

The Paf1 complex is required for efficient transcription elongation by RNA polymerase I

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Regulation of RNA polymerase I (Pol I) transcription is critical for controlling ribosome synthesis. Most previous investigations into Pol I transcription regulation have focused on transcription initiation. To date, the factors involved in the control of Pol I transcription elongation are poorly understood. The Paf1 complex (Paf1C) is a well-defined factor that influences polymerase II (Pol II) transcription elongation. We found that Paf1C associates with rDNA. Deletion of genes for Paf1C subunits (*CDC73*, *CTR9*, or *PAF1*) reduces the rRNA synthesis rate; however, there is no significant alteration of rDNA copy number or Pol I occupancy of the rDNA. Furthermore, EM analysis revealed a substantial increase in the frequency of large gaps between transcribing polymerases in *ctr9Δ* mutant cells compared with WT. Together, these data indicate that Paf1C promotes Pol I transcription through the rDNA by increasing the net rate of elongation. Thus, the multifunctional, conserved transcription factor Paf1C plays an important role in transcription elongation by Pol I in vivo.

gene expression | ribosome

Synthesis of eukaryotic ribosomes is an important and complex process that involves 78 ribosomal proteins, 4 rRNA species, and ≈200 processing factors (1, 2). rRNA transcription accounts for ≈60% of total transcriptional activity in growing yeast cells, and ≈50% of polymerase II (Pol II)-dependent transcription is devoted to ribosomal protein genes (3). Given the energetic commitment that cells make to ribosome synthesis, proper control of this process is critical.

A major target for the regulation of ribosome synthesis is transcription of rDNA by polymerase I (Pol I). Although most previous investigations into the control of Pol I activity have focused on the transcription initiation step (e.g., refs. 4 and 5), recent data reveal that the elongation step of transcription by Pol I is also an important regulatory target (6). Furthermore, recent data demonstrate that the efficiency of transcription elongation by Pol I is important for optimal processing of rRNA (7). Thus, transcription elongation by Pol I is important for both regulation of rRNA synthesis and its processing.

Despite the importance of transcription elongation by Pol I, little is known about the factors involved. To date, only 5 factors [UBF (6); TFIIS (8); Fcp1p (9); Ctk1p (10); Spt4p/Spt5p (11)] have been implicated in Pol I transcription elongation. By comparison, dozens of factors that influence Pol II elongation have been identified (for a review see ref. 12). It is likely that roles for several additional factors in Pol I transcription elongation remain to be determined.

Paf1 complex (Paf1C) is a well-characterized complex that influences transcription elongation by Pol II. Paf1C contains 5 core subunits: Cdc73p, Ctr9p, Leo1p, Paf1p, and Rtf1p (13, 14). Based on Paf1C's association with transcribed genes (13, 15), genetic interactions with other elongation factors (13, 16, 17), and biochemical activity in vitro (18), it seems clear that Paf1C directly affects Pol II elongation, but the mechanism of this effect has not been determined.

Paf1C is a multifunctional complex. In addition to its direct role in Pol II transcription elongation, Paf1C affects chromatin structure, transcription initiation, and RNA processing. In yeast, Paf1C

induces ubiquitination of histone H2B [by activation of Rad6p and Bre1p (19)] and methylation of histone H3 [by recruiting COM-PASS (19, 20)]. Recent data suggest similar potential roles for Paf1C in *Drosophila* (21). Furthermore, the Rtf1p subunit of Paf1C has been shown to influence TATA box-binding protein association with the TATA box of a Pol II promoter to modulate the efficiency of transcription initiation (22). Finally, Paf1C subunits physically interact with factors involved in mRNA processing (23). Consistent with these interactions, deletion of *PAF1* leads to shortened poly(A) tails on many mRNAs (15), defective 3' processing of small nucleolar RNAs (24), and possibly impaired mRNA export from the nucleus (25). Almost all aspects of mRNA metabolism are affected by Paf1C.

In this study, we identify an additional role for Paf1C in Pol I transcription elongation. Paf1C associates with rDNA in vivo, and the rate of Pol I transcription is reduced in *paf1Δ*, *cdc73Δ*, and *ctr9Δ* mutants. However, this inefficient transcription is not caused by alteration in Pol I occupancy of the rDNA or changes in rDNA copy number, suggesting a role for Paf1C in Pol I transcription elongation. EM analysis of *ctr9Δ* cells supports a role for Paf1C in modulating the net Pol I transcription elongation rate and suggests a specific role for Paf1C in clearing transient pause sites during transcription elongation.

Results

Subunits of Paf1C Associate With rDNA. To determine whether Paf1C associates with rDNA in vivo, we constructed strains expressing his₇-(HA)₃-tagged Cdc73p, Ctr9p, Paf1p, and A135 (the second largest subunit of Pol I; as a positive control; Table 1). ChIP assays were performed on these strains to measure each protein's association with the rDNA, using 10 primer pairs located throughout the entire rDNA repeat (Fig. 1A).

As expected, our ChIP data clearly detected strong binding of Pol I to the transcribed regions of the rDNA (Fig. 1B). The large decrease in ChIP signal observed in the nontranscribed sequence (NTS) and 5S regions demonstrates the specificity and sensitivity of the assay. Using the same primer pairs and same antibody for immunoprecipitation (IP), we also detected significant association of Cdc73p, Ctr9p, and Paf1p with the rDNA (Fig. 1C). The association of Cdc73p, Ctr9p, or Paf1p with the rDNA in the 35S rRNA coding region was ≈10-fold less than that of Pol I, but several-fold higher than background signal from the untagged negative control samples. This reduced signal relative to Pol I is expected for an elongation factor that may not bind DNA directly and is consistent with previous observations (11, 26).

Unlike Pol I, Paf1C proteins also associated with the NTS regions and the 5S rDNA. This finding is consistent with previous observations for Chd1p and Isw1p (26) and the Spt4/Spt5 complex (D.A.S., unpublished results). The reason for positive ChIP signal

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Table 1. Strains or plasmids used in this work

Name	Description
Strains	
NOY396	<i>MATα ade2-1 ura3-1 trp1-1 leu2-3, 112 his3-11,15 can1-100</i>
NOY1051	<i>MATα ade2-1 ura3-1 trp1-1 leu2-3, 112 his3-11,15 can1-100 fob1Δ::HIS3 rpa135Δ::LEU2; pNOY117 rDNA copy number \approx143 (34)</i>
NOY1064	<i>MATα ade2-1 ura3-1 trp1-1 leu2-3, 112 his3-11,15 can1-100 fob1Δ::HIS3; rDNA copy number \approx190 (42)</i>
NOY1071	Same as NOY1064, but rDNA copy number \approx 25 (42)
NOY886	Same as NOY1051, but rDNA copy number \approx 42 (34)
DAS317	Same as NOY396, but <i>PAF1-his7-(HA)$_3$::HIS3</i>
DAS477	Same as NOY396, but <i>MATα RPA135-his7-(HA)$_3$::TRP1</i>
DAS507	Same as NOY396, but <i>CDC73-his7-(HA)$_3$::HIS3</i>
DAS509	Same as NOY396, but <i>CTR9-his7-(HA)$_3$::HIS3</i>
DAS511	Same as NOY396, but <i>cdc73Δ::HIS3</i>
DAS513	Same as NOY396, but <i>ctr9Δ::HIS3</i>
DAS516	Same as NOY396, but <i>paf1Δ::HIS3</i>
Plasmids	
PNOY117	pRS314 (pBluescript, CEN6, ARSH4, TRP1) derivative carrying RPA135
pRS316	pBluescript, CEN6, ARSH4, URA3; see ref. 43

in the regions of rDNA that are devoid of Pol I remains unknown. However, it is known that Pol II transcripts originate from the NTS regions of the rDNA, and Pol III is active at the 5S rRNA genes (27, 28). Thus, this signal may result from association of these factors with the other polymerases that are clearly active in the spacers between 35S rRNA genes. Overall, these data confirm that Paf1C associates with the rDNA in vivo. Thus, these factors are poised to affect Pol I transcription elongation.

Deletion of CDC73, CTR9, or PAF1 Decreases rDNA Transcription in Vivo. To characterize the role that Paf1C plays in Pol I transcription, we constructed 3 strains carrying *cdc73 Δ* , *ctr9 Δ* , or *paf1 Δ* mutations (Table 1). The 3 mutants grew more slowly than the parental WT strain (Fig. 2A) and exhibited temperature sensitivity at 37 °C. To estimate Pol I transcription rates in vivo, the total RNA synthesis rate was measured by pulse-labeling yeast cells for 5 min with 3 H-uridine. Because most RNA synthesis during exponential growth is Pol I-derived, this is a reliable method for approximating Pol I activity. Uridine incorporation into total RNA was reduced \approx 3-fold in the *cdc73 Δ* strain and \approx 10-fold in both the *ctr9 Δ* and *paf1 Δ* strains relative to WT (Fig. 2A). This trend is consistent with the observed decreases in growth rate and suggests that Paf1C may play a significant role in Pol I transcription in vivo.

To quantify the Pol I transcription rate, we pulse-labeled cells with 3 H-methylmethionine for 5 min, chased them with excess cold methionine for 5 min (to permit completion of rRNA processing), isolated RNA, and measured the 3 H incorporation into 25S and 18S rRNA (Fig. 2B). Because rRNA is methylated cotranscriptionally in yeast and the cellular pool of methylmethionine is very low, this method is a reliable way to quantify Pol I transcription in vivo (29). We observed a small reduction in the Pol I transcription rate in the *cdc73 Δ* mutant compared with WT (69% of WT activity) and a larger reduction in *ctr9 Δ* and *paf1 Δ* strains (29% and 38% of WT activity, respectively). These values for inhibition of Pol I activity in the mutant strains are more reasonable for the detected defects in growth, and the greater level of inhibition of total RNA synthesis likely reflects an additional defect in uridine uptake in the mutant strains. Nevertheless, these data confirm that Pol I transcription is inhibited in the Paf1C mutant strains, consistent with their reduced growth rates and lower total RNA synthesis rates (Fig. 2A).

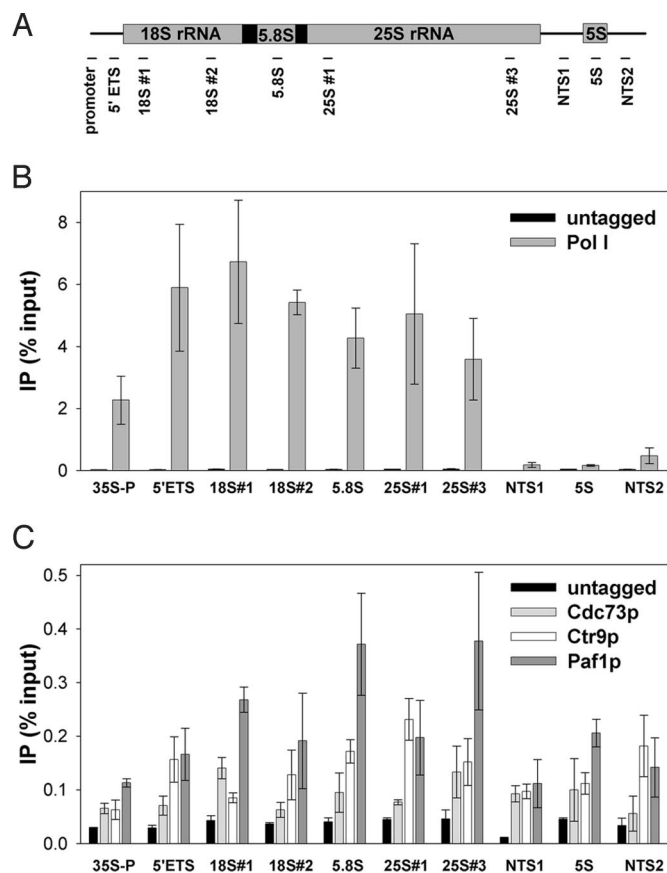


Fig. 1. Paf1C associates with rDNA. (A) Positions of 10 pairs of primers used in real-time PCR analyses are indicated by horizontal bars. (B) ChIP signal of Pol I (RPA135-his₇-HA₃; DAS477) across rDNA repeats is compared with untagged control (NOY396). Anti-HA antibody 12CA5 was used for IP of tagged proteins. IP data are expressed as a percentage of input DNA. (C) His₇-(HA)₃-tagged Cdc73p, Ctr9p, and Paf1p (DAS507, DAS509, DAS317) associate with the rDNA repeat. Data were quantified from at least 2 10-fold dilutions per sample from duplicate cultures. Error = \pm 1 SD.

Deletion of CDC73, CTR9, or PAF1 Does Not Affect Pol I Occupancy of the rDNA. Deletion of Paf1C subunits severely reduced Pol I transcription in vivo (Fig. 2); however, ChIP analysis detected no significant change in Pol I occupancy of the rDNA in Paf1C mutants relative to WT (Fig. 3). Additionally, no ChIP signal for Pol I was observed in the NTS1 region, indicating that termination of Pol I transcription was not significantly impaired in Paf1C mutants.

Processivity of Pol I was also unchanged in Paf1C mutants. Processivity describes the ability of a polymerase to elongate throughout the length of a gene. Relative processivity is estimated by comparing polymerase densities near the 5' end of the gene with the 3' end. Previous investigations have identified defects in Pol II processivity upon deletion of candidate elongation factors (30). Because there is no apparent decrease in the Pol I ChIP signal at the 3' end of the rDNA, it is clear that there is no significant decrease in the relative processivity in Paf1C mutants compared with WT.

Taken together, these data indicate that deletion of Paf1C subunits does not lead to a detectable reduction in Pol I loading or processivity, despite large decreases in rRNA synthesis rates. Thus, the simplest interpretation of these data are that deletion of *CDC73*, *CTR9*, or *PAF1* reduces the Pol I transcription elongation rate.

Effects of Deletion of CDC73, CTR9, or PAF1 on rDNA Copy Number. Because the rDNA in eukaryotic cells exists as a tandemly-repeated array, recombination events can lead to variation in the rDNA copy

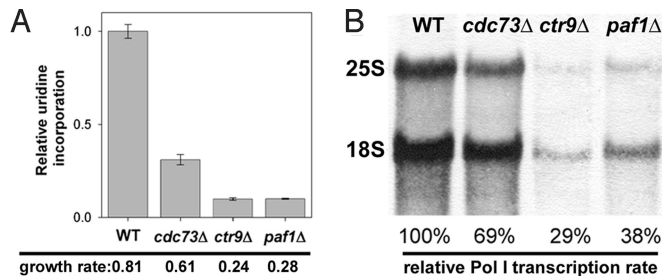


Fig. 2. Pol I transcription is reduced in strains defective in Paf1C function. (A) Relative RNA synthesis rates were measured by using ^3H -uridine incorporation in WT (NOY396), *cdc73Δ* (DAS511), *ctr9Δ* (DAS513), and *paf1Δ* (DAS516) strains. Growth rates are expressed as doublings per h. WT and Paf1C deletion mutants, carrying the pRS316 plasmid, were grown in SD-Ura media to $A_{600} \approx 0.3$. Duplicate samples from duplicate cultures were quantified, averaged, and expressed relative to WT. Error = ± 1 SD. (B) RNA was purified from cells pulse-labeled with ^3H -methylmethionine. After electrophoresis, RNA was transferred to a membrane and detected by autoradiography. 25S and 18S rRNA were cut from the membrane, quantified with a scintillation counter and expressed relative to WT. Data shown are from 1 of 3 independent experiments. Error was $<3\%$.

number (11, 28, 31). A potential decrease in the rDNA copy number could reduce rRNA synthesis rate. To control for this possibility, we measured the rDNA copy number in Paf1C mutants compared with reference strains with known rDNA copy numbers.

We observed no decrease in the rDNA copy numbers in Paf1C mutants. The rDNA copy numbers were estimated by using contour-clamped homogenous field electrophoresis (CHEF) followed by Southern blot hybridization. Because $\approx 60\%$ of chromosome XII in yeast is rDNA sequences (32), the migration distance of chromosome XII through a gel is heavily influenced by changes in the rDNA array. To quantify the rDNA copy number in the Paf1C mutants and the WT control, we compared the mobility of chromosome XII with that from strains with known rDNA copy numbers (Fig. 4). Because the Paf1C mutants are *Fob1*⁺, recombination between rDNA repeats is efficient, and there is significant variation in rDNA copy number within a culture (33). Migration of chromosome XII was measured from Southern blot hybridizations by using an rDNA probe to clearly detect the location of rDNA (Fig. 4A). From a linear regression of chromosome migration distance versus known rDNA copy number in reference strains, we estimated that *ctr9Δ*, and *paf1Δ* mutants carry approximately an equal number of rDNA copies as WT, and the rDNA copy number was increased $\approx 40\%$ in the *cdc73Δ* mutant (Fig. 4B). This increase in rDNA copy number may contribute to the reduced severity of the *cdc73Δ* mutation on Pol I transcription (Fig. 2).

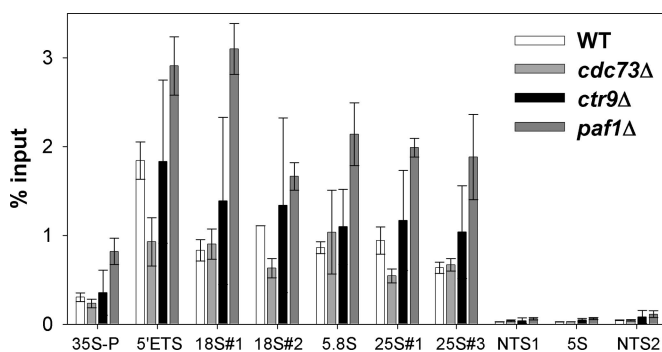


Fig. 3. ChIP analysis of Pol I occupancy of the rDNA in WT (NOY396), *cdc73Δ* (DAS511), *ctr9Δ* (DAS513), and *paf1Δ* (DAS516). The primer pairs used are diagrammed in Fig. 1A. Polyclonal anti-A190 antibody was used to immunoprecipitate Pol I. At least 2 10-fold dilutions of each sample were averaged from duplicate cultures. Error = ± 1 SD.

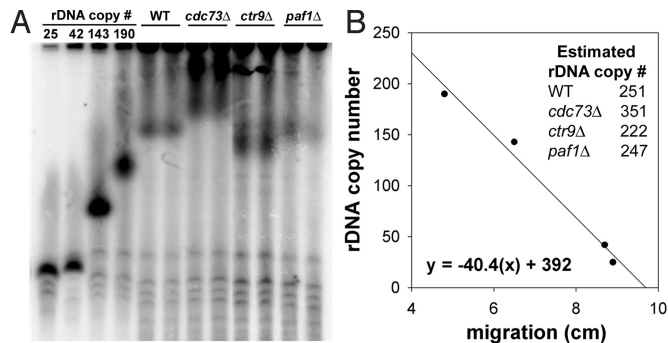


Fig. 4. rDNA copy number was measured by CHEF and Southern blot hybridization. (A) To visualize chromosome XII, we used a ^{32}P -labeled rDNA probe in Southern blot hybridization. Reference strains with known rDNA copy numbers (NOY1071, 25 copies; NOY886, 42 copies; NOY1051, 143 copies; and NOY1064, 190 copies) were included. DNA was isolated from 2 independent cultures of WT (NOY396), *cdc73Δ* (DAS511), *ctr9Δ* (DAS513), and *paf1Δ* (DAS516) and analyzed as described (see *Materials and Methods*). (B) A linear regression was generated by plotting copy number of reference strains versus the migration distance from the wells to the middle of the chromosome XII signal. From this regression, rDNA copy number was estimated for WT, *cdc73Δ*, *ctr9Δ*, and *paf1Δ*.

Taken together, the effects of Paf1C on Pol I transcription are independent of changes in processivity and number of engaged polymerases or rDNA copy number, which is consistent with Paf1C affecting the Pol I transcription elongation rate.

EM Analysis Revealed Impaired Transcription Elongation in *ctr9Δ* Cells.

To further test our model for Paf1C's role in Pol I transcription, we analyzed Pol I transcription *in vivo* by using EM analysis of Miller chromatin spreads of the *ctr9Δ* strain. EM analysis detected a relatively small decrease in the Pol I density per gene relative to WT (representative raw data in Fig. 5A). Quantification of these data showed that the average number of polymerases per gene was reduced 37% in the *ctr9Δ* strain compared with WT (Fig. 5B and C). Thus, the initiation rate per gene is lower in the mutant strain than in WT and the overall defect in transcription initiation is slightly greater than the net defect in transcription elongation (see *Discussion*).

Not all of the rDNA repeats are transcriptionally active in growing yeast cells (34). The fraction of active versus inactive rDNA genes was also quantified by EM. We found a 32% increase in the fraction of active rDNA genes in *ctr9Δ* strain relative to WT (Fig. 5B), thus offsetting the decrease in initiation rate per gene. From these data, we can calculate the average number of polymerases engaged in transcription in the WT and mutant cells [(rDNA copy number)(% active)(no. polys per gene)]. Based on this equation, there are $\approx 6,500$ engaged polymerases per WT cell and $\approx 5,800$ per *ctr9Δ* cell. Because the rRNA synthesis rate in the *ctr9Δ* cells is only 29% of the WT level, the net elongation rate of Pol I in *ctr9Δ* cells is 3.1-fold slower than in WT cells [fold defect in rRNA synthesis (100% divided by 29%) multiplied by the ratio of number of polymerases engaged in transcription in the mutant versus WT (5,800 divided by 6,500) = 3.1].

Processing of rRNA Is Impaired in *ctr9Δ* Cells.

Previous studies have suggested that transcription elongation by Pol I is functionally linked to rRNA processing (7, 11). Our EM analysis revealed defects in rRNA processing in *ctr9Δ* cells. The majority of pre-rRNAs in WT cells are initially processed at sites A_0 , A_1 , and A_2 to release the 20S precursor to 18S RNA. In all control yeast strains examined by EM, the separating cleavage at A_2 can be seen to occur cotranscriptionally as transcripts reach the 3' end of the gene (35). As shown in Fig. 5A, WT cells displayed this cotranscriptional cleavage event (bracketed within Fig. 5A *Top*). However, deletion

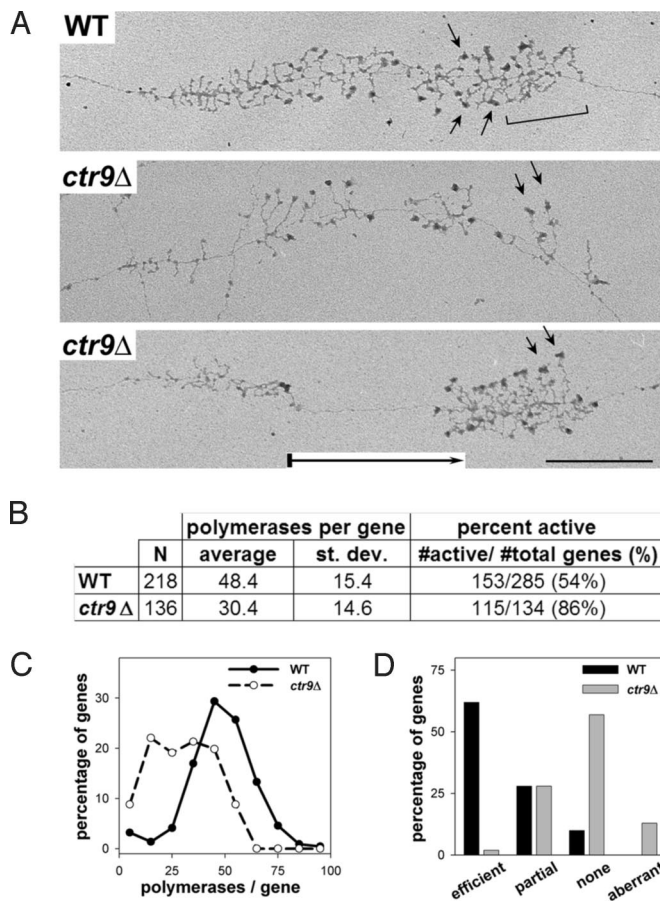


Fig. 5. EM analysis of Miller chromatin spreads reveals defects in Pol I transcription elongation and rRNA processing. (A) Representative rRNA genes from Miller spreads of WT (NOY396) and *ctr9Δ* (DAS513) cells. WT rRNA genes showed cleavage of nascent transcripts, indicated by a bracket (Top). Genes from *ctr9Δ* often exhibited polymerase-free gaps, indicated by a boxed arrow in the gene in Bottom. Arrows indicate uncleaved transcripts. (Scale bar: 0.5 μ M.) (B) Quantification of Pol I densities and percentage of active rDNA genes in WT and *ctr9Δ* strains. Error and size of dataset are indicated. (C) Percentage of genes with different polymerase occupancies is shown for WT and *ctr9Δ* strains. (D) Cotranscriptional cleavage efficiency was classified into 4 categories (efficient, partial, none, and aberrant) and plotted versus the frequency of detection.

of *CTR9* caused a reduction in the efficiency of this cleavage event compared with WT (Fig. 5 A and D), with $\approx 60\%$ of active rDNA genes lacking this normal cotranscriptional cleavage. This observation is consistent with published data from the Jaehning laboratory [showing accumulation of rRNA precursors in *paf1Δ* cells (25)]. Thus, these data are consistent with the model that the efficiency of transcription elongation by Pol I affects rRNA processing.

Polymerase-Free Gaps Are More Frequently Observed in *ctr9Δ* Cells Compared with WT. EM analysis also revealed large gaps between transcribing Pol I complexes in *ctr9Δ* mutant (Fig. 5A Bottom, indicated by boxed arrow). The percentage of genes with large polymerase-free gaps ($\geq 1/4$ of the gene length) was 36% in *ctr9Δ* ($n = 143$) compared with 12% in WT ($n = 302$). These gaps likely reflect sites of paused complexes. Increased susceptibility to pausing/arresting has been shown to correlate with slower elongation rates (36).

The positions of the 5' ends of these gaps were mapped to determine whether there was any evidence for specific sites that Pol I was less capable of clearing in Paf1C mutants (Fig. 6). The

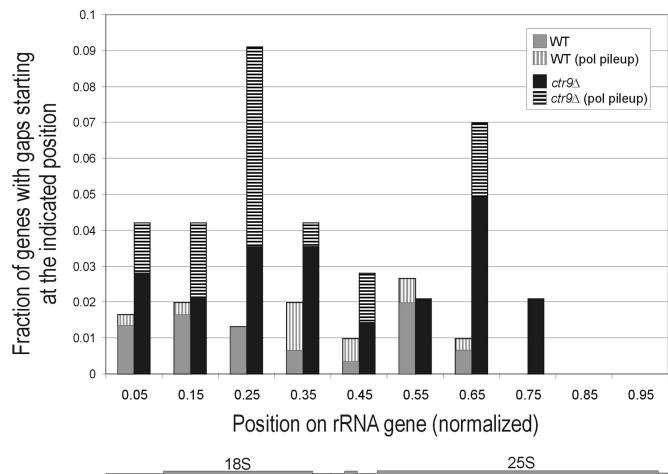


Fig. 6. Gaps between transcribing Pol I complexes are more frequently observed in *ctr9Δ* cells than in WT. Position of 5' ends of gaps (gap start sites) is shown on an rRNA gene normalized in length. Also indicated is the portion of gap start sites with polymerase pileups (see Materials and Methods). The absence of gap start sites in the 3' quarter of the gene is caused by the minimum length requirement ($1/4$ gene length) for gaps to be included in the analysis.

presence or absence of a polymerase pileup at the 5' end of the gap, perhaps caused by a roadblock effect, was also noted. This analysis showed that gap start sites were distributed across the gene in both WT and mutant strains. However, in *ctr9Δ*, there were 2 regions, 1 within the 18S RNA sequence and 1 within the 25S RNA sequence that had relatively more gap start sites. Because these gap sites were significantly more frequent in *ctr9Δ* cells, and a large portion of the gaps clearly involved polymerase blocks or pileups, we propose that Paf1C might play a specific role in transcribing through these regions of the gene.

Discussion

The data presented here demonstrate that subunits of Paf1C (Cdc73p, Ctr9p, and Paf1p) associate with the rDNA and contribute to the mechanism of Pol I transcription elongation in vivo. Deletion of *CDC73*, *CTR9*, or *PAF1* leads to reduced rRNA synthesis rates (Fig. 2), but Pol I occupancy of the rDNA is not reduced as severely (as observed using CHIP and EM; Figs. 3 and 5). The simplest interpretation of these data is that Paf1C plays an important, positive role in Pol I transcription elongation under normal growth conditions. Thus, we have identified an additional role for Paf1C in Pol I transcription in yeast, and this role may be conserved in higher eukaryotes as well.

In addition to defects in Pol I transcription rates in Paf1C mutant strains, we observed defects in rRNA processing. Cotranscriptional cleavage of the 35S pre-rRNA was significantly reduced (as observed with EM; Fig. 5), consistent with previous studies that detected accumulation of rRNA precursors in *paf1Δ* mutants (25). The observation of rRNA processing defects when Pol I transcription elongation is compromised supports the previously proposed model that transcription elongation by Pol I is coupled to rRNA processing (7).

Mechanism by Which Paf1C Influences Pol I Transcription. Genetic and biochemical studies have implicated Paf1C in Pol II transcription elongation, but the mechanism by which Paf1C affects Pol II remains controversial (15). Our ChIP and EM analyses showed that there was little (or no) change in Pol I occupancy of the rDNA or processivity in *cdc73Δ*, *ctr9Δ*, and *paf1Δ* strains compared with WT, despite large decreases in rRNA synthesis rates in the mutant strains. These data suggest that Paf1C positively influences Pol I transcription elongation in vivo.

Our data also indicate that transcription initiation by Pol I is directly or indirectly affected by Paf1C. Because the >3-fold reduction in Pol I elongation rate was not accompanied by a large increase in the polymerase density per gene, the initiation rate was also reduced. Indeed, in the *ctr9Δ* strain, polymerase density per gene was slightly lower than WT (Fig. 5), thus the initiation rate was affected slightly more than the elongation rate. It is not clear whether Paf1C directly affects the transcription initiation rate, or whether promoter clearance and early elongation events indirectly affect the assembly of the initiation complex.

From our EM data, we observed a 3-fold increase in the frequency of detection of gaps between Pol I complexes in the *ctr9Δ* cells compared with WT (Fig. 6). We propose that a major role for Paf1C in vivo is to directly increase the transcription elongation rate of Pol I through the rDNA, making the enzyme less prone to pause. Whether disruption of Paf1C affects the pausing of the transcription elongation complex at specific sites or whether a general decrease in the elongation rate of Pol I renders the complex more prone to pausing remains to be determined, although analysis of gap positions suggests contributions from both.

Immunofluorescence studies have shown that subunits of Paf1C can be detected in the nucleolus (25). However, those analyses suggested that the complex was largely absent from the nucleolus in WT cells, but enriched when the genes for Paf1 subunits (*PAF1* or *RTF1*) were deleted. Our ChIP analyses (Fig. 1) clearly show that Paf1C is in close proximity to the rDNA, even in growing WT cells.

The simplest mechanism we can propose accounting for all of the known data are that Paf1C directly associates with transcribing Pol I complexes and modulates its elongation efficiency. Although this is the simplest model, we cannot exclude more complicated indirect models by which Paf1C might affect Pol I transcription. For example, Paf1C recruits complexes that modify histones in transcribed regions of Pol II genes (19, 20). Thus, it is possible that Paf1C cooperates with other modulators of chromatin to facilitate Pol I transcription. Furthermore, because Paf1C mutations influence Pol II gene expression (37), we cannot exclude potential indirect effects of changes in the expression of other potential transcription elongation factors on Pol I transcription. Detailed in vitro analyses are required to clearly identify direct effects of Paf1C on Pol I transcription elongation.

Paf1C Affects the Chromatin Structure of Inactive rDNA. We observed an increase in the percentage of active rDNA repeats in the *ctr9Δ* strain (Fig. 5B) relative to WT. This observation may reflect a role for Ctr9p in maintenance of silent chromatin structure within the rDNA. Previous studies have shown that *PAF1* is required for efficient rDNA silencing (38). Both of these observations are consistent with the model that Paf1C directly or indirectly affects the maintenance of silent chromatin within the rDNA. The mechanism by which this occurs is a topic for future investigation.

Does Paf1C Regulate Pol I Transcription? Recent evidence suggests that transcription elongation by Pol I is regulated to control rRNA synthesis, but the mechanisms responsible for this regulation are largely unknown. The most detailed study of Pol I elongation as a regulatory target showed that the transcription factor UBF affects transcription elongation by Pol I in mammalian cells and that this effect on elongation was regulated in response to growth factor stimulation (6). The data presented here do not address whether Paf1C serves as a regulatory target for the control of rRNA synthesis. However, preliminary data suggest that deletion of *CTR9* or *PAF1* also leads to inappropriate regulation of Pol I transcription in yeast cells.

In higher eukaryotes, the homologues of Paf1C have been identified (39, 40). Overexpression of hPaf1 enhances cell growth and induces tumor formation; however, transient overexpression of HRPT-2 (the human homologue of *CDC73*) suppresses cell proliferation (40, 41). The involvement of Paf1C in dysregulation of cell

growth during cancer development suggests that Paf1C directly or indirectly contributes to regulation of ribosome synthesis in mammalian cells. Transcription elongation by Pol I as a target for the control of rRNA synthesis is a topic that is rapidly gaining interest and one that will require significant attention in future studies.

Relationship Between Spt4p and Paf1C at the rDNA. We have shown that both Spt4p/Spt5p and Paf1C affect Pol I transcription elongation in vivo, in addition to their known roles in Pol II transcription (11). It has been reported that Spt4p recruits Paf1p to transcribing Pol II complexes (17). Is it possible that Spt4p plays a similar role in Pol I transcription? Phenotypic data suggest this may not be the case. Deletion of *SPT4* had a modest effect on Pol I elongation (11). In contrast, deletion of *CTR9* or *PAF1* induces large decreases in the Pol I elongation rate and in overall cell growth rate compared with WT cells. Although both Spt4p/Spt5p and Paf1C contribute to Pol I transcription elongation, it seems that the two complexes function differently. However, more detailed experiments are required to determine the relative contributions of Paf1C and Spt4p/Spt5p to Pol I transcription in vivo and in vitro.

Conclusions

We have identified an important role for the conserved Paf1C in transcription elongation by Pol I. The importance of transcription elongation by Pol I to regulation of rRNA synthesis (6) and processing of rRNA (refs. 7 and 26 and this study) is becoming clear. However, by comparison to the list of factors known to influence transcription elongation by Pol II, very little is known about the control of Pol I elongation. Thus, many questions remain to be addressed regarding the mechanisms that control transcription elongation of Pol I through rDNA.

Materials and Methods

Strains and Media. Strains and plasmids used in this study are listed in Table 1. Cells were cultured in either yeast extract/peptone/dextrose or synthetic glucose complete (SD) media and were grown at 30 °C with aeration (4). Epitope tags [*his*₇-(HA)₃] were incorporated into Cdc73p, Ctr9p, and Paf1p by using standard methods (11).

ChIP. ChIP analysis was performed as described (11) except that cells were treated with formaldehyde for only 6 min to cross-link in vivo. ChIP signals were quantified by real-time PCR using an ABI 7900HT system (Applied Biosystems). Each reaction contained 1× GeneChoice buffer (GeneChoice), 200 μM dNTPs, 100 μg/ml BSA, 0.01× SYBR green (Invitrogen), 25 units/ml *Taq* polymerase (NEB), and 450 nM each primer. We note that our quantification was performed over at least a 10-fold range of input and IP DNA concentrations. Because this analysis takes into account the true precision of the experiment over a range of DNA concentrations, error bars are larger than would be expected for simple triplicate analysis.

Measurement of RNA Synthesis in Vivo. Duplicate cultures of WT, *cdc73Δ*, *ctr9Δ*, and *paf1Δ*, carrying pRS316 were grown in SD-Ura medium overnight to exponential phase (OD₆₀₀ ≈ 0.3). ³H-uridine (5 μCi) was mixed with 500 μL of each culture and incubated at 30 °C for 5 min with aeration. Then the samples were treated with 2.5 mL of 10% trichloroacetic acid (TCA) with 2.5 mg/ml of uridine. After filtration through nitrocellulose, each membrane was washed with 5% TCA, dried, and counted in a Beckman LS 6500 multipurpose scintillation counter.

To measure Pol I transcription rates, we grew cells in SD-met medium and pulse-labeled cells for 5 min with 20 μCi/ml ³H-methyl methionine (see also ref. 29). We followed that with a chase of cold methionine (500 μg/ml) for 5 min. RNA was prepared from these cells, run on a formaldehyde agarose gel, transferred to a membrane, and detected by autoradiography. Individual RNA species were excised from the blot (together with nearby regions of the blot for assessment of background) and were quantified with a scintillation counter.

rDNA Copy Number Determination. rDNA copy number was determined by using CHEF followed by Southern blot hybridization as described (31). Four reference strains (NOY886, NOY1051, NOY1064, and NOY1071) bearing known rDNA copy number were included. For Southern hybridization, an rDNA probe was amplified by using a forward primer (5'-GGGGCACCTGTCACTTTGGAAA-3') and a reverse primer (5'-GCTGATTTGAGAGGAGTTAC-3'). The resulting 600-bp probe

was then labeled with ^{32}P -dCTP (Redi Prime kit; GE Health U.K. Limited) and hybridized as described (31). A linear regression was constructed by plotting the migration distance of chromosome XII in known copy number reference strains versus the rDNA repeat number. From this regression, the average rDNA copy number was calculated for Paf1C deletion mutants and the WT control.

EM Analysis. EM Miller chromatin spreading analysis was performed as described (11). Polymerase-free gaps that were at least $\frac{1}{4}$ of the total gene length were analyzed by mapping their position within the gene. For each gap, it was also noted whether or not there were 3 or more very closely

positioned (apparently touching) polymerases at its 5' end, indicating a polymerase pileup. When the gaps were at the extreme 5' (or 3') end of the gene, the position of the gene start (or end) site was estimated by using the average length of neighboring genes.

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