DeG strain was generated by fusing the Rpb7 open reading frame in YKL200 strain with the N-degron cassette amplified with the plasmid YKL187 as template and primers (5'-GGT TCT CCT CCT ACA CCA TTC TTA AGG CGC GCC AGA TCT G-3' and 5'-AAG GAC GGA TGA AGG GTA ATA TTA AGC GAA GAA CCT TTA ATA AAA AAC ATG GCA CCC GCT CCA GCG CCT G-3') (30). Strain SC1126 was crossed with the Y07005 (rpb4Δ) strain to obtain rpb4Δ strain containing the TAP-tagged Rpb3 fusion. This haploid transformed with different RPB4 alleles was crossed to the N-DeG strain transformed with different RPB7 alleles and segregrants expressing C-terminal TAP tag Rpb3 and N-deg-Rpb7 fusion proteins in both wild type as well as rpb4Δ background were obtained. The haploids were selected on YPDCuSO4 with 500 μg/ml G418 because the N-deg Rpb7 open reading frame is under the control of CUP1 promoter, and the rpb4Δ is marked with kan resistance. Colony size, rate of growth, and temperature sensitivity were used as parameters for initial screening of rpb4Δ strains and wild type strain because rpb4Δ strains are temperature-sensitive and slow growing with small colony size. The haploid isolates were further confirmed by PCR specific for the N-DeG Rpb7 module and the rpb4Δ locus and by Western blotting with New Zealand White rabbit serum to detect the TAP-tagged moiety in WT and rpb4Δ strains. To check survival of the cells at 37 °C in the presence of high levels of the galactose-inducible Ubr1 protein, both the strains were grown on yeast extract peptone galactose medium and incubated at 37 °C.

Temperature Sensitivity, Sporulation, and Pseudohyphal Growth—These assays were performed essentially as described before (8). The assay for temperature sensitivity was performed on synthetic dextrose plates containing 2% glucose at 25 or 37 °C. The assays for sporulation and pseudohyphal growth were done on 1% potassium acetate plates and on synthetic low ammonia dextrose medium, respectively.

Random Spore Analysis—Sporulated cultures were treated with lyticase (1 mg/ml in 1 M sorbitol) for 30 min to 1 h, vortexed with an equal volume of mineral oil for 2 min, and centrifuged for 30 s. The mineral oil layer enriched with hydrophobic spores was plated on appropriate selection medium.

TAP Tag-based Pol II Pulldowns (31)—The strains with C-terminally TAP-tagged RPB3 in rpb4Δ genetic background carrying either wild type or Rpb4− (33−221) mutant expressing plasmids pNS114 and pVS378 and with rpb7−7 expressing plasmid pVS154 were grown overnight in YPDCuSO4, at 26 °C and diluted to a density of 5 × 10^6 cells/ml in 50 ml of fresh YPD-CuSO4 medium. The cells were pelleted and washed in sterile water, freeze-thawed in liquid nitrogen, and then resuspended in 25 μl of whole cell extract lysis buffer (20 mM HEPES, pH 7.9, 10% glycerol, 0.5 mM EDTA, 300 mM potassium acetate, 2 mM dithiothreitol, and 0.05% Nonidet P-40 with protease inhibitors). The cells were lysed with the addition of chilled glass beads by four cycles of 1 min of vortexing alternating with 5 min cooling on ice. The lysates were clarified at 13,000 rpm for 20 min at 4 °C. The protein concentrations were determined by Bradford assay (Invitrogen). Approximately 1.5 mg of total protein was used for TAP tag purification or immunoprecipitation. TAP-tagged extracts were incubated with 40 μl of rabbit IgG-agarose at 4 °C for 1 h. The beads, equilibrated in TEV protease cleavage buffer, were washed three times with 1 ml of the same buffer. The proteins were eluted from the beads with the addition of 2.5 units of TEV protease in 50 μl of TEV protease cleavage buffer. The eluted proteins were analyzed by Western blotting to identify the interaction of the subcomplex with the rest of the Pol II. The N-deg Rpb7 and rpb7−7 alleles were monitored using α-Rpb7 antibodies generated in the laboratory. The α-Rpb4 antibody procured from Neocline Inc. was raised against the N-terminal region of Rpb4 and hence would not recognize the Rpb4− (33−221) allele described here. Although the Rpb4− (33−221) allele cannot be recognized by the α-Rpb4 antibody, the protein is being made and is stable because it can complement the stress response defects of rpb4Δ (27).

RESULTS

Genetic Screen Allowed Isolation of Conditional Mutants of RPB7—We used a genetic screen to isolate conditional "loss of function" alleles of RPB7. Fig. 1A shows a schematic representation of the screen used to isolate mutants of Rpb7 gene carried on a 2μ plasmid. Briefly, a gapped plasmid was generated that had 650 bp of the 5'− and 3'− untranslated regions of RPB7 at its linearized ends. This gapped plasmid was transformed along with a PCR-mutagenized RPB7 gene pool into the heterozygous RPB7/rpb7Δ strain, SYD7. At the time of carrying out the screen, the only available rpb7Δ mutant was marked with LEU2, and the complementation studies were carried out with the RPB7 gene on a plasmid carrying the URA3 gene. Hence a conventional plasmid shuffle screen was not possible. The transformants were pooled and sporulated, and haploids were generated by random spore analysis. rpb7Δ haploids carrying the in vivo recombined plasmid were selected based on their abilities to grow in the absence of leucine and uracil, ensuring the selection of rpb7Δ cells and the plasmid, respectively. The random spore analysis was standardized to ensure minimal contamination with diploids, and the haploids were confirmed by mating type PCR analysis. These haploids were further screened for temperature sensitivity at 37 °C. The plasmids iso-
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The ability of the plasmid borne alleles to complement the lethality of rpb7Δ strain was tested by retransforming the RPB7/rpb7Δ (SYD7) and carrying out random spore analysis. We defined the percentage of survival as the percentage of haploids carrying a certain genotype among all haploids generated from the sporulated SYD7 strain by random spore analysis. Complementation of the lethality of rpb7Δ by wild type RPB7 results in 50% survival. All of the mutants analyzed showed ability to complement rpb7Δ at room temperature (data not shown), but the sensitivity of the mutants to high temperature varied to a great extent (Fig. 1B).

As mentioned earlier and also seen in Fig. 2, rpb4Δ mutant is temperature-sensitive, defective in sporulation, and predisposed to pseudohyphae formation. Overexpression of RPB7 in rpb4Δ rescues the temperature sensitivity at 34 °C but not at 37 °C. It also rescues the sporulation defect but exaggerates the pseudohyphal morphology. The six mutants analyzed for the rescue of the above phenotypes in rpb4Δ strain showed that except for rpb7−7, all alleles were able to rescue temperature sensitivity, whereas some alleles along with rpb7−7 were unable to rescue sporulation defect. Interestingly, all of them exaggerated the pseudohyphal phenotype. Table 2 summarizes the phenotypes of these mutants.

In this report, we present detailed characterization of the rpb7−7 mutant, which has provided us with a new insight into the interaction between Rpb7 and Rpb4 and the function of the subcomplex. The rpb7−7 mutant was chosen because it has interesting phenotypes in the absence of Rpb4 but behaved like the WT protein in the presence of Rpb4. The plasmid from rpb7−7 mutant was isolated and retransformed into SYD7 to confirm that the lethality of rpb7Δ can be complemented by the mutant as well as RPB7. In addition, the mutant allele does not compromise the growth of rpb7Δ significantly at higher temperature (Fig. 1B). Interestingly, the rpb7−7 allele, when compared with the wild type RPB7 expressed from a similar 2μ plasmid, shows a clear defect in its ability to rescue the temperature sensitivity and sporula-

![Figure 1](image1.png)

**FIGURE 1.** Isolation and characterization of conditional mutants of RPB7. A, scheme of isolation of loss of function mutants of Rpb7. Briefly, the heterozygous rpb7Δ/RPB7 strain was transformed with the PCR-mutagenized pool of RPB7 and a gapped plasmid containing upsteam and downstream sequences. The recombinant plasmid containing the mutagenized RPB7 open reading frame was selected for its ability to complement rpb7Δ and screened for conditional phenotypes. B, the temperature sensitivity assay of loss of function mutants of Rpb7 in rpb7Δ. The haploid rpb7Δ segregants containing the mutant plasmids were replica-plated onto plates incubated at 25 and 37 °C. Photographs were taken after 2 days of incubation.

![Figure 2](image2.png)

**FIGURE 2.** Overexpression of rpb7−7 mutant does not rescue some of the rpb4Δ phenotypes. A, temperature sensitivity assay for the rpb7−7 mutant in rpb4Δ strain background. Diploid rpb4Δ/rpb4Δ strain SYD1011 containing plasmids expressing either wild type or the mutant rpb7 allele was replicaplated onto plates incubated at 25, 34, and 37 °C. Photographs were taken after 2 days of incubation at the respective temperatures. B, the rpb7−7 mutant does not rescue the sporulation defect of rpb4Δ. The diploid rpb4Δ/rpb4Δ strain SYD1011 with plasmids expressing RPB4 or the Rpb7 alleles were pregrown in rich YPD medium and transferred to sporulation medium for 72–96 h. The number of tetrads in a population of at least 500 cells of each strain was counted under an Olympus BX50 microscope and represented as the percentage of sporulation normalized to the wild type sporulation level. Shown here are the average normalized sporulation percentages for three independent experiments with three transformants for each strain and the standard deviations thereof. C, exaggeration of the pseudohyphal morphology of rpb4Δ/rpb4Δ by rpb7−7 mutant. The diploid rpb4Δ/rpb7−7 strain SYD1011 with plasmids expressing the Rpb7 mutants were assayed for pseudohyphal morphology as described under “Experimental Procedures.” Empty vector plasmid (rpb4Δ) and RPB4-expressing plasmid were used as negative and positive controls, respectively, for all of the above phenotypic assays.

lated from the putative mutants were analyzed for various stress phenotypes associated with rpb4Δ and rpb7Δ strains.

Of the 6000 transformants screened, 70 haploids showed the temperature-sensitive phenotype. Of these, six alleles that showed consistent phenotypes after multiple rounds of screening were used for further characterization. These mutants were compared with wild type RPB7 in various assays in rpb7Δ and rpb4Δ strains.
TABLE 2

Summary of phenotypes of the different loss of function mutants isolated in the genetic screen in rpb7Δ and rpb4Δ strains

| Allele | Sequence changes | rpb7Δ Growth at 38 °C | Protein level | rpb4Δ Growth at 34 °C | Sporulation | Pseudohyphal growth | Protein level |
|--------|-----------------|-----------------------|---------------|-----------------------|-------------|---------------------|---------------|
| RPB7   | None            | + (+)                 | +             | + (+)                 | + (+)       | + (+)               | +             |
| rpb7-7 | F70L, V72M, A123E, S125T | -                     | -             | -                     | -           | -                  | -             |
| rpb7-18| D50A, T138P     | -                     | -             | -                     | -           | -                  | -             |
| rpb7-34| I147G           | -                     | -             | -                     | -           | -                  | -             |
| rpb7-61| S162T           | ±                     | +             | +                     | ±           | +                  | ±             |
| rpb7-62| S162T           | ±                     | +             | +                     | ±           | +                  | ±             |
| rpb7-63| ND              | ±                     | +             | +                     | ±           | +                  | ±             |

interaction defects of rpb4Δ strain (Fig. 2, A and B). However, its ability to enhance the pseudohyphal morphology of rpb4Δ strain is not affected (Fig. 2C). The steady state protein level of the rpb7Δ allele in rpb4Δ and rpb7Δ strains is stable and expressed at similar levels as wild type Rpb7 in both rpb4Δ and rpb7Δ strains (data not shown). Thus the defective sporulation and temperature sensitivity seen for the rpb7Δ allele in rpb7Δ is not the result of reduction in the level of the mutant protein.

The RNP Fold of Rpb7 Is an Important Functional Determinant—Rpb7 folds into two domains: an N-terminal truncated RNP fold and a C-terminal OB fold (Fig. 3A). Sequencing of the rpb7Δ allele revealed that it had four mutations (F70L, V72M, A123E, and S125T). The first two mutated residues are located on a conserved β-strand of the RNP fold, whereas the other two mutated residues are on the three-stranded antiparallel β-sheet insertion between strands B3 and B4 of the OB fold domain (Fig. 3A). The phenotype of rpb7Δ could be the consequence of a disrupted RNP fold or could be caused by alterations in the interactions mediated by the solvent-exposed C-terminal β-sheet or both. To distinguish between these possibilities, we swapped the N- and C-terminal domains of the mutant with the wild type Rpb7 to construct rpb7Δ N allele carrying only the F70L and V72M mutations and the rpb7Δ C allele carrying only the A123E and S125T mutations. Both of these mutant alleles could complement the lethality of rpb7Δ as well as the original rpb7Δ mutant (data not shown). On analysis of the phenotypes in rpb4Δ, it was obvious that the N-terminal mutations are responsible for both the temperature sensitivity (Fig. 3B) and sporulation defects (Fig. 3C) of rpb7Δ. For both the phenotypes tested, the behavior of the rpb7Δ C allele is similar to that of RPB7. Thus the RNP fold is important for the role of Rpb7 in rescuing the temperature sensitivity and sporulation defect of rpb4Δ.

The rpb7Δ Allele Is Dependent on Rpb4—The rpb7Δ allele can rescue the lethality of rpb7Δ but cannot rescue some of the phenotypes associated with rpb4Δ. One hypothesis to explain this would be that the mutant protein is able to function in the presence of Rpb4 (as in rpb7Δ strains) but not in its absence (as in rpb4Δ strain). Another hypothesis is that the rpb7Δ can still perform the essential function of Rpb7 but not the rpb4Δ-specific phenotypes. To distinguish between these possibilities, we tested the ability of rpb7Δ to rescue the lethality of rpb7Δ in the absence of Rpb4.

We constructed a diploid strain heterozygous for Rpb4 and RPB7 (RPB4/rpb4Δ::HIS3, RPB7/rpb4Δ::LEU2) expressing either the wild type RPB7 or rpb7Δ allele from a plasmid. If the first hypothesis were true, then rpb7Δ would have a reduced ability to rescue the lethality of rpb4Δ rpb7Δ haploids than rpb7Δ haploids. If the second hypothesis were true, then rpb7Δ should be able to complement lethality of both the rpb4Δ rpb7Δ and the RPB4 rpb7Δ haploids. On sporulation and random spore analysis, strains carrying plasmid borne WT RPB7 generated ~50% spores (of the total) that carry the rpb7Δ allele, indicating full complementation of the lethality of rpb7Δ (Fig. 4). Of these, roughly half are rpb4Δ rpb7Δ, indicating that the wild type RPB7 is able to rescue the lethality of rpb7Δ irrespective of the presence or absence of Rpb4. On the other hand, although the rpb7Δ allele generates ~50% spores that are rpb7Δ, none of them are rpb4Δ rpb7Δ (Fig. 4). Thus the rpb7Δ is unable to complement an rpb4Δ rpb7Δ strain, indicating a complete loss of activity of the Rpb7-7 in the absence of Rpb4.
Interaction of Rpb4-Rpb7 Subcomplex with Core Pol II

The Subcomplex Requires at Least One Functional Point of Interaction with the Core Pol II to Ensure Its Recruitment in the Polymerase—The latest refinement of the crystal structure of the 12-subunit yeast Pol II shows that Rpb7, through its N-terminal conserved tip region in the RNP fold, interacts with the linker region of Rpb1 (20). It also predicts that the N-terminal extension in Rpb4 (aa 1–46) interacts with the rest of the Pol II through Rpb2. We reasoned that because rpb7–7 allele with an altered RNP fold requires Rpb4 for survival, Rpb4 might be required to stabilize the interaction of the mutant Rpb7-7 protein with the Pol II through the N-terminal Rpb4-Rpb2 interaction. If so, an N-terminal deletion of Rpb4, like rpb4Δ, should drastically reduce the ability of rpb7–7 allele to complement the lethality of rpb7Δ.

We have previously shown that the N-terminal deletion of 32 aa of Rpb4 (RPB4-(33–221)) does not affect the ability of Rpb4 to function in stress response phenotypes (27). We performed similar experiments as above using a heterozygous RPB4/rpb4Δ RPB7/rpb7Δ strain carrying either the WT or the N-terminal deletion of RPB4 or the empty vector in combination with either the RPB7 or the rpb7–7 mutant plasmid. We calculated the percentage of survival as before, for rpb4Δ rpb7Δ haploids carrying (a) RPB4, (b) RPB4-(33–221), or (c) the empty vector with RPB7 or rpb7–7. Because the double deletion would be roughly 25% of the total segregants, we expected about 25% survivors only if the combination of RPB7 and RPB4 alleles is viable. As shown in Fig. 5, wild type RPB7 can generate 25% viable haploids in the presence or absence of the N-terminal region of Rpb4 (compare bars 1 and 4 in all three panels). However, the rpb7–7 allele is unable to complement the lethality of rpb7Δ in the absence of the N-terminal extension of Rpb4, whereas it is able to function in the presence of full-length Rpb4 (compare bars 2 and 5 in the left panel with bars 2 and 5 in the middle and right panels). This result was also mimicked by the rpb7–7N derivative, suggesting again that the N-terminal RNP fold mutations in rpb7–7 are responsible for its phenotypes.

To further confirm the observations made above, we constructed a haploid strain where the genomic copy of RPB7 is tagged with a temperature-sensitive N-degron module (see “Experimental Procedures”) (30). A temperature shift to 37 °C destabilizes the degron-tagged Rpb7 and is then targeted for degradation by induced levels of the ubiquitin-protein ligase, UBR1, from a galactose-inducible promoter. Thus under inducing conditions at 37 °C, only the strains containing plasmid-borne Rpb7 alleles that can perform the essential functions of Rpb7 will be able to survive. As expected, rpb4Δ in such a strain background shows temperature sensitivity, which can be rescued by overexpression of RPB7 (Fig. 6A, first row) or RPB4 (Fig. 6A, third row). It can be seen that rpb7–7 allele is able to function even under inducing conditions at 37 °C in the presence of RPB4 but is unable to do so when the N-terminal 32 aa of Rpb4 are deleted or there is no Rpb4 in the cell (Fig. 6A compare fourth row with sixth and second rows).

To investigate whether the essential function of Rpb7 is due to its association with the rest of the Pol II, we immunoprecipitated Pol II complexes using Rpb3-TAP tag from the N-degron-Rpb7 strains used above. Under normal conditions, the N-degron-Rpb7 is associated with the Pol II in the presence or absence of WT Rpb4 (Fig. 6B, lanes 1 and 7). It is interesting to note that the N-degron-Rpb7 interaction with the rest of the Pol II is destabilized in the absence of Rpb4 even under noninducing conditions (Fig. 6B, lanes 4 and 5). In perfect correlation with the viability and complementation analysis, the Rpb7-7 protein is associated with Pol II in the presence of Rpb4, even when the WT Rpb7 from the genomic copy is degraded (Fig. 6B, lane 3). However, when there is no Rpb4 in the cell (Fig. 6B, lane 6) or the N-terminal 32 aa of Rpb4 are deleted (Fig. 6B, lane 9),
The results presented here show clearly that the N-terminal deletion of 32 aa of Rpb4 renders the Rpb7-7 mutant protein unable to function in complementing the rpb7Δ strain (Figs. 5 and 6). Further, they indicate that the rpb7Δ-7 mutant allele encodes a protein unable to stably interact with the rest of the Pol II in the absence of a functional N-terminal region of Rpb4. There are two possibilities by which this could happen, either the N-terminal region of Rpb4 somehow stabilizes the Rpb7-7 mutant protein or stabilizes the interaction of Rpb7 with the rest of the Pol II. Because the stability of the Rpb7-7 protein is not significantly altered in the absence of Rpb4 protein, by extension, these results show that the N-terminal region of Rpb4 also functions as a contact point for the subcomplex with the rest of the Pol II. This is in excellent agreement with the crystal structure of the 12-subunit Pol II (20). In this structure, the N-terminal extension of Rpb4 is not very well defined, and only a model for the main chain could be built into the electron density map. This region is seen to make a weak interaction with Rpb2 (Fig. 7A), although biochemical data suggest that it is not required for the in vitro interaction with Pol II. Our studies confirm that there are at least two contact points for the sub-

**DISCUSSION**

Crystal structures of ScRpb7, HsRpb7, and their archaeal homolog RpoE show the presence of a truncated RNP fold in the N terminus and an OB fold in the C terminus of Rpb7 (20–22). As shown by UV cross-linking studies, the function of the OB fold region is thought to be in binding to the nascent RNA released from the transcribing complex (26). Furthermore, mutations in many of the conserved solvent-exposed residues in the OB fold region abolish binding to single-stranded RNA in vitro (23). The RNP fold on the other hand encompasses a highly conserved tip region that interacts with the core

Pol II and acts as the main point of anchorage for the subunit with the rest of the Pol II (20). The random mutagenesis screen described here was designed to identify residues in Rpb7 that contribute to its function in the variety of phenotypes known to be affected by the Rpb4-Rpb7 subcomplex. Additionally, we hoped to use these mutants to correlate the observed phenotypes to association of the subcomplex with the rest of the Pol II.

We identified several mis-sense mutants in both the domains of Rpb7. Most of these mutants conferred a conditional phenotype such as temperature sensitivity or inability to rescue one of the other phenotypes of rpb4Δ (Table 1). Sequence analysis of many of these mutants shows that the mutations affects highly or moderately conserved residues of Rpb7, predicting a conserved role for Rpb7 in stress response. The mutant allele rpb7Δ-7 was particularly interesting because it appeared to require the presence of Rpb4 for its function. Interestingly, of the four mutations identified in rpb7Δ-7 allele, the two that mapped in the conserved RNP fold in the N terminus appeared to be responsible for the phenotypes of the original mutant. The inability of rpb7Δ-7 to function in certain stress responses (Figs. 2 and 3) implicates the RNP fold as the functional end of the protein with respect to these phenotypes. Further, the inability of rpb7Δ-7 to complement the lethality of rpb7Δ in the absence of Rpb4 (Figs. 3 and 4) corroborates the structural observations that the RNP fold of Rpb7 is required for maintaining interaction of the subcomplex with the Pol II. Our results might also help to explain the observation that the Pol II purified from an rpb6 Q100R strain has lowered levels of the Rpb4-Rpb7 subcomplex (32). Armache et al. (20) observed hydrogen bonding between the Rpb7 Gly66 (in the RNP fold) and Rpb6 Gln100. The rpb7Δ-7 mutations map to nearby Phe70 and Val72 residues, especially Val72, being highly conserved among Rpb7, Rpb2, and the archaeal homolog rpoE (33). Because the rpb6 Q100R mutation maps to the region of the core Pol II that interacts with the tip of the RNP fold, the mutation in Rpb7Δ-7 could lead to reduced interaction between Rpb6 and Rpb7, leading to the loss of the subcomplex from the Pol II.

The results presented here show clearly that the N-terminal deletion of 32 aa of Rpb4 renders the Rpb7-7 mutant protein unable to function in complementing the rpb7Δ strain (Figs. 5 and 6). Further, they indicate that the rpb7Δ-7 mutant allele encodes a protein unable to stably interact with the rest of the Pol II in the absence of a functional N-terminal region of Rpb4. There are two possibilities by which this could happen, either the N-terminal region of Rpb4 somehow stabilizes the Rpb7-7 mutant protein or stabilizes the interaction of Rpb7 with the rest of the Pol II. Because the stability of the Rpb7-7 protein is not significantly altered in the absence of Rpb4 protein, by extension, these results show that the N-terminal region of Rpb4 also functions as a contact point for the subcomplex with the rest of the Pol II. This is in excellent agreement with the crystal structure of the 12-subunit Pol II (20). In this structure, the N-terminal extension of Rpb4 is not very well defined, and only a model for the main chain could be built into the electron density map. This region is seen to make a weak interaction with Rpb2 (Fig. 7A), although biochemical data suggest that it is not required for the in vitro interaction with Pol II. Our studies confirm that there are at least two contact points for the sub-

**Interaction of Rpb4-Rpb7 Subcomplex with Core Pol II**

![Image](image-url)
Interaction of Rpb4-Rpb7 Subcomplex with Core Pol II

A closer look at the Rpb7 structure in the context of the entire Pol II model (Fig. 7, B and C) shows clearly that both the Rpb7 and Val72 residues are involved in hydrophobic interactions that hold the Rpb7 N-terminal domain together. Both residues are therefore critical to maintain the conformation of the tip that interacts with the core of the RNA Pol II. The important structural role of both residues is highlighted by the fact that they are well conserved from archaea to human, with Val72 almost invariant and Phe70 occasionally substituted by tyrosine or histidine residues. Although the mutations in rpb7-7 are chemically subtle (F70L and V72M), the change in size can potentially disrupt the interaction within the Rpb7 hydrophobic core, as a consequence, the contacts between the Rpb4-Rpb7 heterodimer, and the RNA polymerase 10-subunit core.

Our results also suggest that the essential role for Rpb7 in the cell might be through its interaction with the rest of the Pol II. Reduction in the strength of this interaction by mutating the N-terminal tip region as well as the N-terminal region of Rpb4 has deleterious consequences for the viability of the cell. The Rpb4-Rpb7 subcomplex in the 12-subunit RNA Pol II is located very close to the flexible linker connecting the highly conserved C-terminal domain of Rpb1. Although the C-terminal domain is known to play a crucial role in transcription, the Rpb4-Rbp7 subcomplex has been shown to affect recruitment of C-terminal domain modifying proteins such as Fcp1 and Ess1 (4).

An interesting corollary from our study has been that the role for Rpb7 in certain stress responses seems to be independent of its interaction with the Pol II. The Rpb7-7 mutant protein, which is unable to interact with the Pol II in the absence of Rpb4 is still able to exaggerate pseudohyphal growth (Fig. 2C), suggesting that this phenotype involves interactions of Rpb7 outside of the context of the Pol II. Such Pol II-independent functions might also explain the role of Rpb4 in a myriad of seemingly unrelated phenotypes like RNA export and RNA decay.

In conclusion, we have shown that the N termini of both Rpb4 and Rpb7 contribute to the interaction of the subcomplex with the rest of the Pol II, and at least one functional interaction point is required for the subcomplex to be recruited to the core Pol II. It is thus possible that in some of the systems where the Rpb4-Rpb7 homologs do not interact stably outside the context of Pol II structure as tested by two-hybrid or co-immunoprecipitation studies, they may actually be able to interact within the context of the Pol II. It is possible that the Rpb4-Rpb7 subcomplex from other eukaryotic systems uses similar modes for interaction with the Pol II. In fact, the N-terminal regions that are involved in interaction are highly conserved in Rpb7 and to a limited extent in Rpb4. The N-terminal region of Rpb4 thought to be unique to Sc Rpb4 does however share some homology in the first 32 aa (27), and these residues could contribute to interaction with the Pol II in other eukaryotic systems. The present studies thus exemplify evolution of the subcomplex of two subunits where the points of interaction with the core Pol II have been conserved and the nonconserved portions of the proteins have evolved to cater to specific requirements of the individual RNA polymerases I, II, or III. Thus even

\[5\text{. Singh and P. Sadhale, unpublished results.} \]
if they exhibit weaker interactions outside the context of the polymerase, they can function as the subcomplex in all the DNA-dependent RNA polymerases from archaea to the eukaryotes.

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