Leo1 Subunit of the Yeast Paf1 Complex Binds RNA and Contributes to Complex Recruitment*□

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The Paf1 complex (Paf1C) affects RNA polymerase II transcription by coordinating co-transcriptional chromatin modifications and helping recruit mRNA 3’ end processing factors. Paf1C cross-links to transcribed genes, but not downstream of the cleavage and polyadenylation site, suggesting that it may interact with the nascent mRNA. Paf1C purified from Saccharomyces cerevisiae binds RNA in vitro, as do the purified Leo1 and Rtf1 subunits of the complex. In vivo cross-linking and immunoprecipitation of RNA associated with Paf1C (RNA-IP) show that Leo1, but not Rtf1, is necessary for the complex to bind RNA. Cells lacking Leo1 have reduced Paf1C recruitment as well as decreased levels of histone H3 and trimethylated H3 Lys4 within transcribed chromatin. Together, these results suggest that association of Paf1C with RNA stabilizes its localization at actively transcribed regions where it influences chromatin structure.

Eukaryotic transcription by RNA polymerase II (RNAPII)2 is a multistep process involving initiation, elongation, and termination, with a multitude of factors often directly interacting with the polymerase during each stage. One such factor, Paf1 (RNA polymerase-associated factor 1), was originally identified based on its ability to associate with RNAPII (1, 2). Paf1 is found in a complex with Cdc73, Ctr9, Leo1, and Rtf1, designated the Paf1 complex (Paf1C) (3, 4). Although originally implicated in initiation, Paf1C has since been shown to be involved in a variety of processes, including elongation, modification of chromatin, and 3’-processing of mRNAs (5–11). It remains unclear how Paf1C affects these diverse processes.

Paf1C purifies separately from the Mediator form of RNAPII, suggesting that it likely joins the transcription complex after initial recruitment of RNAPII (1). In agreement with this, chromatin immunoprecipitation (ChIP) experiments show that Paf1C is present at low levels near the promoter of active genes, cross-links strongly in transcribed regions corresponding to the mRNA, and is greatly reduced downstream of the cleavage and polyadenylation site (12–15). Consistent with its association with elongating RNAPII, Paf1C genetically and physically associates with other elongation factors (4, 15, 16). Although none of the Paf1C subunit genes is essential, deletion of certain subunits results in sensitivity to 6-azauracil and mycophenolic acid, phenotypes that are often associated with defects in transcription elongation (4, 13, 16, 17). Indeed, extracts from cells lacking Paf1 or Cdc73 exhibited reduced efficiency of transcription elongation in an in vitro assay (18). Additionally, deletion of CDC73, CTR9, or PAI1 resulted in reduced rRNA synthesis without altering the rDNA copy number or RNA polymerase I (RNAPI) occupancy at the rDNA region, suggesting a role for Paf1C in RNAPI elongation (19). In contrast, an in vivo study of transcription elongation and processivity did not detect a strong effect from deleting RTFI or CDC73 (20). Loss of Paf1C does not affect recruitment of other elongation factors, such as Spt16 and Spt4 (15, 21). Consequently, it remains unclear whether Paf1C directly or indirectly stimulates elongation or instead influences transcription through other means.

Although Paf1C has been shown to associate with the transcription machinery as well as other elongation factors, it is unclear how Paf1C is recruited to sites of transcription. Because Paf1C is not a part of the Mediator-RNAPII complex at promoters of genes, it presumably associates with RNAPII sometime after initiation (22). Interestingly, Paf1C displays a ChIP pattern similar to the THO/TREX complex, an RNA-binding complex that also affects transcription elongation (12). Cross-linking of both complexes is greatly reduced downstream of the cleavage and polyadenylation site (12, 13, 15). Although RNAPII continues transcribing past this site, the downstream RNA is degraded rapidly. Two subunits of the TREX complex, Sub2 and Yra1, directly bind RNA, and their association with actively transcribing genes decreases upon degradation of the nascent RNA transcript (23–26). Thus, Paf1C could be recruited and/or stabilized at actively transcribed genes via the nascent RNA in a manner similar to THO/TREX. Although none of the Paf1C proteins contains a canonical RNA-binding domain, Rtf1 contains a Plus-3 domain, named for its three conserved positively charged residues (27). Recently, the Plus-3 domain of human Rtf1 was shown to bind single-stranded DNA in vitro (28). Additionally, Leo1 is highly charged, which could similarly facilitate an interaction with nucleic acids (29), and the C-terminal 210-amino acid region of Ctr9 can bind triple-helical DNA in vitro (30).

In an attempt to understand better the Paf1C role in gene expression, we tested purified Paf1C for effects on RNAPII elongation and RNA interactions. Although no enhancement

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2The abbreviations used are: RNAPII, RNA polymerase II; H3K4me3 and H3K36me3, histone H3 Lys4 trimethylation and Lys56 trimethylation, respectively; Paf1C, Paf1 complex.
of elongation was observed with purified RNAPII, Paf1C does exhibit RNA binding activity. Recombinant Leo1 and Rtf1 bind RNA in vitro, and Leo1 is important for efficient Paf1C recruitment to sites of transcription in vivo. Furthermore, the association between Paf1C and chromatin is sensitive to RNase treatment. We propose that at least part of Paf1C association with an elongation complex is mediated by binding to the nascent RNA transcript.

**MATERIALS AND METHODS**

**Plasmids and Yeast Strains**—Yeast strains used in this study are listed in supplemental Table S1. All recombinant Paf1C subunits were C-terminally tagged with hexahistidine (His$_6$) using the pET-28a (+) vector (Novagen). To generate pET28a (+) plasmids with CDC73, CTR9, LEO1, PAF1, and RTF1, the open reading frame of each gene was PCR-amplified using genomic DNA from YF336 for template. PCR products for and unlabeled pBlue RNA probe (67 nucleotides) was made by in vitro transcription of Apol-linearized pBlueScript II KS+ (Stratagene) using the T3 phage polymerase according to the manufacturer’s instructions (Promega). Unlabeled GAL7 RNA (74 nucleotides) was made by in vitro transcription of Apol-linearized pJCGAL7-1 (33) using T3 phage polymerase. A double-stranded DNA (166 bp) probe was made by PCR using the T3 and T7 primers on pBlueScript II KS+. Single-stranded DNA probes were oligonucleotides of the T3 promoter region (19 nucleotides) and sequence from the coding region of Rpb1 (88 nucleotides) was used in E. For all gels, the asterisk denotes migration of the unbound RNA probe. Lanes marked Paf1C indicate reactions containing 28 nM Paf1C with no unlabeled competitor, and lanes marked with (−) indicate reactions lacking Paf1C.

**FIGURE 1.** Paf1 complex binds RNA in vitro. A, RNA EMSA was performed using purified Paf1C and radiolabeled pBlue RNA. Concentrations of Paf1C are indicated above the gel, and the asterisk shows the position of the free probe. B, RNA EMSA reactions containing radiolabeled pBlue RNA and 28 nM Paf1C were incubated with increasing concentrations (0.2–2 μM) of unlabeled competitor RNA. The pBlue competitor is the same sequence as the radiolabeled probe whereas the GAL7 competitor is a completely different sequence. C, Paf1C RNA binding was challenged with unlabeled competitor dsDNA that contains the same sequence as the radiolabeled RNA probe. DNA was titrated from 0.05 to 2 μM. D and E, Paf1C binding was challenged with unlabeled competitor ssDNA. DNA was titrated from 0.05 to 5 μM in D and 0.05 to 2 μM in E. The T3 promoter primer oligonucleotide (19 nucleotides) was used in D and an oligonucleotide corresponding to the coding region of Rpb1 (88 nucleotides) was used in E. For all gels, the asterisk denotes migration of the unbound RNA probe. Lanes marked Paf1C indicate reactions containing 28 nM Paf1C with no unlabeled competitor, and lanes marked with (−) indicate reactions lacking Paf1C.

**Purification of Endogenous Protein Complexes and Recombinant Proteins**—RNAPII, Paf1C, and Spt4/5 were TAP-purified from whole cell extract prepared from Rpb9 (RNAPII), Paf1 (Paf1C), and Spt5 (Spt4/5) epitope-tagged strains (31, 32). Protein purifications are described in detail in supplemental Materials and Methods. All purified endogenous protein complexes were analyzed by SDS-PAGE and silver staining (see supplemental Materials and Methods) and concentrated using Ultrafree-0.5 centrifugal filters (Millipore). Paf1C was also analyzed by mass spectrometry. All purified recombinant proteins were analyzed by SDS-PAGE, immunoblotting with anti-His$_6$ antibody (Covance), and Coomassie staining. All protein concentrations were determined using the Bio-Rad protein assay.

**RNA Electrophoretic Mobility Shift Assay (EMSA)**—Both radiolabeled and unlabeled pBlue RNA probe (67 nucleotides) was made by in vitro transcription of Apol-linearized pBlueScript II KS+ (Stratagene) using the T3 phage polymerase according to the manufacturer’s instructions (Promega). Unlabeled GAL7 RNA (74 nucleotides) was made by in vitro transcription of Apol-linearized pJCGAL7-1 (33) using T3 phage polymerase. A double-stranded DNA (166 bp) probe was made by PCR using the T3 and T7 primers on pBlueScript II KS+. Single-stranded DNA probes were oligonucleotides of the T3 promoter (19 nucleotides) and sequence from the coding region of RPB1 (88 nucleotides). The sequences of all oligonucleotides used are listed in supplemental Table S2.
RNasin. For competition reactions, protein was incubated with labeled probe for 15 min followed by the addition of unlabeled DNA or RNA and incubation for an additional 20 min. Binding reactions were loaded directly onto a 5% polyacrylamide gel (acrylamide:bisacrylamide 37.5:1, 1×TBE) and run at 120 V in 0.5×TBE at room temperature. Gels were dried and visualized by phosphorimaging.

For UV cross-linking reactions, RNA binding reactions were incubated as for EMSA and then UV-irradiated in an Ultra-lum/UVC-515 ultraviolet multilinker set at 1800 J. After irradiation, unbound RNA was removed by digestion with RNase A and RNase T1 for 30 min at 37 °C. Loading buffer was added, and reactions were resolved by 10% SDS-PAGE. Gels were dried and exposed to a PhosphorImager screen.

Chromatin Immunoprecipitation (ChIP)—Cells were grown in YPD at 30 °C to an A_{600 nm} of 0.5. Formaldehyde cross-linking, chromatin preparation, and immunoprecipitation were performed as described previously (12, 34, 35). TAP-tagged proteins were immunoprecipitated with IgG-Sepharose (GE Healthcare), Rpb3 using a monoclonal antibody (Neoclon), and histones using anti-H3 (Abcam), anti-H3K4me3 (Upstate), or anti-H3K36me3 (Abcam) with protein A-Sepharose or protein G-Sepharose (GE Healthcare). For the H3K4me3 and H3K36me3 immunoprecipitations, binding and washes were performed as described (35). For RNase-treated ChIP samples, chromatin was incubated with 25 units of RNase A (Sigma) and 1000 units of RNase T1 (Fermentas) for 30 min at room temperature prior to immunoprecipitations. Multiplex PCR of precipitated DNA was performed as described (36). Reactions were done in triplicate, and error bars represent standard deviation. Sequences for oligonucleotides used in PCR are listed in supplemental Table S2.

RNA Immunoprecipitation—RNA-IP was performed as described above for ChIP, with the following changes. All lysis, binding, and elution buffers contained 40 units/ml RNasin, whereas all wash buffers contained 20 units/ml RNasin. TE in all buffers was pH 7.5. For DNase I treatment, 60 μl of 10× DNase I buffer (250 mM MgCl₂, 50 mM CaCl₂), 36 μl of FA lysis buffer (see Ref. 34) + 40 units/ml RNasin, and 1000 units of DNase I (250 units/μl; Sigma) were added to 50 μl of extract and incubated for 30 min at 30 °C. Digestion was stopped by the addition of EDTA to 20 mM and heating at 65 °C for 10 min. The extract was centrifuged to remove precipitate and subjected to immunoprecipitation for the TAP tag as for ChIP. After de-cross-linking, RNA was isolated by adding 0.10 volume 3M NaOAc, pH 5.5, and 20 μg of glycogen, then phenol-chloroform-extracted and ethanol-precipitated. DNase I-treated extract (50 μl) was processed in parallel for input RNA and diluted 1:10 prior to RT-PCR. RNA was reverse-transcribed using Superscript II reverse transcriptase according to the manufacturer’s instructions (Invitrogen). PCR was performed as for ChIP, but with 35 cycles using the following primers: primer pair CDS for ADH1, primer pair #3 for PMA1, primer pair #2 for PYK1, and primer pair #3 for YEF3 (see supplemental Table S2). Reactions were run on a 2.5% agarose gel and visualized with ethidium bromide.

RESULTS

Paf1C Binds RNA in Vitro—Given the similarity of ChIP cross-linking patterns of Paf1C and the THO/TREX complex (12, 14, 21), we speculated that Paf1C might be recruited to the transcribed region of a gene via association with the RNA transcript itself. To determine whether Paf1C interacts with RNA, TAP-
Paf1 Complex Subunit Leo1 Binds RNA

**FIGURE 3. Paf1 complex lacking Rtf1 retains RNA binding activity.** A, RNA EMSA was performed as in Fig. 1A using TAP-purified Paf1C isolated from either a wild-type or rtf1Δ strain. A single-shifted band (white arrowhead) is observed in reactions containing Paf1C(rtf1Δ) compared with the doublet seen in reactions with wild-type Paf1C. B, RNA EMSA was performed using radiolabeled RNA and Paf1C(rtf1Δ), and recombinant Rtf1 was added in increasing concentrations from 0.1 to 2 μM as indicated above the gel. Addition of Rtf1 led to the formation of a Paf1C-RNA complex that included Rtf1, which migrates similarly to the upper band of the wild-type Paf1C-RNA doublet (black arrow). The black arrowhead indicates formation of Rtf1-RNA complexes. The asterisks denote migration of the unbound RNA.

**FIGURE 4. Paf1C cross-links to mRNA transcripts.** RNA-IP was performed for the YEF3, PYK1, ADH1, and PMA1 mRNAs using strains expressing the indicated TAP-tagged proteins. Proteins and associated RNA were precipitated with IgG-agarose (TAP IP) or anti-Rpb3 (Rpb3 IP) and analyzed using RT-PCR (IP). All five subunits of Paf1C co-precipitate RNA from all tested genes (lanes 3–7). Paf1 association with RNA is decreased in a leolΔ strain (lane 8) but not in an rtf1Δ strain (lane 9). IP from an untagged strain (No tag; YF336) is shown as a negative control, and IP from an Rpb3Δ-TAP (YF924) strain is shown as a positive control (lanes 1 and 2). Rpb3 association with RNA is unchanged in both a leolΔ strain and an rtf1Δ strain (compare lane 10 with lanes 11 and 12). RT-PCR from total RNA is shown as the Input.

purified Paf1C (supplemental Fig. S1) was incubated with radiolabeled RNA probe and assayed by EMSA. Paf1C-RNA complexes can be seen as a slower migrating doublet (Fig. 1A). These complexes were competed by increasing concentrations of two unlabeled RNAs with completely different sequences, arguing against sequence specificity (Fig. 1B). No competition was observed with linear dsDNA (Fig. 1C), or ssDNA of 19 (Fig. 1D) or 88 nucleotides (Fig. 1E) in length. Therefore, Paf1C binding appears specific to RNA. The purified Paf1C was tested for effects on transcription elongation using purified RNAP and a naked DNA template. Although the two protein complexes interacted, no stimulation or inhibition of elongation was observed (supplemental Figs. S2 and S3).

**Leo1 and Rtf1 Subunits of Paf1C Bind RNA in Vitro**—To identify which of the Paf1C subunits associate with RNA, the Paf1C binding reactions were UV irradiated to crosslink proteins closely associated with the labeled RNA. Four distinct bands were resolved by SDS-PAGE after UV cross-linking and RNase treatment (Fig. 2A). The molecular mass band near 120 kDa corresponds to Ctr9 (124 kDa), whereas the band near 50 kDa is Cdc73 (45 kDa). The bands near 90 kDa and 80 kDa are likely Rtf1 and Leo1, respectively, because both of these proteins are known to migrate slower than their calculated molecular masses of 66 kDa (Rtf1) and 54 kDa (Leo1) (29, 37) (supplemental Fig. S1). This assay shows that these four subunits are close to the RNA, but does not necessarily indicate direct binding.

To identify which Paf1C components bind directly to the RNA, recombinant proteins for each subunit were purified separately (supplemental Fig. S1) and tested using the same EMSA conditions as those for Paf1C. Recombinant Ctr9 had extremely poor expression in *Escherichia coli* and could not be assayed (data not shown). No specific complex was observed for Paf1 protein (Fig. 2D), consistent with its inability to UV cross-link to RNA. The binding reactions with Cdc73 were inconclusive because increasing protein concentrations resulted in disappearance of free probe and retention in the wells, but a discrete complex was not seen (Fig. 2B). This behavior probably indicates aggregation of protein and RNA, but it is unclear whether it reflects specific RNA binding. In contrast, discrete complexes were seen for Leo1 and Rtf1 (Fig. 2, C and E). Assuming that the majority of protein is active, the apparent dissociation constants for both proteins would be in the micromolar range, indicating a moderate affinity. These results identify Leo1 and Rtf1 as good candidates for the RNA binding activity of Paf1C.

**Paf1C Lacking Rtf1 Can Bind RNA**—To test whether Leo1 and Rtf1 contribute to RNA binding in the context of the complex, Paf1C was purified via Paf1-TAP from strains deleted for either LEO1 or RTF1. We were unable to isolate an intact complex from a leolΔ strain; the purified fractions contained only Paf1-TAP and a little Ctr9, but no detectable Rtf1 or Cdc73 (data not shown). The lack of Leo1 results in a partial reduction of the protein levels of the other subunits (supplemental Fig. S4), but this is more likely to be an effect rather than a cause of the complex instability.

Paf1C purified from a strain lacking Rtf1 clearly retained the remaining four subunits (supplemental Fig. S1). EMSA analysis of Paf1C(rtf1Δ) revealed that the complex binds RNA with
affinity similar to the wild-type Paf1C, but showed only a single sharp band (Fig. 3A, lanes 4–7) rather than the doublet seen with the wild-type complex (Figs. 1A and 3A, lanes 1–3). Paf1C(Δrtf1Δ) migrated with the lower band, suggesting that the doublet may represent complexes with (upper band) and without (lower band) Rtf1. It has been reported that Rtf1 is loosely associated with Paf1C (6). As predicted by this hypothesis, addition of recombinant Rtf1 to reactions containing Paf1C(Δrtf1Δ) resulted in the appearance of the upper band as well as the Rtf1-RNA complex (Fig. 3B).

**Paf1C Is Associated with RNA in Vivo**—To investigate whether Paf1C binds to RNA in vivo, RNA-IP was performed using strains with each of the TAP-tagged Paf1C subunits. In this assay, proteins and RNA are cross-linked, and each subunit is precipitated from an extract treated with RNase inhibitors and DNase I. All subsequent steps are similar to ChIP except that a reverse transcriptase reaction preceded the final PCR analysis (34, 38). No signal is seen when the reverse transcriptase step is omitted (data not shown), indicating that the amplification is specific for RNA associated with the precipitated protein. Transcripts from several constitutively expressed genes were analyzed.

All five subunits of Paf1C co-precipitated the tested mRNAs, as did the positive control protein Rpb3 (Fig. 4, lanes 2–7). A negative control using an untagged strain showed that the background signal was low (lane 1). To determine whether Leo1 is important for the ability of Paf1C to bind RNA in vivo, RNA-IP via Paf1-TAP was performed in a leo1Δ strain. The amount of RNA associated with Paf1-TAP in the absence of Leo1 is greatly reduced (compare lanes 6 and 9). Although loss of Leo1 causes some reduction in levels of other Paf1C subunits (supplemental Fig. 4), this drop does not appear sufficient to account for loss of RNA binding. In contrast to Leo1, there is no decrease in Paf1C association with RNA in an rtf1Δ strain (compare lanes 6 and 9). Additionally, Rpb3 association with RNA was unchanged in strains lacking either Leo1 or Rtf1 compared with wild-type (Fig. 4, lanes 10–12). Therefore, as assayed by RNA cross-linking, Leo1 is important for the ability of Paf1C to associate stably with RNA in vivo.

**Leo1 Promotes Association of Paf1C at Sites of Transcription**—Cdc73 and Rtf1 have previously been shown to stabilize the interaction of Paf1C with actively transcribed genes as shown by ChIP, leading to the model that both Cdc73 and Rtf1 are important for the association of the complex with RNAPII (6, 15, 21). Because Leo1 may promote Paf1C association with RNA, we tested whether deletion of Leo1 would also affect the ability of the complex to associate with transcriptionally active genes. Strains bearing TAP-tagged subunits of the Paf1C were grown in both leo1Δ backgrounds. As expected, strains lacking LEO1 grew at nearly wild-type rates (supplemental Fig. S4) (4, 39). Additionally, the tagged leo1Δ strains grew the same as untagged, indicating that the TAP tag does not dramatically interfere with protein function (supplemental Fig. S4). There was a modest decrease in the protein levels of the other Paf1C subunits in the leo1Δ background (supplemental Fig. S4).

To determine whether deletion of LEO1 affects Paf1C occupancy at transcribed genes, Paf1 was monitored by ChIP at two constitutive genes (YEF3 and PYK1) in LEO1 and leo1Δ strains (Fig. 5A). In wild-type cells expressing Paf1-TAP, we observe Paf1 occupancy along the length of each gene, with increased cross-linking within the open reading frame (Fig. 5B). As pre-
increased in a manner similar to Cdc73, Ctr9, and Rtf1. Cross-linking of Cdc73, Ctr9, and Rtf1 are also decreased in a leo1 strain, Paf1 occupancy is dramatically reduced (Fig. 5A). Previous studies have shown that RNase treatment decreases the association of Paf1 with chromatin preparation with or without RNase treatment prior to immunoprecipitation. PCR products are numbered, and occupancy values are calculated as for Fig. 5. C, levels of Paf1-TAP were normalized to the levels of Rpb3 at both YEF3 and PYK1.

FIGURE 6. RNA transcript contributes to Paf1 recruitment. A, B, and D, ChIP was performed for TAP-tagged Paf1 (A) as well as Rpb3 (B) and TBP (D) in wild-type (YFB62) and leo1Δ (YSB176) strains using the same chromatin preparation with or without RNase treatment prior to immunoprecipitation. PCR products are numbered, and occupancy values are calculated as for Fig. 5. C, levels of Paf1-TAP were normalized to the levels of Rpb3 at both YEF3 and PYK1.

Histone H3 Methylation Is Altered in a leo1Δ Strain—Because loss of Leo1 results in decreased association of the Paf1C, and this complex has previously been shown to be required for co-transcriptional trimethylation of lysine 4 and lysine 36 of histone H3 (9, 40–42), we assayed for changes in both of these modifications in cells lacking Leo1. As expected, a decrease in H3K4me3 at promoter regions was observed (Fig. 7A). Surprisingly, after normalizing for total H3, the levels of H3K36me3 appeared to increase in the leo1Δ strain (Fig. 7B, lower panel). However, this change was actually due to a decrease in the amount of H3 cross-linking at both YEF3 and PYK1 compared with a nontranscribed region of chromosome VI (Fig. 7C). In contrast, the absolute amount of H3K36me3 was roughly equivalent in both leo1Δ and leo1Δ (Fig. 7B, upper panel), indicating that Leo1 is not required for H3K36 trimethylation. We note that Chu et al. also reported no change in H3K36me3 levels in a leo1Δ strain (42). We interpret our results to mean that Leo1 helps to maintain histone levels in transcribed regions, perhaps helping to replace nucleosomes displaced during transcription. This would be consistent with the described role of the Paf1 complex in suppressing cryptic internal initiation (42).

DISCUSSION

Since its initial characterization, the molecular functions of Paf1C have remained obscure. Although the complex has been implicated in chromatin modification and RNA processing, it is unknown how Paf1C functions at the molecular level. The association of Paf1C with RNAPII is almost certainly critical for its activity. Multiple subunits have been shown to be essential for Paf1C association with RNAPII and/or chromatin (6, 15, 21, 43). Although the complex has been suggested to be required for both RNAPII and RNAPII elongation (18, 19), we did not observe a direct stimulation of RNAPII elongation activity by Paf1C using a purified in vitro transcription system (supplemental Fig. S2). Previous studies suggest that Paf1C recruitment and interaction requires the elongation factor DSIF (Spt4/Spt5), however there was no overt effect on RNAPII elongation in the presence of Paf1C and Spt4/5 (supplemental Fig. S3). This
suggests that the complex may affect elongation indirectly via another elongation factor or through chromatin-mediated effects.

We show here that Paf1C can directly interact with RNA both in vitro and in vivo, mediated at least in part through the Leo1 subunit. Loss of Leo1 results in lower levels of Paf1C subunits, reduced ability of Paf1C to localize to transcribed genes, and changes in chromatin structure at sites of transcription. We propose that Paf1C association with the nascent RNA helps stabilize its interaction with elongating RNAPII and is important for Paf1C functions.

Using recombinant proteins, at least two Paf1C subunits exhibit RNA binding activity. Both Rtf1 and Leo1 can form discrete complexes in a native gel EMSA. Although Rtf1 is clearly important for transcription and chromatin modifications (16, 43), it does not appear to be required for the association of the complex with RNA in vivo (Fig. 4). Additionally, purified Paf1C lacking Rtf1 can still form a stable complex with RNA (Fig. 3). Indeed, wild-type Paf1C forms two shifted bands in the RNA EMSA, one with Rtf1 and one without. This is consistent with multiple observations that indicate that Rtf1 is more peripherally associated with the complex (6, 21). Also consistent with our results is the finding that purified mammalian Paf1C does not contain Rtf1 as a stoichiometric subunit and instead is associated with the RNA degradation factor Ski8 (44). Although we cannot exclude the possibility that both Leo1 and Rtf1 contribute to Paf1C RNA binding activity, our in vivo data suggest a model in which Leo1 is a major contributor to this function. Detailed mutational analysis of both Rtf1 and Leo1 is required to clearly define the RNA-binding regions of both proteins.

Leo1 is important for efficient Paf1C recruitment. The remaining four subunits of Paf1C have reduced ChIP signals at actively transcribed genes in a leo1Δ strain. However, some Paf1C function must remain because deletion of LE01 has relatively mild phenotypes compared with loss of some of the other subunits. It is interesting to note that Paf1C levels closest to promoters are least affected, suggesting that Leo1 may be less important for initial Paf1C recruitment than for maintenance during elongation (Fig. 5 and supplemental Fig. S5).

Paf1C recruitment to genes is at least partially mediated by RNA interactions because cross-linking in

FIGURE 7. Loss of Leo1 alters H3 occupancy and H3K4 trimethylation. A, H3K4me3 levels are reduced in a leo1Δ strain. ChIP for H3K4me3 and H3 was performed in wild-type (YF336) and leo1Δ (YF347) strains. The upper panel shows absolute levels of H3K4me3 at YEF3 and PYK1, normalized to a nontranscribed region. The lower panel shows levels for H3K4me3 on both genes normalized to total H3. Note that the high level of H3K4me3 at the 3′ end for YEF3 is due to the proximity of this primer pair to the promoter of the downstream gene. B, H3K36me3 levels are maintained in a leo1Δ strain. ChIP for H3K36me3 and H3 was performed in wild-type and leo1Δ strains. The levels for H3K36me3 were normalized to a nontranscribed region (upper panel) and to total H3 levels (lower panel). C, H3 occupancy is decreased in a leo1Δ strain as determined by ChIP when normalized to a nontranscribed region. All values are shown with S.D. for three independent experiments. Quantification was done as for Fig. 6.

ChIP experiments is reduced when using chromatin treated with RNase (Fig. 6). Similarly, significantly less RNA cross-links to Paf1C in strains lacking Leo1. We were unable to purify an intact Paf1C from a strain lacking Leo1 even though the cells still contained substantial, albeit reduced, levels of the other subunits. Because a leo1Δ strain does not exhibit the same severe growth phenotypes as a strain with either PAF1 or CTR9 deleted, other Paf1C functions that do not require Leo1 apparently persist even at reduced protein levels. Leo1 contributes to complex stability, but it is unclear whether this is a cause or effect of reduced recruitment of Paf1C to the transcription elongation complex.

Multiple studies have implicated Paf1C in regulation of chromatin modifications. Paf1 and Rtf1 are required for ubiquitylation of histone H2B by the Rad6-Bre1 complex (10, 11). This modification in turn is required for trimethylation of H3K4 by the Set1 complex COMPASS (45, 46). Similarly, reducing levels of the human Paf1C leads to decreased levels of H3K4me3, indicating that this function is conserved (44). A
subset of the Paf1C subunits is required for recruitment of Set1 and Set2 to chromatin and H3K36me3 (40–42). Here, we show that cells lacking LEO1 have decreased levels of H3K4me3 at several strongly transcribed genes. In agreement with Chu et al. (42), we see normal levels of H3K36me3 in leo1Δ strains, suggesting that Paf1C activity in the absence of LEO1 is sufficient for recruitment of Set2.

ChIP experiments suggest that Paf1C promotes rapid histone removal upon induction of the GAL1 and GAL10 genes (47). Histone H3 levels within transcribed regions are decreased in a leo1Δ strain (Fig. 7), suggesting that the complex may also be important for nucleosome reassembly in the wake of the elongating polymerase. There are extensive interactions between Paf1C and factors important for histone displacement and replacement. LEO1 and other members of Paf1C genetically interact with ASFI and RTT106, histone chaperones that exchange H3/H4, and Paf1C physically interacts with FACT (Spt6/Pob3), a complex that exchanges H2A/H2B dimers during transcription (4, 13, 48–51). Although Paf1C is not required for SpT6 recruitment (21), Paf1C loss may affect additional components of the elongation complex that promote nucleosome remodeling. For example, SPT2, which is required for maintaining wild-type histone H3 levels on actively transcribed chromatin, genetically interacts with Paf1C genes, and SpT2 recruitment to transcribed regions is decreased in a paf1Δ strain (6). Additionally, Rtf1 genetically interacts with SPT6, which is required for maintenance of histones in transcribed regions as well as recruitment of Spt2, SpT5, Ctr9, Paf1, and Rtf1 (16, 52–54). Thus, decreased Paf1C association along the chromatin may lead to inefficient histone chaperone activity, resulting in defective nucleosome reassembly after passage by RNAPII.

In conclusion, our findings provide evidence that Paf1C interacts with the nascent RNA, at least in part via LEO1, and this function contributes to proper chromatin structure in transcribed regions. Although contacts with RNAPII are made through Rtf1 and Cdc73, it remains to be seen whether these interactions are important for the initial recruitment of Paf1C to the transcription complex, continued association during elongation, or both. Although strains lacking LEO1 do not display strong phenotypes related to elongation and chromatin modifications, this is likely because the remaining subunits provide decreased but sufficient Paf1C function. It is likely that interactions with the RNA (via LEO1 and potentially Rtf1) cooperate with interactions between RNAPII and Rtf1 and Cdc73 to maintain Paf1C at sites of transcription. The higher occupancy of Paf1C within transcribed regions upstream, but not downstream, of the polyadenylation site (12, 15) suggests that interaction with nascent RNA becomes important for efficient and stable recruitment of Paf1C. It is unclear whether Paf1C is released from the transcription machinery after cleavage of the transcript or instead remains associated with the released transcript to modulate RNA processing. Although LEO1 has not been studied extensively, our findings suggest that this subunit is important for Paf1C stability and recruitment, interactions with the RNA transcript, modulation of co-transcriptional nucleosome reassembly, and H3K4 trimethylation.

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Paf1 Complex Subunit Leo1 Binds RNA

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