The Rpb4 Subunit of RNA Polymerase II Contributes to Cotranscriptional Recruitment of 3' Processing Factors^{▽†}

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The RNA polymerase II enzyme from the yeast *Saccharomyces cerevisiae* is a complex of 12 subunits, Rpb1 to Rpb12. Crystal structures of the full complex show that the polymerase consists of two separable components, a 10-subunit core including the catalytic active site and a heterodimer of the Rpb4 and Rpb7 subunits. To characterize the role of the Rpb4/7 heterodimer during transcription in vivo, chromatin immunoprecipitation was used to examine an *rpb4Δ* strain for effects on the behavior of the core polymerase as well as recruitment of other protein factors involved in transcription. Rpb4/7 cross-links throughout transcribed regions. Loss of Rpb4 results in a reduction of RNA polymerase II levels near 3' ends of multiple mRNA genes as well as a decreased association of 3'-end processing factors. Furthermore, loss of Rpb4 results in altered polyadenylation site usage at the *RNAI4* gene. Together, these results indicate that Rpb4 contributes to proper cotranscriptional 3'-end processing in vivo.

The synthesis of eukaryotic mRNA by RNA polymerase II (RNAPII) is a multistep process involving initiation, elongation, and termination. During the transcription cycle, RNAPII associates with many proteins involved in the regulation of these processes, including the basal transcription factors, coactivators, elongation factors, and factors involved in 3'-end formation and termination (6). This regulation of transcription by RNAPII and its associated proteins is critical for gene expression.

The RNAPII enzyme from the yeast *Saccharomyces cerevisiae* is a complex of 12 subunits, Rpb1 to Rpb12, that can dissociate into a 10-subunit core and a heterodimer consisting of Rpb4 and Rpb7 (6). Rpb4 is not essential during optimal growth conditions, although the deletion strain grows very slowly (32). RNAPII purified from an *rpb4Δ* strain lacks Rpb4 but also contains no detectable level of Rpb7 (8). It has been shown that Rpb7, a subunit that is essential for cell viability (22), can interact with polymerase independently of Rpb4, but this interaction is weak and can easily be detected only when Rpb7 is overexpressed (28). RNAPII lacking Rpb4/7 is catalytically active for polymerization, but the heterodimer is required for promoter-dependent transcription in vitro (8).

Crystal structures show the location of the Rpb4/7 heterodimer in the context of the complete RNAPII complex (2, 3). Rpb4 makes very little contact with the core subunits; the dimer is held primarily through contacts between Rpb7 and core subunits Rpb1 and Rpb6. Rpb4/7 is found near both the transcript-exit groove and the linker to the C-terminal domain (CTD) of Rpb1, a location that would allow for interactions with the nascent RNA transcript as well as protein factors

involved in transcription regulation. In fact, Rpb7 contains a potential oligonucleotide-binding domain that faces the presumed RNA exit site (6) and has recently been shown to cross-link to the emerging RNA transcript (31).

Recent reports also indicate that the heterodimer can interact with several transcription factors. Rpb7 from the fission yeast *Schizosaccharomyces pombe* physically interacts in vitro with Seb1, a homolog of the *S. cerevisiae* CTD-binding protein and termination factor Nrd1 (23). Rpb4 has also been shown to physically interact with Fcp1, a CTD phosphatase (10, 17). Both the capability of Rpb4/7 to interact with these factors and the proximity of the heterodimer to the CTD suggest that Rpb4/7 might play a role in the recruitment of some CTD-binding proteins to transcribing RNAPII.

Because *RPB4* is nonessential in *S. cerevisiae*, it is possible to examine the role of the Rpb4/7 heterodimer in vivo by using an *rpb4Δ* strain. Cells that lack *RPB4* are both heat and cold sensitive and also grow more slowly than wild-type strains at permissive temperatures (~24 to 30°C) (32). The sensitivity of *rpb4Δ* cells to high temperatures has been associated with a general RNAPII transcription defect (20, 24). In an attempt to better understand the in vivo effects of Rpb4 loss on the transcribing polymerase, we used chromatin immunoprecipitation (ChIP) to map the cross-linking patterns of polymerase and multiple associated factors along transcribed genes in both wild-type and *rpb4Δ* strains. Deletion of *RPB4* results in decreased polymerase occupancy at the 3' end of mRNA genes. Additionally, Rpb4 is required for the association of the 3'-end processing factors Rna14 and Rna15 with RNAPII and 3' ends of genes. Our results therefore indicate an in vivo role for Rpb4 in the coupling of transcription and mRNA 3'-end processing.

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MATERIALS AND METHODS

***S. cerevisiae* strains.** *S. cerevisiae* strains used in this study are listed in Table S1 in the supplemental material.

ChIPs. Cells were grown in YPD medium (1% yeast extract, 1% Bacto peptone, 2% glucose) at 23°C to an optical density (600 nm) of 0.8. Formaldehyde cross-linking, chromatin preparation, and immunoprecipitation were performed as described previously (11, 15). TAP-tagged proteins were precipitated with immunoglobulin G (IgG)-agarose (Sigma-Aldrich). Rpb3 and Rpb4 immunoprecipitations were done with commercially available mouse monoclonal antibodies (Neoclone). The Rna15 antibody was a gift from C. Moore (Tufts Medical School). Immunoprecipitations of hemagglutinin-tagged proteins were done with monoclonal 12CA5 antibody (Roche Molecular Biochemicals). All oligonucleotide sequences used are listed in Table S2 in the supplemental material. PCR conditions for *SNR13* ChIPs and ChIPs with results shown in Fig. 6 were described previously (19). For *ADH1*, *PYK1*, and *PMA1* ChIPs with results shown in other figures, multiplex PCRs were done using a protocol developed by TaeSoo Kim (Harvard Medical School). PCR conditions were as follows: 60 s at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C, followed by a final 2 min at 72°C.

Protein analysis and immunoprecipitations. Whole-cell extracts were prepared from *S. cerevisiae* grown in YPD medium at 23°C until an optical density (600 nm) of ≈ 0.7 . Glass beads were used to disrupt cells in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10% glycerol, 0.05% NP-40, 0.5 mM EDTA, 0.5 mM dithiothreitol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, and 1 μ g/ml benzamide) and phosphatase inhibitors (1 mM NaF and 0.5 mM NaVO₃). Protein concentrations were determined by the Bradford assay (Bio-Rad), and 1.5 mg was used for IP in a 1-ml volume. TAP-tagged proteins were precipitated overnight at 4°C with rabbit IgG-agarose in either the absence or the presence of 0.1 mg/ml RNase A to determine whether interactions were RNA dependent. Precipitates were washed three times with the IP lysis buffer, dissolved in sample buffer, and separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting was performed using standard methods. Immunoblotting of Rpb1 was performed using the 8WG16 anti-CTD antibody (Covance), and peroxidase antiperoxidase (Sigma-Aldrich) was used to recognize the TAP tag.

Northern blot analysis. Total RNA was prepared using hot phenol extraction from *S. cerevisiae* grown in YPD medium at 23°C until an optical density (600 nm) of ≈ 0.7 . Either 20 or 40 μ g of total RNA was separated on a 1.2% agarose formaldehyde-MOPS (morpholinepropanesulfonic acid) gel and transferred to a nylon membrane (Hybond-N+; Amersham). To create probes for the *RPB1* and *RNA14* analyses, DNA fragments were PCR amplified (see oligonucleotide sequences in Table S2 in the supplemental material) from the genomic loci and used for random hexamer labeling. After hybridization and washing, membranes were exposed to X-ray film or analyzed by phosphorimager.

RESULTS

Rpb4/7 travels with core RNAPII along actively transcribed genes in vivo. Due to the ease with which Rpb4/7 can be dissociated from the 10-subunit core of RNAPII (8), as well as the finding that immunoprecipitated RNAPII contains only substoichiometric amounts of Rpb4/7 (18), it has been suggested that Rpb4/7 may interact with core RNAPII only during certain stages of transcription, for example, only during initiation (4). To examine the involvement of Rpb4/7 during transcription in vivo, the occupancy of yeast Rpb4 and Rpb7 along three representative genes (*ADH1*, *PYK1*, and *PMA1*) was assayed by ChIP (Fig. 1A). In wild-type cells expressing TAP-tagged Rpb7, we find that Rpb4 and Rpb7-TAP cross-link at equal levels throughout the promoter, coding region, and 3' end of each gene. This matches the cross-linking pattern seen for the core of the polymerase, determined by ChIP of Rpb3 as a representative subunit (Fig. 1B). As expected, an *rpb4* Δ strain showed no Rpb4 cross-linking. Cross-linking of Rpb7-TAP was reduced in an *rpb4* Δ strain compared to the level for the wild type, but the level of Rpb3 cross-linking was correspondingly reduced (Fig. 1C). Therefore, it appears that Rpb4/7 remains stoichiometrically associated with core polymerase throughout the transcription cycle. In agreement with

our conclusions, human Rpb7 was recently shown to remain associated with core RNAPII into the early elongation stage of transcription (5).

Immunoblotting of whole-cell extracts shows that Rpb7 levels are significantly decreased in the *rpb4* Δ strain (Fig. 1D). Normalized to total RNAPII levels as measured by the Rpb3 subunit, loss of Rpb4 results in reduction of Rpb7 protein levels by 25 to 50%. Therefore, Rpb7 appears to be stabilized by binding to Rpb4 and this may largely account for the lack of Rpb7 in RNAPII purified from *rpb4* Δ cells. Surprisingly, Rpb1 protein levels actually increase in the *rpb4* Δ strain. The increase in Rpb1 appears to be at least partly mediated by an increase in *RPB1* mRNA levels (Fig. 1E) and may be a response of the cell to low levels of transcribing RNAPII.

Decreased levels of RNAPII are found near 3' ends of mRNA genes in an *rpb4* Δ strain. In vitro experiments show that RNAPII lacking Rpb4/7 is capable of promoter-independent transcription but that the heterodimer is required for promoter-driven transcription (8). Interestingly, RNAPII lacking Rpb4/7 still assembles into initiation complexes in vitro but seems unable to transition into elongation (8, 25). Since Rpb4/7 travels with core RNAPII subunits along transcribed genes in vivo, it was important to test if loss of Rpb4 affected transcription elongation or termination. The ChIP assay was used to monitor the Rpb3 subunit of RNAPII throughout mRNA genes in the context of both wild-type polymerase and polymerase lacking Rpb4 (Fig. 2A; also see later figures). At *ADH1* and *PYK1*, Rpb3 cross-linking in an *rpb4* Δ strain is slightly reduced along the genes compared to wild-type levels, but this difference is most pronounced at the 3' ends of the genes. At the *PMA1* gene, the Rpb3 levels in the wild-type and *rpb4* Δ strains are roughly equal until the 3' end of the gene, where there is a drop in Rpb3 levels in the *rpb4* Δ strain. It is interesting to note that the strongest decreases in Rpb3 levels coincide with the locations of the polyadenylation sites of all three genes tested. The occupancy of Rpb3 along the *SNR13* gene was also analyzed. *SNR13* is a snoRNA gene that is also transcribed by RNAPII; like most snoRNA genes, it is much shorter than most mRNA genes. In contrast to the mRNA genes tested, Rpb3 is found at similar levels along *SNR13* in the wild-type and *rpb4* Δ strains (Fig. 2B). These results suggest that Rpb4 might be important for RNAPII processivity, particularly near 3' ends of mRNA genes.

Rpb4 is required for association of the 3'-end processing factor Rna14 with transcribed chromatin and RNAPII. To determine how the loss of Rpb4 affects the recruitment of various transcription factors involved in initiation, elongation, and 3'-end processing, various TAP-tagged fusion proteins were analyzed by ChIP in wild-type and *rpb4* Δ strains. Levels of cross-linking of initiation factors TATA-binding protein and TFIIF were equivalent at the 5' ends of genes in wild-type and *rpb4* Δ strains (data not shown). Therefore, in agreement with in vitro results, Rpb4 is not necessary for preinitiation complex formation in vivo. There was also no difference in recruitment of the snoRNA termination factor Nrd1 (data not shown). Nrd1 is the homolog of *S. pombe* Seb1, which was shown to interact with Rpb7 in vitro (23). The lack of effect of *rpb4* Δ on Nrd1 may be due to the fact that Rpb7 is still associated with transcribing RNAPII in this strain.

One factor that showed a marked reduction in recruitment

FIG. 1. Relationship between the Rpb4/7 heterodimer and core of RNAPII. (A) Schematic representation of the *ADH1*, *PYK1*, and *PMA1* genes used in ChIP experiments. Numbers are nucleotide positions relative to the open reading frame beginning at +1. Bars above the genes represent the PCR products assayed by ChIP (see Table S2 in the supplemental material for primer sequences). Arrows indicate the major polyadenylation sites of each gene. (B) Rpb4/7 is associated with all transcribed regions. ChIP was performed using a strain expressing TAP-tagged Rpb7 (YF981). DNA coprecipitated with antibodies against Rpb3 or Rpb4 or with IgG-agarose (to precipitate TAP-tagged Rpb7) was analyzed by PCR using primers shown in panel A. The asterisk-marked PCR product is an internal background control from a nontranscribed region on chromosome VI. The input control is used to normalize for the PCR amplification efficiency of each primer pair. (C) Rpb7 associates with transcribing RNAPII in the absence of Rpb4. ChIP analysis was performed as described for panel B, using an *rpb4Δ* strain expressing TAP-tagged Rpb7 (YSB2224). (D) Rpb7 protein levels are decreased and Rpb1 protein levels are increased in an *rpb4Δ* strain. Whole-cell extracts from wild-type (WT) and *rpb4Δ* strains expressing TAP-tagged Rpb7 were analyzed by immunoblotting with PAP to detect the TAP tag, with 8WG16 antibody (to detect Rpb1), and with anti-Rpb3. (E) *RPB1* transcript levels are increased in an *rpb4Δ* strain. Northern blot analysis was performed for *RPB1* in wild-type (YSB1140) and *rpb4Δ* (YSB1755) strains. Methylene blue-stained 18S and 26S rRNAs are shown as a loading control.

in the *rpb4Δ* strain was Rna14, a core component of the 3'-end processing factor cleavage factor IA (CF IA) (14). Figure 3A shows ChIP results for Rna14-TAP in the wild-type and *rpb4Δ* strains at the *ADH1*, *PYK1*, and *PMA1* genes. Rna14-TAP cross-linking, like other mRNA 3'-end processing factors, is strongest near the polyadenylation site of a gene in a wild-type strain (1). This signal is lost in the *rpb4Δ* strain at all three genes tested. To further confirm a loss of 3'-end processing factor recruitment in the *rpb4Δ* strain, ChIP was also performed using an antibody against the Rna15 subunit of the CF

IA complex. As with Rna14, deletion of *RPB4* also results in the loss of recruitment of Rna15 (Fig. 3B).

To address the possibility that loss of Rna14 and Rna15 recruitment in the *rpb4Δ* strain is due either to slow growth of the strain or to a general defect in RNAPII, ChIP of Rna14 and Rna15 was performed with an *rpb9Δ* strain. Rna14 and Rna15 recruitment is unaffected by deletion of the Rpb9 subunit of RNAPII (see Fig. S1 in the supplemental material). Normal levels of Rna14 and Rna15 at the *ADH1* and *PYK1* genes were also seen (data not shown) in a strain containing

FIG. 2. RNAPII levels are reduced toward the 3' ends of mRNA genes in an *rpb4Δ* strain. (A) ChIP was performed on the *ADH1*, *PYK1*, and *PMA1* genes using anti-Rpb3 in wild-type (WT) (YSB772) and *rpb4Δ* strains (YSB2225). The left panel shows PCR products, and the right panel shows quantification of the results. The occupancy value is the ratio of the signal from specific primer products to the internal negative control after normalizing to the input controls. The values shown represent the averages and standard errors from three independent experiments. The asterisk-marked PCR product is an internal background control from a nontranscribed region on chromosome VI. (B) ChIP of Rpb3 shows that RNAPII levels are equal along the *SNR13* gene in wild-type and *rpb4Δ* strains. A schematic of *SNR13* showing positions of PCR primers is shown below the gel. The asterisk-marked PCR product is an internal background control from a nontranscribed region on chromosome V.

the slow-growing TFIIF mutant Kin28(T17D) (12, 27). This indicates that a general defect in growth or elongation is not sufficient to explain the loss of Rna14 and Rna15 recruitment in the *rpb4Δ* strain.

Because cross-linking for RNAPII is also decreased at the 3' ends of mRNA genes in the *rpb4Δ* strain, it was possible that

the loss of 3'-end processing factors was simply due to the drop in total RNAPII. Because Rna14 has previously been shown to cross-link to the *SNR13* snoRNA gene (16) and RNAPII occupancy at this gene is unaffected by *RPB4* deletion (Fig. 2B), Rna14 recruitment at *SNR13* was assayed. Although equivalent amounts of Rpb3 cross-linked along *SNR13* in the wild-

FIG. 3. Loss of Rpb4 results in decreased occupancy of Rna14 and Rna15 at 3' ends of mRNA genes. (A) ChIP was performed for Rna14-TAP and Rpb3 in wild-type (WT) (YSB1147) and *rpb4Δ* (YSB2033) strains. Quantification is shown as described in the legend for Fig. 2. The values shown represent the averages and standard errors from three independent experiments. The asterisk-marked PCR product is an internal background control from a nontranscribed region on chromosome VI. (B) ChIP was performed using antibodies against Rna15 and Rpb3 in wild-type (YSB1140 and YSB772) and *rpb4Δ* (YSB1755 and YSB2225) strains. Quantification is shown as described in the legend for Fig. 2.

FIG. 5. Association of Rna14 with RNAP II is dependent on Rpb4. IgG-agarose was used to precipitate TAP-tagged Rna14 from whole-cell extracts of wild-type (YSB1147) and *rpb4Δ* (YSB2033) strains. An untagged wild-type strain (YSB1140) was also included as a control. Immunoprecipitations were done both in the absence (–) and in the presence (+) of RNase A. Immunoblot analysis was then performed using 8WG16 antibody against Rpb1 (top) as well as the PAP antibody to detect expression of TAP-tagged Rna14 (bottom).

Fig. 7A) produces three different transcripts (2.2, 1.5, and 1.1 kb) as a result of processing at different polyadenylation sites (30). A *ctk1Δ* strain produced reduced amounts of the shortest transcript (1.1 kb), while the amount of the largest transcript (2.2 kb) increased (1). This pattern is similar to that observed for strains with mutations in *RNA14* or *RNA15* (21), and this change may be part of a feedback mechanism to increase amounts of Rna14 in response to low levels of CF IA. The *rpb4Δ* strain showed a pattern very similar to that of the *ctk1Δ* strain, consistent with inefficient cotranscriptional polyadenylation (Fig. 7B and C). The results seen for the *rpb4Δ* strain are

not caused by a defect in recruitment of the Ctk1 kinase, as ChIP experiments show that Ctk1 occupancy is normal in the *rpb4Δ* strain (Fig. 6).

DISCUSSION

In vitro studies using the 10-subunit core of RNAP II indicate that the Rpb4/7 heterodimer is required for promoter-dependent transcription initiation but not initiation complex assembly or promoter-independent elongation (8, 25). Here, we explore the in vivo role of Rpb4 and find that it contributes

FIG. 6. Loss of Rpb4 does not compromise serine 2 phosphorylation of the CTD. (A) Ctk1 kinase occupancy at the *PMA1* gene is unaffected by loss of Rpb4. ChIP for Ctk1-hemagglutinin was performed with wild-type (WT) (YSB772) and *rpb4Δ* (YSB2225) strains. A schematic of *PMA1* showing positions of PCR primers is shown below the gel. Quantification is shown in the right panel. Values represent the averages and standard errors from three independent experiments. (B) Loss of Rpb4 does not result in a decrease of cellular levels of serine 2-phosphorylated CTD. Whole-cell extracts from wild-type (YSB1140) and *rpb4Δ* (YSB1755) strains were analyzed by immunoblotting with H5 antibody (to detect serine 2-phosphorylated CTD [CTD-S2p]). The increase was similar to that in total Rpb1 levels (Fig. 1 and data not shown).

FIG. 7. Cotranscriptional 3'-end processing is compromised in an *rpb4Δ* strain. (A) Schematic representation of the *RNA14* gene and its transcripts. ORF, open reading frame. (B) Northern blot analysis of *RNA14* transcripts in wild-type (WT) (YSB1140) and *rpb4Δ* (YSB1755) strains. A *ctk1Δ* strain (YSB1142) is also shown as a control for compromised cotranscriptional 3'-end processing. Methylene blue-stained 18S and 26S rRNAs are shown as a loading control. (C) Quantification of the Northern blot experiment with results shown in panel B. Bars represent a ratio of the abundance of the two short transcripts to the abundance of the long transcript.

to cotranscriptional recruitment of 3'-end processing factors. In an *rpb4Δ* strain, fewer RNAPII complexes are present at the 3' ends of multiple mRNA genes than in a wild-type strain (Fig. 2A). This pattern of polymerase cross-linking has some similarity to that seen in cells treated with 6-azauracil, which inhibits transcription elongation by unbalancing the cellular nucleoside triphosphate concentrations (13, 33). Therefore, RNAPII lacking Rpb4 may be partially defective in transcription elongation in vivo. This would be consistent with the fact that an *rpb4Δ* strain is sensitive to both 6-azauracil and mycophenolic acid, phenotypes often correlated with an elongation defect (7).

Coincident with lower Rpb3 occupancy at 3' ends of mRNA genes, *rpb4Δ* strains do not show cotranscriptional recruitment of 3'-end processing factors Rna14 and Rna15. The decrease in polymerase may partially explain the drop in 3'-end processing factor recruitment. However, it is also possible that it is the loss of polyadenylation factor recruitment that leads to lower RNAPII cross-linking at 3' ends, particularly given findings

suggesting that 3'-end signals trigger pausing of elongating polymerase (26). At each of the three mRNA genes tested, the most substantial reduction in RNAPII levels in the *rpb4Δ* strain coincides with the polyadenylation site.

Several observations argue that Rpb4 is more directly involved in recruiting 3'-end processing factors. First, although polyadenylation factors are normally recruited to snoRNA genes (16), Rna14 recruitment to *SNR13* is lost in the *rpb4Δ* strain (Fig. 4). This is not due to loss of RNAPII, because levels of Rpb3 at *SNR13* in the *rpb4Δ* strain are the same as levels in the wild type. The snoRNA genes are relatively short and therefore would be less susceptible to elongation defects. Second, alternative polyadenylation site usage at the *RNA14* gene is altered in the *rpb4Δ* strain (Fig. 7). The altered usage pattern is very similar to that observed in yeast strains lacking Ctk1, a kinase necessary for cotranscriptional recruitment of polyadenylation factors (1, 21), or strains with mutations in *RNA14* or *RNA15* (1, 21).

A role for Rpb4 in 3' processing factor recruitment is consistent with its location within the RNAPII complex. Located near the CTD of Rpb1 as well as the transcript-exit groove (2, 3), Rpb4 is potentially in position to stabilize an interaction of 3'-end processing factors with the RNAPII CTD and the polyadenylation site sequences on the emerging transcript. Such stabilization could be mediated by a direct interaction of Rpb4/7 with a polyadenylation factor, with the RNA, or with an intermediary factor. Alternatively, Rpb4/7 could be involved in modulating the CTD phosphorylation required for polyadenylation factor recruitment. In this respect, we did not observe any defects in recruitment of the CTD kinase Ctk1 (Fig. 6A) or in whole-cell levels of serine 2-phosphorylated CTD (Fig. 6B). However, others have demonstrated interactions of Rpb4/7 with the CTD phosphatase Fcp1 (10, 17).

In conclusion, our results demonstrate a new function for Rpb4 in the coupling of transcription with 3'-end processing. Combined with previous findings that Rpb4 is required for promoter-dependent transcription (8, 25) and a recent study suggesting that Rpb4 has a role in mRNA export (9), it is clear that Rpb4 plays an integral role in various stages of transcription.

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