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Molecular evidence indicating that the yeast PAF complex is required for transcription elongation

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PAF is a five-subunit protein complex composed of Paf1, Cdc73, Leo1, Rtf1 and Ctr9, which was purified from yeast in association with RNA polymerase II and which is believed to function in transcription elongation. However, no direct proof exists for this yet. To assay whether PAF is required in elongation, we determined the *in vitro* transcription-elongation efficiencies of mutant cell extracts using a DNA template containing two G-less cassettes. *paf1*Δ or *cdc73*Δ cell extracts showed reduced transcription-elongation efficiencies (16–18% of the wild-type levels), whereas *leo1*Δ and *rtf1*Δ showed wild-type levels. *In vivo* transcription efficiency was diminished in the four mutants analysed, as determined by their abilities to transcribe *lacZ*. Our work provides molecular evidence that PAF has a positive role in transcription elongation and is composed of at least two functionally different types of subunits (Paf1-Cdc73 and Leo1-Rtf1).

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INTRODUCTION

Eukaryotic RNA polymerase II (RNAPII)-mediated transcription is a highly coordinated process with three major steps: initiation, elongation and termination. During elongation, specific factors associate with RNAPII to help to overcome situations derived from transient pausing or to traverse through nucleosome structures (Hartzog, 2003; Shilatifard *et al*, 2003). Eukaryotic factors with a function in transcription elongation are TFIIS, TFIIF, FACT/Spt16-Pob3 and DSIF/Spt4-Spt5 found in yeast and humans as well as the yeast 19S subunit of the proteasome and human Elongin, 11–19 lysine-rich leukemia (ELL) and NELF (Shilatifard *et al*, 2003). However, there are a number of factors that associate with RNAPII *in vivo*, for which different experimental evidence suggests a function in transcription elongation, but no direct proof of that function exists yet. One such factor is the PAF complex from *Saccharomyces cerevisiae* (Krogan *et al*, 2002; Pokholok *et al*, 2002).

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PAF is a five-subunit complex containing Paf1, Cdc73, Ctr9, Rtf1 and Leo1 (Krogan *et al*, 2002; Mueller & Jaehning, 2002; Squazzo *et al*, 2002). Although initially implicated in transcription initiation, there is evidence suggesting a functional role for PAF in elongation. Thus, PAF is physically and genetically connected to the Spt4-Spt5 and Spt16-Pob3 elongation factors (Krogan *et al*, 2002; Squazzo *et al*, 2002) and localizes to transcriptionally active open reading frames, as determined by chromatin immunoprecipitation (ChIP) (Krogan *et al*, 2002; Pokholok *et al*, 2002). Also, deletions of the PAF genes confer 6-azauracil (6-AU) sensitivity in certain genetic backgrounds, a hallmark phenotype for mutations in transcription-elongation factors (Costa & Arndt, 2000; Krogan *et al*, 2002; Squazzo *et al*, 2002). Recently, PAF has been connected to histone H3 methylation. In active genes, the Lys 4 and Lys 36 residues of histone H3 are methylated by the action of the Set1 and Set2 methyltransferases, respectively (Briggs *et al*, 2001; Santos-Rosa *et al*, 2002; Strahl *et al*, 2002). It has been shown that the interaction of Set1 with RNAPII, its recruitment to actively transcribed chromatin and the Set1-mediated methylation of histone H3 are dependent on PAF (Krogan *et al*, 2003a; Ng *et al*, 2003). Similarly, *in vivo* histone methylation by Set2 is PAF-dependent (Krogan *et al*, 2003b).

Paf1 and Cdc73 were initially pulled down together with Hpr1 and Ccr4 as protein associated with RNAPII (Chang *et al*, 1999). Hpr1 is a component of the THO complex that functions at the interface between transcription elongation, mRNA metabolism and mRNA export, and which is required for efficient transcription elongation and for preventing transcription-associated genetic instability (Chávez *et al*, 2000; Rondón *et al*, 2003b). The observation that *paf1*Δ and *cdc73*Δ mutants had hyper-recombination phenotypes between direct repeats similar to THO mutants, although to a lesser extent, and that *paf1*Δ *hpr1*Δ double mutants are lethal (Chang *et al*, 1999) suggests *a priori* a functional relationship between the PAF and THO complexes.

In this study, we show that PAF is required for efficient transcription elongation, as determined by *in vitro* assays of whole-cell extracts (WCEs). Interestingly, whereas *paf1*Δ and *cdc73*Δ mutations strongly reduced transcription-elongation efficiency *in vitro*, *leo1*Δ and *rtf1*Δ had no effect. All mutants are impaired in *lacZ* transcription *in vivo*, the effect being stronger in *paf1*Δ and *cdc73*Δ mutants. This *in vivo* transcription defect was similar to that of the THO mutants, but, in contrast, was not

linked to hyper-recombination. Our work provides molecular evidence that PAF has a positive role in transcription elongation and is formed by two functionally different subunits (Paf1-Cdc73 and Rtf1-Leo1).

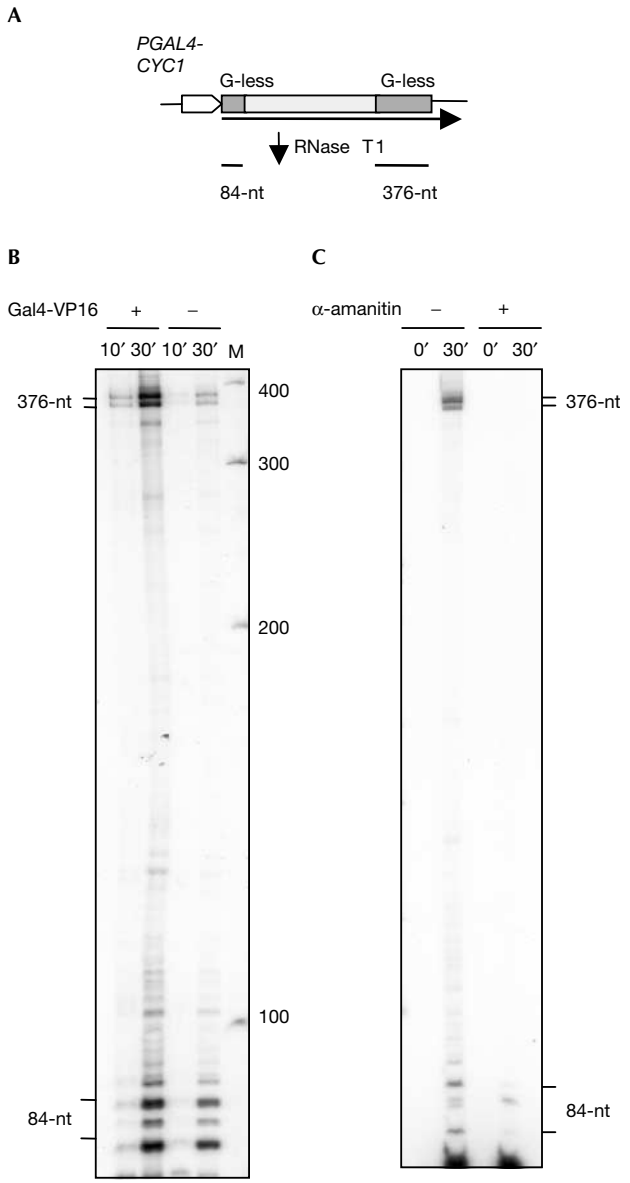

Fig 1 | Characterization of the *in vitro* transcription assays in wild-type WCEs. (**A**) Scheme of the double G-less cassette system used for the analysis of *in vitro* transcription elongation. RNase T1 treatment of the mRNA renders two fragments corresponding to the G-less cassettes. (**B**) *In vitro* transcription assay of WCEs from BY4741 (WT) with or without addition of the Gal4-VP16 activator to the reaction. Each reaction was stopped after 10 or 30 min, treated with RNase T1 and run in 6% PAGE. Two bands from each G-less cassette were obtained probably due to incomplete action of RNase T1. (**C**) *In vitro* transcription assay of WCEs from BY4741 (WT) treated or not with α-amanitin. Each reaction was stopped after 10 or 30 min. The 376- and 84-nt-long fragments produced by RNAPII are α-amanitin sensitive, but fragments below 84 nt are α-amanitin insensitive; therefore, they are not the result of RNAPII transcription.

RESULTS

Defective transcription in *paf1* and *cdc73* WCEs

To determine whether PAF has a positive role in transcription elongation, we used our newly reported *in vitro* assay for the analysis of transcription elongation (Rondón *et al*, 2003a). Briefly, the efficiency of transcription elongation of WCEs is assayed in a DNA template, carrying two G-less cassettes of different sizes (84 and 376 nt), which is transcribed *in vitro* from the hybrid *GAL4–CYC1* promoter. The 84-nt G-less cassette is immediately downstream of the promoter and the 376-nt cassette is 1.48 kb away from it. Treatment of the *in vitro*-produced transcript with RNase T1 degrades all G-containing mRNA sequences, rendering the two G-less cassettes intact (Fig 1A). Transcription-elongation efficiency is measured as the ratio between the 367- and 84-nt G-less RNA fragments.

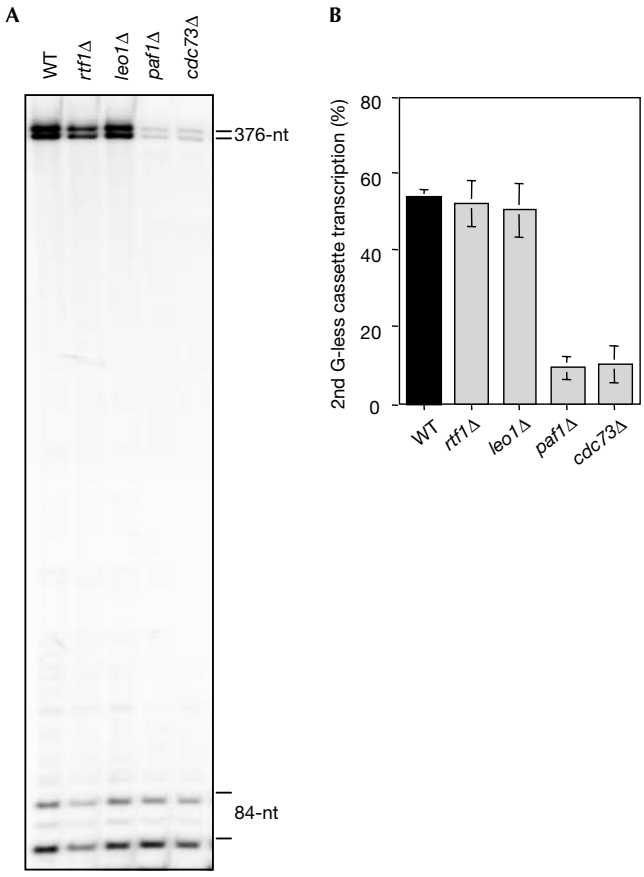

Fig 2 | *In vitro* transcription assays of wild-type and PAF mutant WCEs. (**A**) *In vitro* transcription assay of WCEs from BY4741 (WT), YGL244w (*rtf1*Δ), YOR123c (*leo1*Δ), YBR279w (*paf1*Δ) and YLR418c (*cdc73*Δ) strains. Each reaction was stopped after 30 min, treated with RNase T1 and run in 6% PAGE. **(B)** Efficiency of transcription elongation determined as the percentage of transcripts that reached the 376-nt G-less cassette versus those covering the 84-nt cassette. Bands were quantified in a Fuji FLA3000 and normalized with respect to their C content. The mean value and standard deviations of three independent experiments are shown.

We first confirmed that the transcripts detected in these assays initiated at the GAL4–CYC1 fusion promoter, by showing that both the 367- and 84-nt G-less RNA fragments accumulated more efficiently when exogenous Gal4-VP16 activator was added to the reaction (Fig 1B). In addition, we confirmed that these transcripts were made by RNAPII, because they did not accumulate in the presence of α-amanitin concentrations at which RNAPII is specifically inhibited (Fig 1C).

We assayed the transcription-elongation capacity of WCEs of the PAF null mutants, *paf1*Δ, *cdc73*Δ, *leo1*Δ and *rtf1*Δ. As can be seen in Fig 2, after 30 min, *paf1*Δ and *cdc73*Δ cell extracts fully transcribed the 376-nt G-less cassette with 16 and 18% of the efficiency of the wild type, respectively. However, *rtf1*Δ and *leo1*Δ mutants showed wild-type transcription-elongation efficiencies. This suggests that PAF could be functionally divided into two types of subunits. For further confirmation, we determined the kinetics of transcription elongation in time-course experiments in the mutants of one subunit of each class, *paf1*Δ and *rtf1*Δ. As shown in Fig 3, the transcription-elongation efficiency of *paf1*Δ was dramatically decreased, reaching 7–11% of the wild-type efficiency depending on the reaction time. However, the kinetics of transcription elongation was the same in *rtf1*Δ and wild-type cells, with only a slight decrease after 40 min of reaction, in which *rtf1*Δ efficiency was 73% of the wild type. Therefore, we can conclude that PAF is required for efficient transcription elongation and is formed at least by two functionally different subunits, Paf1-Cdc73 and Leo1-Rtf1.

PAF mutants show *in vivo* transcription defects

Next, we assayed the effect of PAF mutations on gene expression *in vivo*. We analysed the ability of *paf1*Δ, *cdc73*Δ, *rtf1*Δ and *leo1*Δ to transcribe *lacZ*- and *PHO5*-coding regions, which differ in length and GC content, driven from the *GAL1* promoter. In mutants showing elongation defects, such as *spt4*Δ and *hpr1*Δ, *lacZ* (long and GC-rich gene) is poorly transcribed whereas *PHO5* (short and low-GC gene) is transcribed at wild-type levels (Chávez *et al*, 2000; Rondón *et al*, 2003). As can be seen in Fig 4A, in galactose-containing media, β-galactosidase activity in *paf1*Δ and *cdc73*Δ was 19% of wild-type levels but 44–51% in *rtf1*Δ and *leo1*Δ mutants, whereas acid phosphatase activity was only slightly affected. The latter can reflect a partial defect in their ability to activate the *GAL1* promoter, as has been shown for *paf1*Δ (Shi *et al*, 1996). Northern analysis revealed that the accumulation of *lacZ* mRNA was strongly reduced in *paf1*Δ and *cdc73*Δ mutants and less strongly in *leo1*Δ and *rtf1*Δ mutants (Fig 4B), whereas *PHO5* transcription was either slightly decreased or unaffected in the PAF mutants tested (Fig 4B), consistent with the enzymatic analyses. Therefore, even

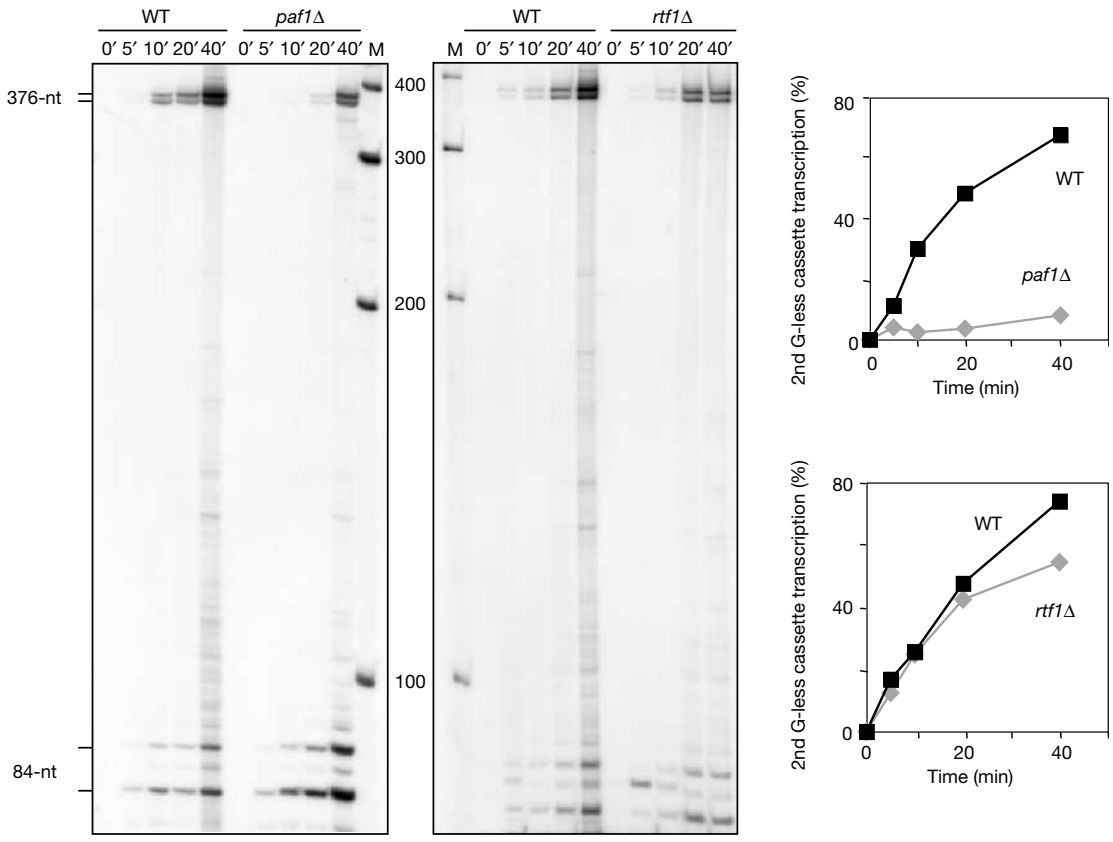

Fig 3 | *In vitro* assays of time-course transcription-elongation experiments in wild-type and PAF mutant WCEs. *In vitro* transcription assay of 100 μg of WCEs from wild-type BY4741 and isogenic *paf1*Δ and *rtf1*Δ strains. Reactions were stopped at 0, 5, 10, 20 and 40 min, treated with RNase T1 and run in 6% PAGE. Strains and other details are as in Fig 2.

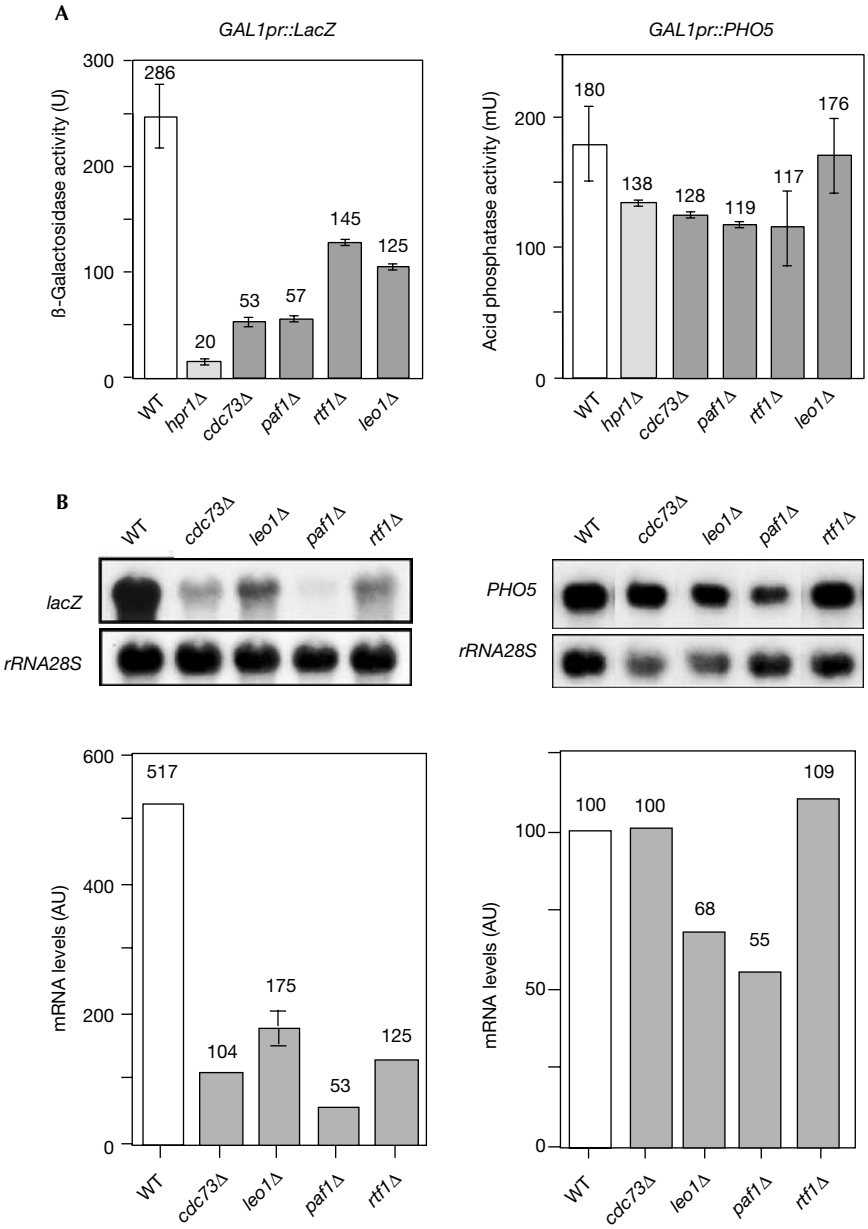

Fig 4 | Effect of PAF mutations on gene expression. **(A)** ß-Galactosidase and acid phosphatase activities of wild type and isogenic *paf1*Δ, *cdc73*Δ, *rtf1*Δ and *leo1*Δ transformed with p316*GAL1lacZ* and pSCh202 as compared to hpr1Δ. The mean and standard deviations of two induced cultures of different transformants are shown. No detectable activity was observed in repressed cultures (2% glucose). **(B)** Northern analysis of *lacZ* and *PHO5* mRNAs driven from the *GAL1* promoter. Mid-log-phase cultures were induced with 2% galactose, and samples were taken after 2 h. The 3 kb *Bam*HI–*Bgl*II *lacZ* fragment and an internal 589 bp 25S rRNA fragment obtained by PCR were used as ^{32}P-labelled DNA probes. Northern blots were quantified in a Fuji FLA 3000. RNA levels are given in arbitrary units (AU) normalized with respect to the rRNA levels of each sample. Strains are as in Fig 2.

though efficient *lacZ* transcription requires the entire PAF complex, Paf1 and Cdc73 have a more prominent role in its transcription.

To confirm that the low mRNA levels detected by northern analysis in the PAF mutants were not due to a higher decay of *lacZ* mRNA, we analysed the kinetics of degradation of the *lacZ* mRNA. As can be seen in Fig 5, degradation curves were similar in wild type and PAF mutants.

Paf-transcription defects are not hyper-recombinogenic

Mutants of PAF have similar transcription-elongation defects as THO mutants, but with different intensities. *paf1*Δ and *cdc73*Δ mutants also increased recombination in the *leu2-k::ADE2-URA3::leu2-k* direct-repeats system, though to a lesser extent than THO mutants (Chang *et al*, 1999). Consequently, we wondered whether the transcription-elongation impairment of PAF mutants led to transcription-dependent hyper-recombination.

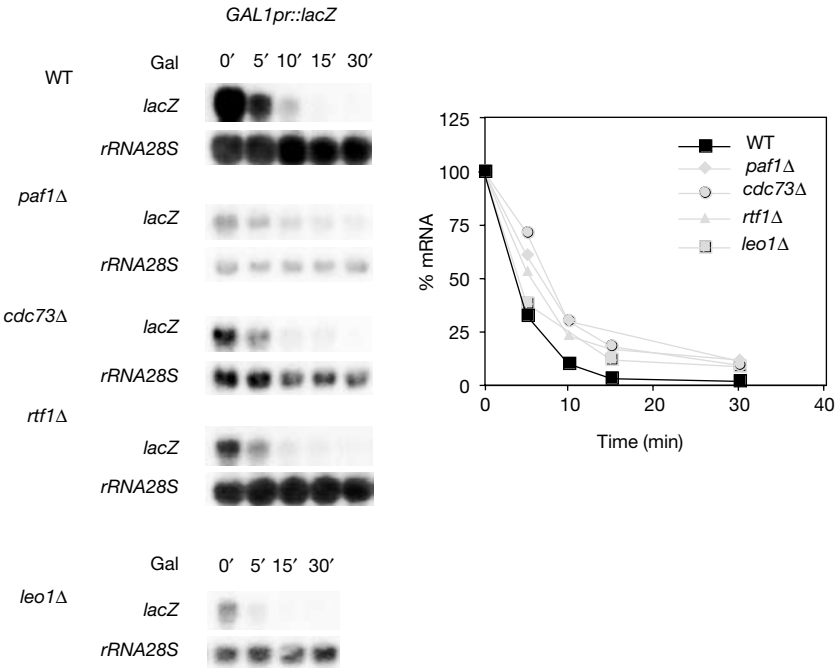

Fig 5 | Kinetics of degradation of *lacZ* mRNAs in PAF mutants. **(A)** Northern analysis of *lacZ* mRNA decay in wild-type BY4741 and isogenic *paf1*Δ, *cdc73*Δ, *rtf1*Δ and *leo1*Δ cells harbouring p416*GAL1lacZ*. Mid-log-phase cultures were grown in 2% galactose synthetic medium for 8 h and transferred to 2% glucose synthetic medium before samples were taken. Given the low initial levels of *lacZ* mRNA in *paf1* mutants, *paf1* data were normalized with respect to the steady-state background levels. Strains used and other details are as in Fig 4.

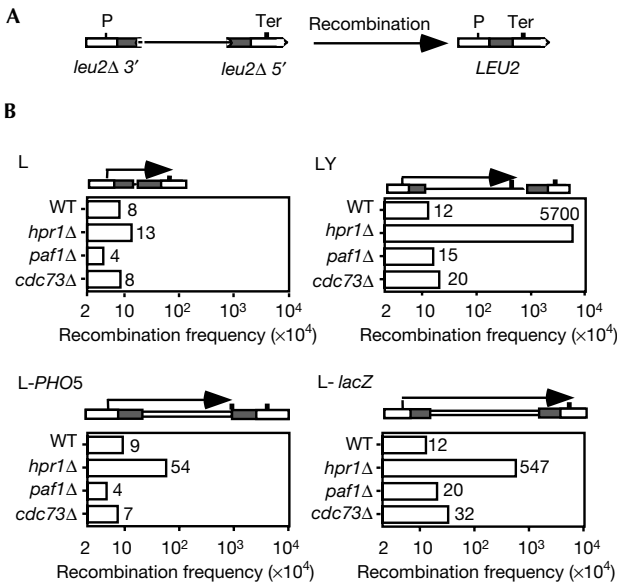

Fig 6 | Analysis of recombination of *paf1*Δ and *cdc73*Δ. **(A)** Scheme of the 0.6 kb *leu2*-based direct-repeat recombination assays used and the deletions resulting from recombination between the repeats. **(B)** Recombination frequencies of strains WMCD-1B (WT), WMCD-5A (*cdc73*Δ) and WMP-3C (*paf1*Δ) transformed with plasmids pRS314-L (L), pRS314-LY (LY), pSCh204 (L-*lacZ*) and pSCh206 (L-*PHO5*). In the scheme of each repeat system, the transcripts driven from the external *LEU2* promoter are indicated by arrows. The mean of 3–4 independent median frequencies, each obtained from six independent cultures, is given for each genotype.

We reasoned that if the hyper-recombination phenotypes of *paf1*Δ and *cdc73*Δ were linked to their transcription defects, these mutants should be hyper-recombinant for direct-repeat systems such as LY and L-*lacZ* but not for systems such as L and L-*PHO5* as was the case for THO mutants. LY and L-*lacZ* are based on two 0.6 kb *leu2* repeats flanking pBR322 and *lacZ*, whereas L and L-*PHO5* are based on the same repeats flanking either no sequence or *PHO5*. LY and L-*lacZ* are the only systems carrying intervening regions through which transcription is THO- or PAF-dependent. The recombination levels of *paf1*Δ and *cdc73*Δ mutants were the same in all systems analysed (Fig 6). The values were similar to wild type and different from the *hpr1*Δ mutant, in which recombination increased 475- and 45-fold in the LY and L-*lacZ* systems, respectively (Fig 4B; Chávez & Aguilera, 1997; Prado *et al*, 1997). Therefore, in contrast to THO mutants, the transcription defect and the previously reported hyper-recombination phenotypes of PAF mutants are not linked.

DISCUSSION

Our work provides molecular evidence that the PAF complex has a positive role in transcription elongation and is composed of at least two functionally different types of subunits.

Our *in vitro* assays showing that *paf1*Δ and *cdc73*Δ cell extracts have a reduced efficiency of transcription elongation (16–18% of the wild-type levels), whereas *leo1*Δ and *rtf1*Δ mutants are not affected, indicate that PAF is formed, in addition to the Ctr9 protein, by two types of subunits. The Paf1 and Cdc73 subunits would have a functional role in transcription elongation that can be detected *in vitro*, whereas Rtf1 and Leo1 would not. However, the observation that in addition to *paf1*Δ and *cdc73*Δ, the *leo1*Δ

and *rtf1*Δ mutants were also impaired in *in vivo lacZ* transcription, although to a lesser extent, suggests that Leo1 and Rtf1 could also have a role in transcription.

It is important to note that our *in vitro* assays are performed with naked DNA templates. Therefore, we cannot discard the possibility that Leo1 and Rtf1 could be required for elongation through chromatin. Indeed, PAF is necessary for Set1 recruitment to the RNAPII in actively transcribed chromatin and for Lys 79 and Lys 4 methylation of histone H3 by Dot1 and Set1, respectively (Krogan *et al*, 2003a; Ng *et al*, 2003). Furthermore, Set2, a methyltransferase of histone H3 Lys 36, needs Rtf1 and Cdc73 for recruitment to actively transcribed genes and for histone methylation (Krogan *et al*, 2003b). In addition, co-immunoprecipitation assays show interaction between PAF and Chd1, a highly conserved chromatin remodelling factor with ATPase-dependent remodelling activity *in vitro* (Simic *et al*, 2003). This interaction could be mediated by the Spt16-Pob3 complex to which PAF and Chd1 bind (Krogan *et al*, 2002). Furthermore, the absence of PAF is lethal in combination with mutants affected in chromatin assembly (Formosa *et al*, 2002). It is possible that PAF has a role in transcription elongation through chromatin. However, our *in vitro* analysis, performed with naked DNA templates, indicates that PAF has a functional role in elongation that is independent of chromatin and is mediated by Paf1-Cdc73 subunits.

There also exists the possibility that Leo1 and Rtf1 had a functional role modulating the transcriptional elongation activity of the complex in response to cell signalling (stress, cell cycle, etc.), which would not be detected *in vitro*. The PAF complex could be a general factor involved in elongation under specific growth conditions or a specific factor involved in elongation of particular genes, for example, cell cycle- or stress-regulated genes. In agreement with this idea, *paf1*Δ decreases the expression of only a small subset of genes, 30% of which are cell cycle regulated (Porter *et al*, 2002), and *paf1*Δ and *ctr9*Δ stop cell cycle progression before budding (Koch *et al*, 1999).

The transcription defects of *paf1*Δ and *cdc73*Δ have also been observed in THO mutants. Copurification of Paf1 and Cdc73 with Hpr1 and the observation that *paf1*Δ and *cdc73*Δ mutants are also hyper-recombinant (Chang *et al*, 1999), although to a lesser extent than THO mutants, could suggest that THO and PAF complexes have similar functions. However, our study shows that this is not the case. Whereas the *in vivo* transcription defects of THO mutants are tightly linked to an increase in recombination, this is not the case for PAF mutants (Fig 6B), indicating that mutants of the THO and PAF complexes cause different types of transcription-elongation defects. Whereas THO is functionally related to mRNA export and THO mutations are synthetic lethal in a *mex67-5* mRNA export mutant background (Jimeno *et al*, 2002), PAF is not related to mRNA export and *paf1*Δ *mex67-5* mutants are viable (data not shown). This is consistent with the absence of detectable subunits of PAF in purified THO complex in silver-stained SDS–PAGE (Chávez *et al*, 2000; Strasser *et al*, 2002). Nevertheless, copurification of Paf1, Cdc73 and Hpr1 in association with RNAPII (Chang *et al*, 1999) is consistent with the idea that both complexes function in actively transcribed chromatin (Krogan *et al*, 2002; Pokholok *et al*, 2002; Strasser *et al*, 2002).

In summary, PAF has a positive role in transcription elongation and is composed, in addition to the Ctr9 subunit, of two functionally different types of subunits (Paf1-Cdc73 and Leo1-Rtf1). Further molecular analysis should contribute to the understanding of the specific role of PAF in transcription.

MATERIALS AND METHODS

Yeast strains and plasmids. Yeast strains used were wild-type BY4741 and the isogenic mutants *paf1*Δ (YBR279w), *cdc73*Δ (YLR418c), *rtf1*Δ (YGL244w) and *leo1*Δ (YOR123c) (Euroscarf, Frankfurt). Strains WMCD-1B (WT), WMCD-5A (*cdc73*Δ) and WMP-3C (*paf1*Δ) were originated from genetic crosses between W303-1B and YLR418c or YBR279w. The plasmids used for expression analyses were p416*GAL1lacZ* (Mumberg *et al*, 1994) and pSCh202 (Chávez & Aguilera, 1997). For recombination assays we used pRS314-L, pRS314-LY, pSCh204 and pSCh206 (Chávez & Aguilera, 1997; Prado *et al*, 1997). Plasmid pRJRGAL4-VP16 (M. Ptashne, Sloan-Kettering Institute, New York) was used to purify the His_6-tagged Gal4-VP16 recombinant protein from *Escherichia coli*. As a template for the *in vitro* transcription-elongation assays we used plasmid pGCYC1-402 (Rondón *et al*, 2003a).

Preparation of yeast WCEs and *in vitro* transcription assays. These were performed as described (Rondón *et al*, 2003). For the inhibition of RNAPII, 5 ng/μl α-amanitin was added to the transcription reaction and preincubated at 30 °C for 15 min.

***In vivo* analysis of gene expression, mRNA decay and genetic recombination.** ß-Galactosidase and acid phosphatase activities, mRNA levels and recombination frequencies were determined as described (Chávez *et al*, 2000; Rondón *et al*, 2003b).

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