Genomewide Recruitment Analysis of Rpb4, a Subunit of Polymerase II in Saccharomyces cerevisiae, Reveals Its Involvement in Transcription Elongation†‡

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The Rpb4/Rpb7 subcomplex of yeast RNA polymerase II (Pol II) has counterparts in all multisubunit RNA polymerases from archaeabacteria to higher eukaryotes. The Rpb4/7 complex in Saccharomyces cerevisiae is unique in that it easily dissociates from the core, unlike the case in other organisms. The relative levels of Rpb4 and Rpb7 in yeasts affect the differential gene expression and stress response. Rpb4 is nonessential in S. cerevisiae and affects expression of a small number of genes under normal growth conditions. Here, using a chromatin immunoprecipitation (“ChIP on-chip”) technique, we compared genomewide binding of Rpb4 to that of a core Pol II subunit, Rpb3. Our results showed that in spite of being nonessential for survival, Rpb4 was recruited on coding regions of most transcriptionally active genes, similar to the case with the core Pol II subunit, Rpb3, albeit to a lesser extent. The extent of Rpb4 recruitment increased with increasing gene length. We also observed Pol II lacking Rpb4 to be defective in transcribing long, GC-rich transcription units, suggesting a role for Rpb4 in transcription elongation. This role in transcription elongation was supported by the observed 6-azauracil (6AU) sensitivity of the rpb4Δ mutant. Unlike most phenotypes of rpb4Δ, the 6AU sensitivity of the rpb4Δ strain was not rescued by overexpression of RPB7. This report provides the first instance of a distinct role for Rpb4 in transcription, which is independent of its interacting partner, Rpb7.

Transcription is the process by which the information encoded in the genome is converted in an accurately timed and spatially regulated manner to RNA. RNA polymerase II (Pol II) is responsible for the synthesis of all eukaryotic protein coding RNAs (mRNAs) and thus is the central component of the eukaryotic transcription machinery. From yeast to human, it is composed of 12 subunits, designated Rpb1 to Rpb12 in order of decreasing molecular weight (52). These subunits exhibit a high degree of sequence similarity across eukaryotic species. In most of the eukaryotes, all these subunits are integral parts of Pol II. Saccharomyces cerevisiae Pol II is an exception in that two of its subunits, Rpb4 and Rpb7, form a subcomplex that is reported to be dissociable from the core structure of 10 subunits (11). Interestingly, the subcomplex has structural (and probably functional) counterparts in RNA Pol I and RNA Pol III, suggesting that this subcomplex may have a more general role to play in the transcription process (29, 40, 46).

Rpb7 is an essential protein, whereas Rpb4 is dispensable under moderate growth conditions in yeast. Cells lacking Rpb4 show various stress-specific phenotypes (7, 40). The rpb4Δ cells are temperature sensitive, lose viability in stationary phase (8), are defective in sporulation, and display enhanced pseudohyphal morphology (30). In the absence of Rpb4, association of Rpb4 with Pol II is reduced (11), suggesting that Rpb4 stabilizes interaction of Rpb7 with the rest of the 10-subunit core. Interestingly, the association of the Rpb4/7 subcomplex with the rest of the core Pol II is reported to vary with growth conditions. In exponentially growing cells, only ~20% Pol II molecules contain this subcomplex, whereas in stationary-phase cells, all Pol II molecules have the Rpb4/Rpb7 subcomplex (8). In contrast, in higher eukaryotes the Rpb4/7 subcomplex has not been reported to be dissociable (18, 21).

In vitro studies exploring the role of the Rpb4/7 subcomplex in transcription have revealed that Pol II lacking a Rpb4/Rpb7 heterodimer is incapable of promoter dependent transcription initiation but elongation of transcription occurs normally on a naked DNA template (11). Subsequently, Rpb4 has been shown to be required for transcription from inducible promoters but not from constitutive promoters (31). Genomewide expression profiling also indicated that Rpb4 plays a role in the transcription of only a small subset of genes under normal growth conditions and following a brief heat shock treatment (30). However, following prolonged heat shock, transcription is severely reduced in the absence of Rpb4 (25). Apart from its involvement in transcription, Rpb4 has also been implicated in mRNA export (13) and RNA stability (24).

The crystal structure of Pol II with all 12 subunits has been solved (2, 5). The Rpb4/7 subcomplex was found near the base of the clamp region and in close proximity with the linker region of Rpb1 leading to the C-terminal domain. In Rpb4/ Rpb7-bound Pol II, the clamp adopts a closed conformation, the state acquired by Pol II during transcription elongation, suggesting that it may play an important role in the clamp movement during the transition between transcription initiation and elongation. The placement of Rpb4/Rpb7 in Pol II
also increases the docking area of Pol II, thereby suggesting a role in the assembly of different components of transcription machinery (3). In agreement with such a role, Rpb4 has been observed to affect recruitment of the C-terminal domain phosphatase Fep1 in the transcription complex (16, 19).

In spite of the genetic, biochemical, and structural data available on various features of Rpb4, the role of Rpb4 in transcription and the dynamics of its association with core Pol II during various stages of transcription still remain poorly understood. In the present study, we examined the genome-wide occupancy of Rpb4 using a chromatin immunoprecipitation (ChIP) assay and found that although Rpb4 is globally recruited, the extent of its recruitment is less than that for Rpb3. Interestingly, the extent of Rpb4 recruitment was found to be significantly greater on coding regions of long genes, hinting toward a role for Rpb4 in transcription elongation. Taken together, this study provides global in vivo evidence for the involvement of Rpb4 in transcriptional elongation.

MATERIALS AND METHODS

Yeast strains. The strain with tandem affinity purification (TAP)-tagged Pol II subunit Rpb3, SC126 (MATa ade2 arg4 leu2-3,112 trpl-289 ura3-52 YIL021W: TAP-KL.URA3) (14), was obtained through EUROSCARF. The TAP-tagged Rpb4 strain was described (24). The wild-type (MATa his3Δ1 leu2Δ200 ura3Δ0) and rpb4Δ (Mara his3Δ1 leu2Δ200 lys2Δ0 ura3Δ0 YIL140w:kanMX4) strains were also obtained from EUROSCARF. These strains were transformed with the plasmid constructs listed in Table 1 to generate the appropriate strains assayed for the DNA amplification by ligation-mediated PCR, labeling, and hybridization.

Microarray slides were obtained from Agilent Technologies (yeast 4 × 44K, whole-genome array). The arrays were comprised of 60-mer oligonucleotide probes at ~250- bp spacing, covering ~12 Mb of the genome. DNA amplification, labeling, and hybridization were performed as described in the DNA microarray protocol (version 9.1, Agilent Technologies). Briefly, the input and immunoprecipitated (IP) DNA fragments were first ligated with linker DNA, followed by amplification in a PCR using linker-specific primers. The amplified input and IP samples were labeled with cyanine 3 and cyanine 5, respectively, using Invitrogen’s CGH labeling kit, and 3.5 μg of each cyanine-3- and cyanine-5-labeled sample was hybridized on microarray slide and incubated in the hybridization oven rotator set at rotation speed to 10 rpm for 40 h at 65°C. Following hybridization, microarrays were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30% to 50% PMT gain.

Data analysis. Data extraction was carried out with Agilent Feature Extraction software (version 9.1), and normalization was done using a linear per-array algorithm according to the manufacturer’s protocol for array CGH that showed a high correlation (r² = 0.9) of bound probes between data for the two biological replicates. Identification of bound probes and genes was made using Agilent ChIP Analytics software (version 1.3) by using the Whitehead Institute Per-array Neighborhood model, version 1.0. In the neighborhood model, if a candidate bound probe set P value was less than 0.05, the central probe was marked as potentially bound and the probe sets were required to pass one of two additional filters: the center probe in the probe set has a single-point P value less than 0.05 or one of the flanking probes has a single-point P value less than 0.25. These two filters cover situations where a binding event occurs midway between two probes and situations where the signal reflects the event, weakly. Correlation analysis was done using the Microsoft Excel and Access software programs. Chromosomal location maps for all probes and gene ontology analysis were generated using the Gene Spring GX software program (Agilent Technologies). The gene annotation file was downloaded from SGD website and built into Gene Spring for the gene ontology analysis.

Quantitative radioactive PCR. For quantitative radioactive PCR, we used same input and IP samples which were used for the labeling reaction for ChIP-on-chip analysis. We selected four genes for these quantitative PCRs, which included two genes which have significantly long open reading frames (ORFs). Primers were designed to yield 150- to 260-bp products covering the region of interest (sequences are available upon request). In addition, three dilutions of input DNA were used to verify that DNA amplification was in the linear range of the PCR (data not shown). Reactions were carried out with 15-μl PCR mixes containing 0.5 μCi of [3H]dATP and 200 μM each of all four deoxynucleoside triphosphates, using 2 μM concentrations of each primer pair for 20 to 25 cycles depending on the linearity of the signal. The optimal annealing temperature was predetermined for each primer set. A volume of 10 μl of the PCR was run on a 7% urea polyacrylamide gel. The gel was dried, and radioactive signals were transferred to a phosphorimagere screen. The signal intensities were quantified with the software program ImageQuant (version 2.3). To calculate the binding ratio, the signal intensity of the IP DNA was divided by the average signal of the input. For each set of data, at least two independent PCRs were used to generate average binding ratios represented in Fig. 3C and D and 4B and C.

6AU sensitivity assay. For the 6AU sensitivity assay, strains were grown in yeast extract-peptone-dextrose medium for 24 h and diluted to an OΔD600 of 1.0, and these were further serially diluted (1:10 dilution steps) and spotted on plates.
of synthetic complete medium without uracil (SC--Ura) or SC--Ura with 75 μg of 6AU per ml. Plates were incubated at 28°C for 3 to 6 days before photographs were taken.

RT-PCR analysis. Total RNA was isolated from all the strains by the hot-phenol method. RNA samples were quantified using a nanodrop spectrophotometer. Total RNA (5 μg) isolated from each strain was treated with DNase I (Roche) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (RT) (MBI Fermentas Inc.) using an oligo(dT) primer, and the 3' region of the PUR5 ORF was amplified using the PUR5f and PUR5r primers. Similarly, RT-PCR analysis of lacZ and PHO5 transcript was done using the lacZf and lacZr and PHO5f and PHO5r primers, which amplify a 350-bp region near the 3' end of the respective ORF. RT-PCR for ACT1 was carried out using the ACT1f and ACT1r primers to compare the relative mRNA levels between samples. The PCR products were run on 1.5%-agarose gels. Primer sequences used here are available upon request.

RESULTS
To investigate which genes are transcribed by Rpb4-containing polymerase, we performed genomewide ChIP-on-chip analysis using strains that have either Rpb4 or Rpb3 fused with the TAP tag (14, 24). These strains were checked for the absence of growth defects. Two independent cultures of each tagged strain were grown to logarithmic phase in rich medium. ChIP experiments were followed by amplification and labeling of the input and the IP DNA. The labeled input and IP DNA were then applied to yeast whole-genome high density oligonucleotide microarray slides, and the data were analyzed and validated as described in Materials and methods.

Rpb4 recruitment is similar to that of Rpb3. To obtain a whole-genome view of Rpb3- and Rpb4-bound probes, data for the two biological replicates were averaged to get an enrichment map of all the probes on all the 16 chromosomes. The extent of enrichment for individual probes on three representative chromosomes (chromosomes III, IV, and VI) is shown (Fig. 1A). It was found that enrichment profiles of both Rpb4 and Rpb3 followed a similar pattern across all 16 chromosomes. To further investigate the extent of similarity between genomewide binding of Rpb3 and Rpb4, we examined the correlation between occupancy of Rpb3 and that of Rpb4 on all genes. The scatter plot of the Rpb3 binding ratio versus the Rpb4 binding ratio for all the enriched probes clearly shows that most probes are bound by both the proteins (Fig. 1B). The correlation coefficient of Rpb3 and Rpb4 binding was found to be strikingly high at 0.86, confirming that Rpb4, like Rpb3, is present in most of the Pol II molecules engaged in transcription throughout the genome.
genes with intermediate expression levels (medium). The cutoff used for significantly bound genes was a \( p \) value of \(<0.05\), as obtained by analyzing the data with the Chip Analytics (version 1.3) software program (Agilent Technologies). (B) Moving average plot showing binding trend of Rpb3 and Rpb4 as a function of transcription rate (Fig. 2B).

Rpb4 is bound on both promoter and coding region. In vitro studies have implicated Rpb4 in initiation of promoter-dependent transcription (11), but the in vivo role of Rpb4 in different stages of transcription has yet to be explored. To test if there is any stage specificity of the function of Rpb4 in transcription, we carried out a finer analysis of binding events along the length of the transcription units, including the promoter and the gene coding region (GCR). We examined the binding trend of Rpb4 and Rpb3 on different regions of all genes by plotting a moving average of binding ratios for the promoter and coding regions for Rpb3 and Rpb4 (Table 2).

TABLE 2. Gene ontology categories enriched for Rpb3- and Rpb4-bound genes

| Identifier and biological process | \( P \) value |
|----------------------------------|-------------|
| GO:43037: translation             | 3.46E–202   |
| GO:44249: cellular biosynthesis   | 3.28E–179   |
| GO:9058: biosynthesis             | 2.11E–173   |
| GO:9059: macromolecule biosynthesis | 6.07E–154  |
| GO:6412: protein biosynthesis     | 1.55E–145   |
| GO:6007: glucose catabolism       | 6.17E–82    |
| GO:19320: hexose catabolism       | 9.71E–77    |
| GO:46365: monosaccharide catabolism | 1.29E–72   |
| GO:6096: glycolysis               | 1.35E–68    |
| GO:46164: alcohol catabolism      | 1.00E–67    |
| GO:16052: carbohydrate catabolism | 2.01E–59    |
| GO:44275: cellular carbohydrate catabolism | 2.01E–59 |
| GO:6092: main pathways of carbohydrate metabolism | 5.88E–57 |
| GO:44260: cellular macromolecule metabolism | 1.39E–56 |
| GO:19538: protein metabolism      | 5.13E–52    |
| GO:44267: cellular protein metabolism | 1.39E–50  |
| GO:6006: glucose metabolism       | 5.03E–47    |
| GO:19318: hexose metabolism       | 9.81E–38    |
| GO:44238: primary metabolism      | 4.22E–37    |
| GO:5996: monosaccharide catabolism | 3.01E–36   |
| GO:44237: cellular metabolism     | 6.62E–36    |
| GO:6082: organic acid metabolism  | 6.77E–36    |
| GO:19752: carboxylic acid metabolism | 6.77E–36  |
| GO:8152: metabolism               | 7.96E–35    |
| GO:15980: energy derivation by oxidation of organic compounds | 4.05E–34 |
| GO:6066: alcohol metabolism       | 9.65E–34    |
| GO:44262: cellular carbohydrate metabolism | 1.11E–33 |
| GO:6633: fatty acid biosynthesis  | 4.49E–33    |
| GO:43170: macromolecule metabolism | 1.58E–32 |
| GO:6091: generation of precursor metabolites | 1.19E–31 |

The distribution of both Rpb4 and Rpb3 throughout the genome appears to be consistent with what is expected for Pol II occupancy. The enrichment of these subunits is reduced near the telomeres, which is consistent with the phenomenon of transcriptional silencing at subtelomeric positions (34). Based on the strain background and glucose-rich growth conditions, genes like FLO11 and the genes at the GAL locus (GAL1, GAL7, and GAL10) were expected to be transcriptionally inactive. We found that all the four genes analyzed showed very low enrichment of both the subunits (Fig. 1C and D).

Rpb4 binding correlates with transcriptional activity. Both Rpb3 and Rpb4 showed significant binding (\( P \) value of \(<0.05\)) to \( \sim 15\% \) of total genes represented on the array, and it was of interest to examine whether the extent of Rpb3 and Rpb4 binding, by virtue of their being Pol II subunits, correlates with the transcriptional levels. Using the available transcription activity profile of the \( S. cerevisiae \) wild-type strain in log phase (1), we categorized all 5,828 genes based on expression levels into three broad classes: high (top 20% = 1,165 genes), low (bottom 20% = 1,165 genes), and medium (remaining 3,498 genes). Although such a categorization is arbitrary, it allows a clear distinction to be made between the two extremes of the spectrum of the transcriptional level. As shown in Fig. 2A, we found that both Rpb3 and Rpb4 displayed higher levels of binding to genes whose transcriptional activity falls in the “high” category. Functional classification revealed that genes belonging to ribosomal biogenesis and metabolism, which are generally highly expressed, are represented among the genes significantly enriched by both Rpb3 and Rpb4 (Table 2). The converse scenario also holds true, since the genes which display low transcription activity also showed relatively poor enrichment of the two Pol II subunits. We also compared our genomewide recruitment data with the transcription rate (http://web.wi.mit.edu/young/pub/holstege.html) using the average moving plot method and found that the binding trends of both Rpb3 and Rpb4 showed a positive correlation with the transcription rate (Fig. 2B).

FIG. 2. Rpb3 and Rpb4 binding correlates with transcription activity. (A) Bar graph showing fraction of Rpb3- and Rpb4-bound genes according to gene expression levels. Genes were separated into three groups: the 1,165 most transcribed genes (high, consists of top 20% of highly expressed genes), the 1,165 least transcribed genes (low, bottom 20% genes), and the 3,498 genes with intermediate expression levels (medium). The cutoff used for significantly bound genes was a \( p \) value of \(<0.05\), as obtained by analyzing the data with the Chip Analytics (version 1.3) software program (Agilent Technologies). (B) Moving average plot showing binding trend of Rpb3 and Rpb4 as a function of transcription rate. Binding ratios of all the probes of all genes were averaged out to get a single binding ratio to represent the gene, and genes were ranked according to transcription rate. The binding trend was generated by taking the average of a sliding window of binding ratios for 100 genes from low to high transcription rates.
and GCR with respect to their transcription rate (Fig. 3A and B). For both the promoter and GCR, the trend of Rpb3 and Rpb4 binding was found to increase with transcriptional activity, confirming the positive correlation presented in the previous section. Interestingly, on both the promoter and GCR, the extent of binding of Rpb4 was always less than that of Rpb3 (Fig. 3A). To illustrate this trend in our ChIP-on-chip experiment, two examples of highly transcribed genes, viz, RPL42B and ILV5, are shown (Fig. 3C and D, left panels). This trend was further validated by conventional radioactive PCR for enrichment of Rpb3 and Rpb4 on promoter and coding regions of these genes. The radioactive PCR results correlated well with the enrichment seen in the genomewide array (Fig. 3C and D, right panels).

**Rpb4 enrichment increases with gene length.** Since we observed significant enrichment of both the subunits on coding regions, we analyzed their enrichment as a function of gene length. The average moving plot (Fig. 4A) clearly indicates that the occupancy of Rpb4, like that of Rpb3, varies with the gene length. Interestingly, on shorter genes (<2,000 bp), the Rpb4 binding is less than that of Rpb3, but this difference narrows down with an increase in gene length. For instance, the binding ratios of Rpb4 and Rpb3 throughout the coding region of long and highly expressed genes like FKS1 (5.6-kb ORF) and ACC1 (6.7-kb ORF) were found to be very similar (Fig. 4B and C, left panels), which is in contrast to the binding ratios of these two proteins on relatively shorter genes (Fig. 3C and D). The enrichment of Rpb4 and Rpb3 on FKS1 and ACC1 was vali-
dated by radioactive PCR as represented in the bar graphs (Fig. 4B and C, right panels). These results indicate that Rpb4 recruitment on the coding region of long genes might have some functional significance.

**rpb4** mutant is defective in transcription of long and GC-rich coding regions. Transcription of long and GC-rich genes is likely to be most affected by a defect in transcription elongation. This has been observed to be the case with several mutants showing elongation defect (38, 39). In these mutants, the 3-kb-long and GC-rich (56% G+C content) lacZ coding region is poorly transcribed in comparison with a 1.5-kb-long and relatively less GC-rich (40% G+C content) PHO5 coding region. We assayed the ability of the rpb4Δ mutant to transcribe lacZ- and PHO5-coding regions driven from the GAL1 promoter. Since we had previously observed that the expression from the GAL1 promoter is detectable only after prolonged incubation under inducing conditions in rpb4Δ cells (N. Sharma and P. Sadhale, unpublished data), we analyzed the transcript levels corresponding to these two coding regions after 24 h of growth under either inducing or noninducing conditions. Both lacZ and PHO5 transcript levels increased in wild-type cells in galactose-containing medium, but the accumulation of lacZ mRNA was severely compromised compared to that of PHO5 mRNA in the rpb4Δ cells (Fig. 5). The possibility that the lacZ transcript is preferentially degraded in the

![FIG. 4. Relative occupancies of Rpb4 and Rpb3 vary with respect to gene length. (A) Moving average plot of binding ratios of Rpb3 and Rpb4 with respect to gene length was generated as described above (see Fig. 2B). (B and C) Examples of long genes showing comparable binding by both Rpb3 and Rpb4 from genomewide ChIP (left panels) and their validation by radioactive PCR (right panels). Representation is as described in the legend for Fig. 3.](image)

![FIG. 5. Transcript levels of lacZ and PHO5 in wild-type and rpb4Δ cells driven from GAL1 promoter. RT-PCR analyses of wild-type (WT) and rpb4Δ strains transformed with pPS231 and pSCh202 are shown. Mid-log-phase cells grown in SC-Ura containing 2% glucose were diluted to an OD600 of 0.4 in fresh medium containing either 2% glucose (UI) or 2% galactose (I), and samples for RNA isolation were collected after 24 h of growth.](image)
The growth of the *rpb4* strain can be differentiated by checking for the induction of the *PUR5* gene. Induction of the intracellular pools of GTP and UTP (12), and the gene for IMP dehydrogenase, which is transcriptionally coupled with an inability to induce the *PUR5* gene, suggests that it may have a role in transcription elongation.

*A* 6AU sensitivity phenotype of *rpb4Δ* cells. (A) Serial dilutions of the wild type and the *rpb4Δ* mutant were spotted on SC–Ura medium plates lacking or containing 75 μg/ml 6AU. Photographs were taken after 3 days of growth at 28°C in the absence (−6AU) or 6 days of growth in the presence (+6AU) of 6AU. (B) RT-PCR analysis of the *PUR5* gene for the wild type and the *rpb4Δ* mutant grown in the presence of 6AU at indicated time points.

Absence of Rpb4 was ruled out by expressing *lacZ* under CUP1 promoter control. The *lacZ* mRNA was found to be equally stable in the presence or absence of Rpb4 as judged by RT-PCR assay (data not shown). The slightly reduced induction of the *PHO5* transcript in *rpb4Δ* cells compared to results for the wild type might be due to defective transcription activation of the *GAL1* promoter reported earlier (31). The observation that Rpb4 is required for the transcription of long and GC-rich genes suggests that it may have a role in transcription elongation.

**rpb4Δ** cells show 6AU sensitivity, a phenotype associated with a transcriptional elongation defect. The compound 6AU is known to adversely affect transcriptional elongation by reducing the intracellular pools of GTP and UTP (12), and several mutations affecting transcriptional elongation have been reported to confer sensitivity to 6AU (15, 27, 28, 51). We examined the growth of *rpb4Δ* cells in the presence of 6AU. The growth of the *rpb4Δ* strain was highly compromised in the presence of 6AU (Fig. 6A), suggesting that the *rpb4Δ* strain could be defective in transcription elongation. However, sensitivity to 6AU may not always be due to a defect in transcription elongation (35, 50). Whether the 6AU sensitivity-inducing mutation is a result of a transcription elongation defect or not can be differentiated by checking for the induction of *PUR5*, the gene for IMP dehydrogenase, which is transcriptionally induced by exposure of yeast cells to 6AU (44). Mutations that cause both transcription elongation defects and 6AU sensitivity also prevent the induction of *PUR5* transcription in response to 6AU treatment, while mutations that cause only 6AU sensitivity without affecting transcription elongation do not interfere with *PUR5* induction. Thus, a 6AU-sensitive phenotype coupled with an inability to induce *PUR5* in response to 6AU confirms a defect in transcription elongation. We isolated RNA from wild-type and *rpb4Δ* cells before and after 6AU treatment and analyzed *PUR5* expression levels by RT-PCR. As shown in Fig. 6B, treatment with 6AU induces the transcription of *PUR5* in the wild-type strain, but such an induction is not observed in the *rpb4Δ* strain. This analysis clearly demonstrates Rpb4 to be an active player in transcription elongation.

**Conserved domains of Rpb4 are important for its role in elongation.** *S. cerevisiae* Rpb4 is a considerably longer protein (221 amino acids [aa]) than its eukaryotic orthologs (~144 aa). Sequence alignment of different eukaryotic orthologs of Rpb4 suggests that the N- and C-terminal regions of Rpb4 are highly conserved (41), but the central region displays less sequence conservation across species and is rich in acidic and basic residues (Fig. 7A). The role of different domains of Rpb4 under various stress conditions has been studied by our group (41). To examine which domain of Rpb4 is important for its role in transcriptional elongation, we transformed the *rpb4Δ* strain with a plasmid expressing either the full-length Rpb4 protein lacking the N- and C-terminal (1 to 198 aa) or both the C- and N-terminal (33 to 198 aa) conserved domains did not rescue the 6AU sensitivity of the *rpb4Δ* strain. The expression of Rpb4 variants lacking either the C-terminal (1 to 198 aa) or both the C- and N-terminal (33 to 198 aa) conserved domains did not rescue the 6AU sensitivity of the *rpb4Δ* strain (Fig. 7B). The *rpb4Δ* strain expressing Rpb4 lacking the conserved N-terminal (33 to 221 aa) domain was only partially sensitive to 6AU and grew slowly compared to the full-length protein (Fig. 7B). In contrast, expression of Rpb4 lacking the nonconserved residues (66 to 80 aa and 107
to 140 aa) or weakly conserved residues (95 to 105 aa) did not affect the 6AU sensitivity of the rpb4Δ strain (data not shown). These results suggest that the C-terminal domain, and to a certain extent the N-terminal domain, are important for the function of Rpb4 in transcription elongation.

**RPB7 overexpression does not rescue 6AU sensitivity of the rpb4Δ mutant.** The conserved N- and C-terminal domains of Rpb4 are involved in interaction with Rpb7 (41). Previous studies from our group have also shown that some of the phenotypes associated with the absence of RPB4 are partially rescued by overexpression of its interacting partner, RPB7 (31, 43). Therefore, it was of interest to test if overexpression of RPB7 can rescue the 6AU sensitivity of the rpb4Δ strain. As shown in Fig. 8, overexpression of RPB7 from a multicopy plasmid under the control of the constitutive TEF2 promoter did not rescue the 6AU sensitivity of the rpb4Δ strain. Since cells expressing the C-terminally truncated Rpb4 protein also show sensitivity to 6AU (Fig. 7B), we tested if overexpression of RPB7 would rescue this phenotype. We observed that overexpression of RPB7 is unable to rescue the 6AU sensitivity of rpb4Δ cells expressing the C-terminal truncation of Rpb4 (data not shown). Thus, the role of Rpb4 in transcriptional elongation is likely to be a direct function of Rpb4 and not an indirect result of its role in stabilizing the interaction of Rpb7 with the rest of the core Pol II.

**DISCUSSION**

An understanding of the spatial and temporal recruitment of different components of transcription machinery is vital for an insight into the regulation of transcription. We have shown through analysis of genomewide recruitment that Rpb4, a disassociable subunit of yeast RNA Pol II, is actually recruited on all transcription units throughout their length. This was rather unexpected, since (i) genomewide transcription profiling has suggested that transcription of only a small subset of genes is affected in the absence of Rpb4, and (ii) in vitro analyses have suggested that Rpb4 lacking polymerase is not defective for transcription elongation, such as transcription elongation. Several mutants defective in transcription elongation, such as rpb2-21, paf1Δ, spt6-140, rpb2-21, and paflΔ (6, 38, 39), show a similar inability to express long and GC-rich genes fused to the GAL1 promoter. We also observed delayed induction of the GAL1 promoter in rpb4Δ cells, which has also been linked to a transcription elongation defect (44).

The sensitivity to 6AU is often associated with a defect in transcriptional elongation. The enzyme IMP dehydrogenase, encoded by PUR5, is inhibited by 6AU, and its transcription is induced in wild-type cells on exposure to this drug. In mutants defective in transcription elongation, this induction is not observed (10). Our results that the rpb4Δ strain is extremely sensitive to 6AU and defective in induction of PUR5 (Fig. 6A and 6B) further support an in vivo role of Rpb4 in transcription elongation. Additionally, this role of Rpb4 seems to be independent of its interacting partner, Rpb7, since our results show that overexpression of RBP7 cannot rescue the 6AU sensitivity of rpb4Δ cells (Fig. 8), although several phenotypes, such as temperature sensitivity and slow growth of the rpb4Δ strain, can be rescued by overexpression of RBP7 (31, 43, 47). Probably Rpb4 might be involved in the recruitment of other elongation factors during transcription.

It has been observed that there are several components of binding sites in a manner qualitatively similar to that for the core subunit, Rpb3 (Fig. 1A), implying that it is involved in transcription of all transcription units throughout the transcription process. This is unexpected, since the expression level of ~80% of yeast genes remains unaffected in an rpb4Δ strain during normal growth conditions (25, 30, 31). This could lead to the interpretation that Rpb4 is required for transcription of only a small number of genes. The global distribution of Rpb4 is thus, at first glance, counterintuitive. Such a scenario has well-established precedents in transcription machinery. In fact, studies carried out with the core subunits and the Srb8-11 module of mediator have shown global recruitment, although both are thought to independently affect expression of only a small subset of genes (1, 53). The recruitment of human homologs of both Rpb7 and Rpb4 on both promoter and coding regions based on the analysis of a small number of genes agrees well with our genomewide analysis (9).

It is noteworthy that the distribution of Rpb4, while similar in pattern to that of Rpb3, differs from the latter in terms of the extent of occupancy. Although this difference in the binding ratios of Rpb3 and Rpb4 was observed on most transcription units, with Rpb4 having reduced but significant binding through out the length of the genes, the extent of occupancy of Rpb4 increased with increasing gene length (Fig. 4B). Recruitment of Rpb3 represents the total Pol II transcribing a given gene, and the level of Rpb4 recruitment represents the fraction of Pol II involved in transcription of that gene. We see that recruitment of Rpb4 increases relative to that of Rpb3 in the gene coding regions of long genes. These results suggested that Pol II containing Rpb4 contributes more to the transcription of longer genes and raised the possibility of involvement of Rpb4 in transcription elongation. If Rpb4 indeed has a role in transcription elongation, long genes should exhibit a greater dependence on Rpb4. Our result that lacZ, a long gene with higher GC content, is transcribed more poorly than PHO5 in the absence of Rpb4 (Fig. 5) supports the role of Rpb4 in transcription elongation. Several mutants defective in transcription elongation, such as rho2Δ, spt4Δ, elp3Δ, spot6-140, rpb2-21, and paf1Δ (6, 38, 39), show a similar inability to express long and GC-rich genes fused to the GAL1 promoter. We also observed delayed induction of the GAL1 promoter in rpb4Δ cells, which has also been linked to a transcription elongation defect (44).

FIG. 8. RPB7 overexpression does not rescue 6AU sensitivity phenotype of rpb4Δ strain. The serial dilutions of indicated strains were grown on SC–Ura–Leu plates in the absence (3 days of growth) or presence (6 days of growth) of 75 μg/ml 6AU.
the transcription machinery which have a role in transcription elongation (42). In agreement with the proposed role in elongation, Rpb4 also shows genetic interactions (synthetic lethality) with several proteins known to be involved in elongation, such as elongation factors, TFIIIS, Elf1 (33), Paf1 (J. Verma and P. Sadhale, unpublished data), and a Pol II subunit, Rpb9 (4, 49). Many of these elongation factors, e.g., Elf1, Paf1, Rpb4, Rpb9, and TFIIIS, are all individually dispensable for survival, indicating that they may have redundant functions in transcription elongation. Structurally, Rpb4 being placed on the opposite face of yeast Pol II compared to the other known elongation factors, like TFIIIS and Rpb9, prompts us to speculate that Rpb4 may be involved in a different mechanistic pathway of transcription elongation.

Rpb4 has been implicated in the control of transcription initiation (11, 31), but our studies clearly show a role for Rpb4 in transcription elongation. This contradicts a previous observation that Rpb4 is dispensable for transcription elongation in vitro (11). How can we reconcile these observations? The discrepancy between in vitro and in vivo observations may reflect the difference in the nature of the template involved in these studies. The template used to study the role of Rpb4 in elongation in vitro was naked DNA (11), while the in vivo template is packaged in chromatin, which requires a large number of factors for efficient transcription (32, 36, 37). Modification of chromatin by several of these factors plays an important role in transcription elongation (20, 23, 26, 48). Recently a specific requirement of the histone methyltransferase Set2 in transcription of long genes was reported (22). It is possible that Rpb4 might play a role in the recruitment of such factors, thereby affecting transcription elongation in vivo.

The domain analysis of Rpb4 suggests that the elongation role of Rpb4 is mediated by the C-terminal domain, since the deletion of this domain resulted in the 6AU sensitivity of the rpb4Δ strain (Fig. 7B). The C-terminal domain of Rpb4 is highly conserved, and its deletion is known to cause slow growth, temperature sensitivity, and a transcription initiation defect (41). This suggests that the C-terminal domain of Rpb4 might contribute to both the initiation and elongation roles of Rpb4.

There are several examples where a factor(s) which was earlier known to be involved in transcription initiation has also been implicated in transcription elongation, such as the Paf1 complex (45), mediator (49), and transcription regulator CCR4-NOT complex (10). The multiple roles played by other initiation factors in such processes as promoter clearance, transcriptional elongation, polyadenylation, 3′-end formation, and DNA repair suggest that factors controlling initiation can also be utilized in other aspects of gene expression.

Our study presents a new facet of involvement of Rpb4 in transcription through its genomewide recruitment in the cell. The global presence of Rpb4 offers the cell yet another potential regulatory point, adding to the already complex repertoire of control mechanisms known to occur in eukaryotes. The fact that Rpb4 participates in transcription elongation strengthens the emerging paradigm of transcription elongation as an important regulatory event of significance, comparable to transcription initiation. Future studies on the recruitment of Rpb4 under a variety of stress conditions are likely to uncover more details of transcription modulation by Rpb4 and are currently under way in our lab.

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