# Ctr9, Rtf1, and Leo1 Are Components of the Paf1/RNA Polymerase II Complex

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The *Saccharomyces cerevisiae* Paf1-RNA polymerase II (Pol II) complex is biochemically and functionally distinct from the Srb-mediator form of Pol II holoenzyme and is required for full expression of a subset of genes. In this work we have used tandem affinity purification tags to isolate the Paf1 complex and mass spectrometry to identify additional components. We have established that Ctr9, Rtf1, and Leo1 are factors that associate with Paf1, Cdc73, and Pol II, but not with the Srb-mediator. Deletion of either *PAF1* or *CTR9* leads to similar severe pleiotropic phenotypes, which are unaltered when the two mutations are combined. In contrast, we found that deletion of *LEO1* or *RTF1* leads to few obvious phenotypes, although mutation of *RTF1* suppresses mutations in TATA-binding protein, alters transcriptional start sites, and affects elongation. Remarkably, deletion of *LEO1* or *RTF1* suppresses many *paf1Δ* phenotypes. In particular, an *rtf1Δ paf1Δ* double mutant grew faster, was less temperature sensitive, and was more resistant to caffeine and hydroxyurea than a *paf1Δ* single mutant. In addition, expression of the $G_1$ cyclin *CLN1*, reduced nearly threefold in *paf1Δ*, is restored to wild-type levels in the *rtf1Δ paf1Δ* double mutant. We suggest that lack of Paf1 results in a defective complex and a block in transcription, which is relieved by removal of Leo1 or Rtf1.

RNA polymerase II (Pol II) requires both general transcription factors (GTFs) to form a stable initiation complex at promoters (reviewed in references 6, 24, and 60) and mediators and coactivators to communicate signals from transcriptional activators and repressors (reviewed in reference 36). Large, apparently preassembled forms of Pol II including many of these accessory factors may be the actual initiating forms of the enzyme in vivo. However, recent studies have demonstrated that mediator complexes may also function in the absence of Pol II (7, 3a, 40). The best characterized of these holoenzyme forms of Pol II is the Srb-mediator complex from *Saccharomyces cerevisiae* (reviewed in references 34 and 35). In addition to core Pol II, this complex contains the mediator, a collection of about 20 proteins including the Srb proteins, and the GTFs transcription factor IIB (TFIIB), TFIIF, TFIIE, and TFIIH. Complexes similar to the yeast Srb-mediator have also been described in multicellular organisms (reviewed in reference 13).

Our laboratory has isolated and characterized a Pol II complex from yeast containing Paf1, Cdc73, Hpr1, and Ccr4 that is biochemically distinct from the Srb-mediator complex (4, 47, 57). The Paf1 complex contains TFIIF and TFIIB and the coactivators Gal11 and Sin4 that are also present in the Srb-mediator complex. However, the Srb and Med proteins are not found in the Paf1 complex, and Paf1, Cdc73, Hpr1, and Ccr4 are not found with the proteins of the Srb-mediator complex. Although genes encoding Paf1 complex components are not essential, mutations in these genes exhibit a variety of phenotypes indicative of defects in gene expression (4, 47; J. L. Betz,

M. Chang, T. M. Washburn, S. E. Porter, and J. A. Jaehning, submitted for publication). Consistent with these phenotypes, mutation of Paf1 complex genes results in changes in abundance of a small but significant subset of yeast transcripts (4, 47; M. Chang, J. Fostel, and J. A. Jaehning, unpublished data). In contrast, many of the genes encoding Srb and Med proteins are essential (reviewed in reference 34), and loss of at least one of the essential Srb proteins (Srb4) results in diminished expression of most, if not all, yeast genes (18).

Transcripts differentially expressed in strains lacking Paf1 include cell wall biosynthetic genes (4) and many cell cycle-regulated genes (S. E. Porter, R. M. Washburn, M. Chang, and J. A. Jaehning, submitted for publication). Loss of Paf1, Cdc73, or Ccr4 results in cell wall integrity defects, and loss of Paf1, Cdc73, or Hpr1 is correlated with elevated rates of recombination between direct repeats (4). The fact that these two otherwise unrelated phenotypes are also observed for mutations in genes in the protein kinase C–mitogen-activated protein kinase signaling pathway led us to establish that the Paf1 complex plays an important role in the transmission of signals from Pkc1 to downstream target genes (4). Although Paf1 and Cdc73 have not been identified in other complexes, both Hpr1 and Ccr4 are present in other non-Pol II-containing complexes. Hpr1 is part of the Hpr1/THO complex that influences transcription and recombination (5), and Ccr4 is part of the Ccr4/Caf1/NOT complex that functions in both transcript synthesis and turnover (27, 28, 52).

Recently Koch and coworkers identified Paf1 and Cdc73 as factors associated with Ctr9, a protein required for full expression of the $G_1$ cyclin *CLN2* (22). Loss of Paf1 or Ctr9 leads to similar severe pleiotropic phenotypes, and loss of both genes does not result in an enhanced phenotype. These genetic and biochemical observations are consistent with the idea that Ctr9 and Paf1 function in the same pathways. To further character-

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ize the Paf1 complex, and to determine whether Ctr9 is also one of the Pol II-associated proteins in this complex, we have used the tandem affinity purification (TAP) (43) system to isolate the Paf1 complex. We have confirmed the presence of Ctr9 in the Pol II complex, and we have identified new components of the Paf1 complex, including Rtf1 and Leo1. The absence of these newly identified factors from the Srb-mediator complex further establishes the biochemical differences between these two forms of Pol II. Our observation that loss of Rtf1 or Leo1 suppresses many of the pleiotropic phenotypes caused by loss of Paf1 or Ctr9 has led us to a new model of the role of the Paf1 complex in transcription of yeast genes.

## MATERIALS AND METHODS

**Yeast strains, growth conditions, and genetic techniques.** The *S. cerevisiae* strains used in this study were derived from strain YJJ662 (*MAT***a** *leu2Δ1 his3Δ200 ura3-52*) (47) and are all isogenic except for the specific deletion or modification indicated: YJJ577 (*paf1Δ::HIS3*), YJJ1303 (*rtf1Δ::kan^r*), YJJ1326 (*rtf1Δ::kan^r paf1Δ::HIS3*), YJJ1336 (*leo1Δ::kan^r*), YJJ1339 (*trp1Δ::kan^r*), YJJ1361 (*leo1Δ::kan^r paf1Δ::HIS3*), and YJJ1364 (*ctr9Δ::kan^r paf1 Δ::HIS3*). Details of TAP-tagged and hemagglutinin (HA)-tagged strain construction are described below. YJJ1339 was used to create HA-tagged Leo1, resulting in strain YJJ1330 [*LEO1::LEO1^HA6 (KlTRP1)*]. The TAP-tagged strains were derived from YJJ662 and include YJJ1307 [*CDC73::CDC73^CBP-TEV-ProtA(KlURA3)*], YJJ1308 [*SRB5::SRB5^CBP-TEV-ProtA (KlURA3)*], YJJ1329 [*CTR9::CTR9^CBP-TEV-ProtA(KlURA3)*], and YJJ1334 [*HPR1::HPR1^CBP-TEV-ProtA(KlURA3)*]. Strains containing both Leo1-HA and TAP tags are YJJ1309 [*CDC73::CDC73^CBP-TEV-ProtA(KlURA3) LEO1::LEO1^HA6(KlTRP1)*], YJJ1331 [*SRB5::SRB5^CBP-TEV-ProtA(KlURA3) LEO1:: LEO1^HA6(KlTRP1)*], and YJJ1332 [*CTR9::CTR9^CBP-TEV-ProtA(KlURA3) LEO1:: LEO1^HA6(KlTRP1)*]. Strains were grown in YPD with 2% glucose using standard methods (15).

**Construction of TAP-tagged strains.** Yeast chromosomal genes *CDC73*, *SRB5*, *HPR1*, and *CTR9* were epitope tagged with a C-terminal TAP tag (43). The tagging cassette consists of two immunoglobulin G (IgG) binding domains of *Staphylococcus aureus* protein A and a calmodulin binding peptide separated by a tobacco etch virus (TEV) protease site. The *Kluyveromyces lactis URA3* gene located at the 3′ end of the tag served as a selectable marker. PCR fragments were generated using a 5′ oligonucleotide corresponding to the last 51 bp of the gene of interest, excluding the stop codon, plus 17 bp matching the TAP tag, and a 3′ oligonucleotide corresponding to the 51 bp downstream of the stop codon plus 17 bases of the tagging cassette. Oligonucleotide sequences are available upon request. The PCR fragments were transformed into strain YJJ662, generating strains YJJ1307 (Cdc73-TAP), YJJ1308 (Srb5-TAP), YJJ1329 (Ctr9-TAP), and YJJ1334 (Hpr1-TAP). TAP-tagged constructs were confirmed by Western blotting and PCR.

**Construction of Leo1-HA strains.** Leo1 was C-terminally epitope tagged using the pYM3 plasmid (19) containing a six-HA peptide and a *K. lactis TRP1* marker. To permit selection on synthetic complete medium lacking Trp, *TRP1* was deleted in YJJ662, using the KANMX4 cassette (54), resulting in YJJ1339. TAP-tagged constructs were generated as described above. The Leo1-HA PCR fragment was transformed into YJJ1339. The resulting strains containing Leo1-HA were YJJ1330 (Leo1-HA), YJJ1309 (Cdc73-TAP, Leo1-HA), YJJ1331 (Srb5-TAP, Leo1-HA), and YJJ1332 (Ctr9-TAP, Leo1-HA). HA-tagged constructs were confirmed by Western blotting and PCR.

**Extract preparation and purification of TAP-tagged complexes.** The TAP-tagged strains were grown in YPD medium to a density of $2 \times 10^7$ cells/ml, and transcriptionally active whole-cell extracts were prepared as described previously (56). Briefly, protein pellets were resuspended after $NH_4SO_4$ precipitation in buffer A (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 10 mM EGTA, 10 mM $MgSO_4$, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine hydrochloride, leupeptin [0.5 μg/ml], bestatin [0.35 μg/ml], pepstatin [0.4 μg/ml]) to a final protein concentration of 20 to 30 mg/ml. The resuspended proteins were extensively dialyzed against buffer A containing 100 mM $NH_4SO_4$. Approximately 1 g of total protein was incubated with 500 μl of rabbit IgG agarose (Sigma) at 4°C for 2 h. Beads were washed three times with 50 ml of buffer A and then equilibrated in TEV protease cleavage buffer (20 mM HEPES-KOH [pH 8.0], 10% glycerol, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 mM benzamidine hydrochloride, leupeptin [0.5 μg/ml], bestatin [0.35 μg/ml], pepstatin [0.4 μg/ml]). Proteins were eluted from the beads by adding 200 U of

TEV protease in 1 ml of TEV protease cleavage buffer and incubating the mixture at ambient temperature for 2 h. The eluted proteins were concentrated with a Centricon YM-30 filter (Millipore) and applied at 0.2 ml/min to a Superose 6 HR 10/30 (Pharmacia) gel filtration column equilibrated in buffer B [30 mM HEPES-KOH (pH 7.9), 8% glycerol, 2 mM EDTA, 2 mM EGTA, 0.1 M $(NH_4)_2SO_4$, 0.05% NP-40, 5 mM β-glycerophosphate, 1 mM DTT, 1 mM PMSF, 2 mM benzamidine hydrochloride, leupeptin (0.5 μg/ml), bestatin (0.35 μg/ml), pepstatin (0.4 μg/ml)]. Fractions (200 μl) were collected. Fractions were trichloroacetic acid (TCA) precipitated (38) and analyzed by Western blotting. Transcriptional activity was measured by a nonselective transcription assay as described elsewhere (58). To estimate the size of protein complexes, the following markers were used to calibrate the Superose 6 column: aggregated MCM protein (>2,000 kDa; gift from X. Chen, University of Colorado Health Sciences Center), thyroglobulin (669 kDa), apoferritin (443 kDa), and β-amylase (200 kDa).

**Isolation of Leo1-HA complexes.** The C-terminally Leo1 HA-tagged strains were grown in 100 ml of YPD to a density of $1.4 \times 10^7$ cells/ml. Cells were pelleted and washed in sterile water, freeze-thawed in liquid nitrogen, and then resuspended in 1.0 ml of whole-cell extract lysis buffer [0.2 M Tris-HCl (pH 7.9), 0.39 M $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 20% (vol/vol) glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, plus protease inhibitors as above]. Cells were lysed with the addition of 1 ml of glass beads and vortexed four times for 1 min while cooling. Lysates were clarified at 13,000 rpm with a Baxter Biofuge 13 for 20 min at 4°C. Protein concentrations were determined by Bradford assay (GibcoBRL). Approximately 1.5 mg of total protein was used for TAP tag purification or immunoprecipitation. TAP-tagged extracts were incubated with 25 μl of rabbit IgG agarose at 4°C for 2 h. Beads, equilibrated in TEV protease cleavage buffer, were washed three times with 1 ml of buffer B. Proteins were eluted from the beads with the addition of 25 U of TEV protease in 50 μl of TEV protease cleavage buffer. Eluates were TCA precipitated and analyzed by Western blotting.

Strains YJJ662 and YJJ1330 were used for immunoprecipitation studies. To reduce the binding of nonspecific proteins to the protein A-coupled beads, extracts were preincubated with 25 μl of Sepharose 4B (Sigma) for 1 h at 4°C. The beads were removed, 1 μl of 12CA5 antibody (Roche) was added, and extracts were incubated at 4°C for 1 h. To isolate Leo1-HA complexes, protein A immobilized on Sepharose 4B (Sigma) was added to the extract. After binding for 1 h at 4°C, the beads were pelleted and washed four times with buffer B. Beads were suspended in 20 μl of NuPAGE LDS Sample buffer (Invitrogen) and heated to 70°C for 10 min, and aliquots were resolved on a 4-to-12% Bis-Tris NuPAGE gel (Invitrogen).

**Western blotting.** Proteins were resolved on a 4 to 12% bis-Tris NuPAGE gel and then transferred to a polyvinylidene difluoride (Millipore) membrane. Proteins were detected with the following primary antibodies: anti-Rpb1 (8WG16; D. Bentley); anti-Cdc73, anti-Paf1, and anti-TFIIB (X. Shi); anti-Srb5 (R. Young); anti-HA (Roche); anti-Spt5 (G. Hartzog); anti-Rtf1 (K. Arndt); and anti-Rpt5 and anti-Rpt6 (Sug1) (R. Deshaies). Horseradish peroxidase-conjugated antibody was the secondary antibody, and proteins were detected using a Chemiluminescent Reagent Plus kit from NEN.

**Mass spectrometry analysis.** Fractions from the Superose 6 gel filtration column were pooled, TCA precipitated, and resolved on a 4-to-12% Bis-Tris NuPAGE gel. Proteins were visualized by staining with NOVEX colloidal blue. Protein bands were excised and processed as described previously (25). Briefly, samples were reduced and alkylated by incubating with 2.8 mM DTT in 100 mM $NH_4CO_3$ at 60°C for 15 min, followed by the addition of 10 μl of 100 mM iodoacetamide. After a 15-min incubation at ambient temperature in the dark, the gel slices were washed with 100 μl of 50% acetonitrile in 50 mM $NH_4CO_3$ with shaking for 20 min. To dehydrate the gel slices, 100 μl of 100% acetonitrile was added and incubated for 10 min. The solvent was removed, and the gel pieces were completely dried in a vacuum centrifuge. The gel pieces were swollen with the addition of 0.2 μg of modified trypsin (Promega) in 10 μl of 25 mM $NH_4CO_3$. The gel pieces were kept wet during enzymatic cleavage (37°C, overnight) with 25 mM $NH_4CO_3$. The supernatant containing the digested peptides was collected, and the remaining peptides were extracted from the gel twice with 20 μl of 1% trifluoroacetic acid. All supernatants were pooled and dried in a vacuum centrifuge. The sample was reconstituted in 3 to 5 μl of 1% trifluoroacetic acid and desalted using C18 Ziptips (Millipore). Samples were processed on a PE-Biosystems Voyager DE-PRO matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectrometer. The masses of the tryptic peptides were used to search for protein candidates using the programs Protein Prospector and ProFound. Up to two undigested trypsin sites and a mass tolerance of ±1.0 Da were allowed.

**RNA isolation and analysis.** Cells were grown to a density of $1.3 \times 10^7$ cells per ml, and RNA was isolated as described (4). Fifteen micrograms of total RNA

per sample was fractionated by formaldehyde gel electrophoresis according to standard methods (33). RNA was transferred to ZetaProbe GT membranes (Bio-Rad). The *CLN1* probe, a gel-purified 500-bp fragment from a plasmid which contains the 1.2-kb *CLN1* coding region (R. Sclafani), was labeled using the random prime method (RadPrime kit; GibcoBRL). The 18S rRNA oligonucleotide probe (5′-GCTTATACTTAGACATGCAT-3′) was 5′ end labeled. Hybridizations and washes were performed at either 64°C for the *CLN1* probe or at 41°C for the 18S rRNA probe. Blots were exposed to a phosphorimaging screen, and bands were quantitated using Quanity One imaging software. Signals were normalized to 18S rRNA.

## RESULTS

**Affinity purification of the Paf1 complex.** Previous studies of the Paf1 complex used affinity purification techniques to establish the connection to Pol II and to identify some of the components of the complex. Paf1 and Cdc73 were originally identified as RNA Pol II-associated proteins using a monoclonal antibody directed against the C-terminal domain (CTD) of Pol II to isolate the enzyme from yeast transcription extracts (57). The RNA Pol II-associated proteins also included GTFs TFIIF and TFIIB. Subsequently, GST-tagged forms of Paf1, Cdc73, and TFIIF were used to demonstrate that the complex is distinct from the Srb-mediator form of Pol II and that Hpr1 and Ccr4 are also associated with the complex (47). Although the plasmid-borne GST-tagged constructs functionally complemented the genes they replaced (47), they were overexpressed relative to the natural chromosomal copies of the genes. In addition, dimerization of the GST tag and associated proteins (32, 41) causes difficulties in determining the size of the complex. Therefore, to further characterize the composition and function of the Paf1 complex, and to compare it to the Srb-mediator complex, we epitope-tagged the chromosomal copies of *CDC73* and *SRB5* with a TAP tag (43), as described in Materials and Methods. The TAP tag includes two IgG binding domains and a calmodulin binding peptide separated by a TEV protease site. This C-terminal tag should not alter the normal expression of the tagged genes from their native promoter. Consistent with this supposition, we found that the TAP-tagged strains did not display any of the pleiotropic phenotypes (including slow growth and temperature sensitivity) found for deletion mutants of these genes (data not shown).

Yeast whole-cell transcription extracts were prepared from the tagged strains, the TAP-tagged constructs were isolated by binding to IgG agarose, and bound proteins were eluted by TEV protease digestion. We found that both the Cdc73-tagged Paf1 complex and the Srb5-tagged Srb-mediator complex could be isolated by this procedure (see below) but that neither complex was stable to subsequent purification on calmodulin beads. Apparently the interactions with Pol II are not stable to the second stringent affinity step, although they are maintained under the high-salt (100 mM NH$_4$SO$_4$) conditions used for binding to the IgG agarose. Figure 1 shows the results from the IgG agarose isolation of proteins associated with TAP-tagged Cdc73 and Srb5. The input, flowthrough, and eluate fractions from both preparations were probed with antibodies to the largest subunit of Pol II (αRpb1), Paf1 (αPaf1), and Srb5 (αSrb5). Pol II is clearly associated with both tagged proteins, Paf1 is only present in the TAP-tagged Cdc73 eluate, and Srb5 (in its tagged form) is uniquely found in the TAP-tagged Srb5 eluate (Fig. 1). These results establish that the TAP tag pro-
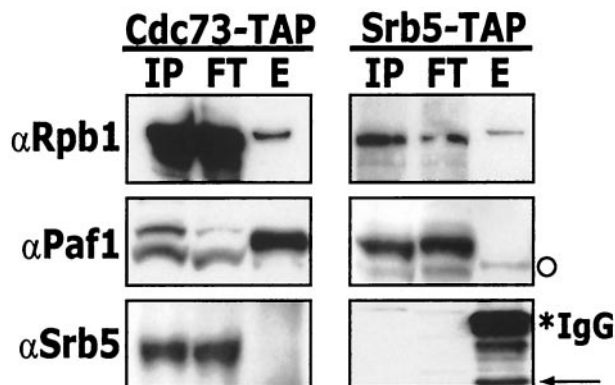


FIG. 1. The Paf1 complex is distinct from the Srb-Med complex. Whole-cell extracts made from a TAP-tagged Cdc73 strain (YJJ1307) and a TAP-tagged Srb5 strain (YJJ1308) were incubated with rabbit IgG agarose. Unbound proteins were washed away, and proteins that associate with TAP-Cdc73 were eluted by the addition of TEV protease. Fractions (IP, input; FT, flowthrough; E, eluate) were loaded onto a 4-to-12% NuPAGE gel and analyzed by Western blotting with antibodies as indicated. The asterisk indicates cross-reacting IgG from the IgG agarose column, and the arrow indicates the TEV protease cleaved form of TAP-Srb5. We observe two cross-reacting bands with the α-Paf1 antibody (see also Fig. 5B and reference 47); however, the faint band indicated by the open circle in the TAP-tagged Srb5 eluate does not correspond to either form of Paf1, and its presence is not reproducible (see Fig. 5B).

tocol specifically and efficiently isolates distinct Pol II complexes from tagged strains.

To further purify the Paf1 complex it was fractionated by chromatography on a Superose 6 gel filtration column (Fig. 2). The elution profile of Paf1 complex components was monitored by immunoblotting using the antibodies indicated in Fig. 2A. A high-molecular-mass complex containing Pol II, Paf1, Cdc73, and TFIIB, but not Srb5, was observed eluting from the column at approximately 1.7 MDa. Cdc73 is present in the high-molecular-mass complex and also in an intermediate position overlapping with a small amount of Pol II. Since Cdc73 is known to bind Pol II directly (47), this may represent a subcomplex of the larger Paf1 complex. We also observed significant amounts of free core Pol II in the lower-molecular-mass fractions (~600 kDa), a position in the fractionation that also contained proteolyzed forms of Paf1 (Fig. 2, see also below). When the TAP-tagged Srb5 complex was fractionated on Superose 6 under identical conditions, we found that all of the Pol II was in the lower-molecular-mass position of free core, indicating that the Paf1 complex may be somewhat more stable to purification than the Srb-mediator complex.

In addition to the antibody identification of Pol II, we also performed a nonspecific transcriptional assay for RNA polymerase activity on the Superose 6 fractions. Transcriptional activity was detected in fractions 39 to 47 corresponding to the high-molecular-mass Paf1 complex and also in fractions 69 to 73 corresponding to core Pol II (data not shown). Similar nonselective transcription assays performed on GST-tagged constructs (see reference 47) revealed that approximately 2% of the total Pol II was associated with the Paf1 complex (data not shown).

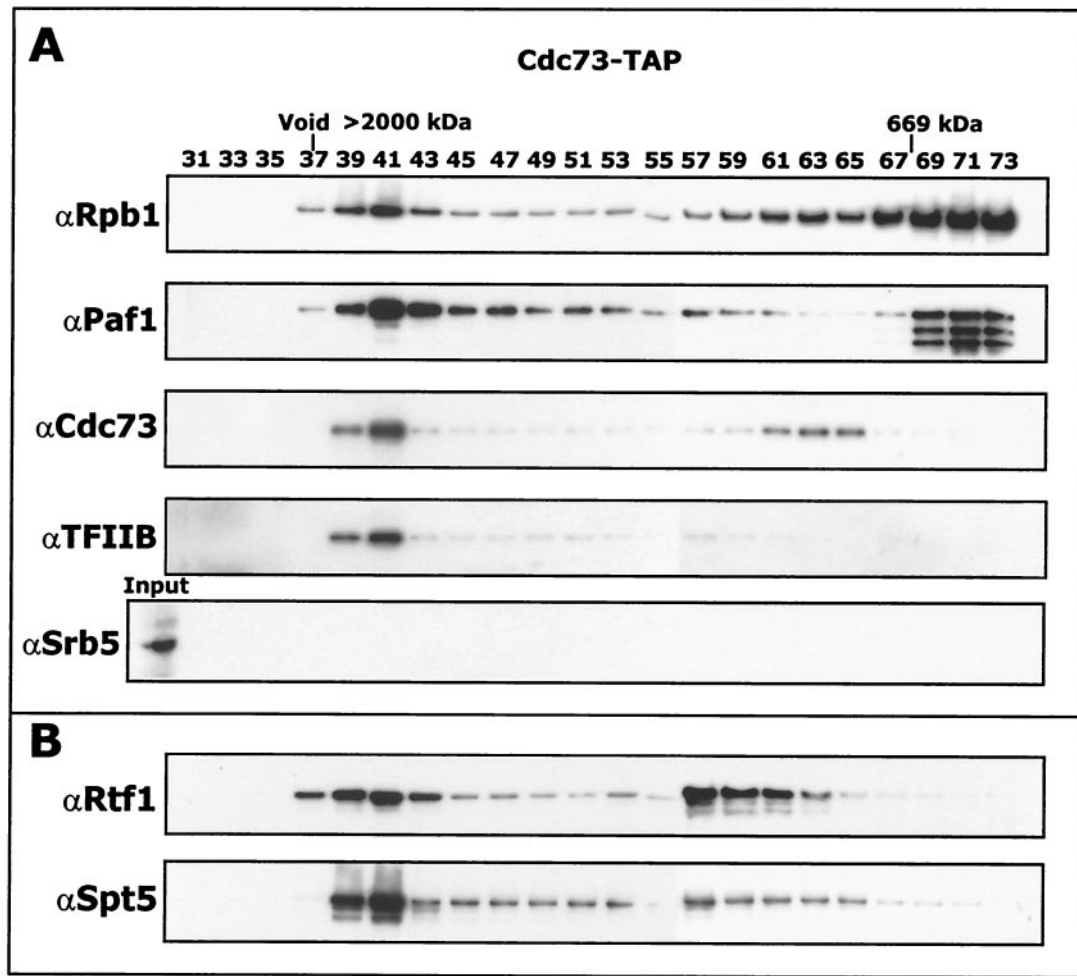**Identification of Paf1 complex components by mass spec-**

FIG. 2. The Paf1 complex is approximately 1.7 MDa. Proteins that associate with Cdc73-TAP after chromatography on IgG agarose and elution with TEV protease were subjected to gel filtration chromatography on a Superose 6 gel filtration column. Fractions were separated on a 4-to-12% NuPAGE gel and analyzed by Western blotting with the indicated antibodies. Sizes were determined by calibrating the gel filtration column with the indicated standards as described in Materials and Methods.

trometry. To identify proteins that associate with the 1.7-MDa TAP-tagged Cdc73 complex, we performed mass spectrometry on individual protein bands excised from a sodium dodecyl sulfate (SDS) gel (Fig. 3). Peptides from the trypsin-digested proteins were eluted and analyzed by MALDI-TOF mass spectrometry as described in Materials and Methods. The tryptic peptide masses were used to search for protein candidates in several Web-based data sets (ProFound, Protein Prospector, and MASCOT). Identification was considered positive if the top candidate had a probability score near 1.0 and the second-best candidate had a significantly lower probability. Proteins that were identified in this way are marked in Fig. 3. Newly identified proteins of the Paf1 complex include Ctr9, Rtf1, and Leo1. The ribosomal proteins identified in Fig. 3 are nonspecific contaminants in the high-molecular-mass fractions, as also observed by others isolating yeast complexes (28, 29, 45). A few peptides corresponding to Spt5 were also identified in the same gel band that contained Rpb2 and Ctr9, but the lower probability score precluded a confident match (indicated by the parentheses in Fig. 3). We also analyzed the proteins in the

lower-molecular-mass fractions containing free Pol II (Fig. 3, 580 to 670 kDa). We were able to unambiguously identify most of the Pol II subunits, plus Leo1 and both intact and proteolyzed forms of Paf1.

**Validation of newly identified Paf1 complex factors.** To confirm the identifications from mass spectrometry, we showed by Western blotting that Rtf1 and Spt5 were present in the same high-molecular-mass complex with Pol II and Paf1 (Fig. 2B). In addition, we observed Rtf1 and Spt5 in an intermediate fraction between the Pol II complex and free core Pol II, suggesting that they might exist in a separate subcomplex.

The Koch laboratory described an association between Ctr9 and Paf1 and Cdc73 but did not establish a connection to Pol II (22). To confirm that Ctr9 is part of the Paf1 complex, we constructed a strain bearing a TAP-tagged form of Ctr9 and confirmed that the tagged strain had a wild-type phenotype. We isolated TAP-tagged Ctr9-associated proteins as described above for TAP-tagged Cdc73. Fractions from the Superose 6 gel filtration column were immunoblotted with antibodies specific for Pol II and Paf1 (Fig. 4A). Both Paf1 and Pol II were
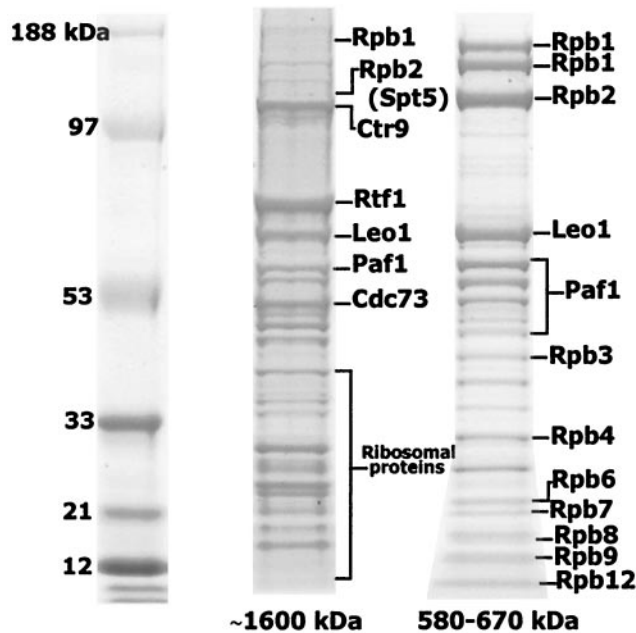
FIG. 3. Ctr9, Rtf1, Leo1, and Spt5 associate with the Paf1 complex. Fractions 37 to 41 and 69 to 73 from the Superose 6 gel filtration column were pooled, TCA precipitated, and separated on a 4-to-12% NuPAGE gel. Protein bands were visualized by colloidal blue staining and processed for MALDI-TOF analysis. Proteins that were accurately identified are marked.

found in these fractions, again in the high-molecular-mass complex and at the position of free core Pol II. Based on these observations, and the mass spectrometry identification, we conclude that Ctr9 is part of the Paf1 complex.

We have previously used a GST-tagged form of Hpr1 to establish that this protein is associated with Pol II and Paf1 (4). Because Hpr1 has also been found in another, non-Pol II-containing complex, the Hpr1/Tho complex (5), we confirmed the Pol II association by analyzing proteins associated with TAP-tagged Hpr1. The comigration of Pol II, Paf1, and Hpr1 in the high-molecular-mass fractions of the Superose 6 column (Fig. 4B) reaffirms that Hpr1 associates with the Paf1 complex.

There is little information about Leo1 other than the fact that it is a very hydrophilic protein encoded by a nonessential gene (30). We therefore constructed strains containing chromosomally HA-tagged Leo1 in wild-type and in various TAP-tagged backgrounds (Materials and Methods). Using an anti-HA antibody to precipitate HA-tagged Leo1 we found both Paf1 and Rtf1, but not Srb5, in the bound fraction (Fig. 5A). However, although Paf1 was not present in the precipitate from the untagged strain, a small amount of Rtf1 was detected. We have observed low levels of nonspecific association of Rtf1 in many of the different affinity assays performed in these studies (see also below); however, the specific association of Rtf1 is clearly apparent in Fig. 5A over this background. To confirm that Leo1 associates specifically with the Paf1 complex, we used double-tagged strains (TAP tags plus Leo1-HA), first isolating the complexes with the TAP tag and then probing for the presence or absence of HA-tagged Leo1 (Fig. 5B). We found that Leo1, like Paf1 and Rtf1, associates with TAP-

tagged Cdc73 and Ctr9, but not with TAP-tagged Srb5. These results confirm that Leo1 is part of the Paf1 complex and is not part of the Srb-mediator.

**Proteasome components are not part of the Paf1 complex.** Paf1, Ctr9, Rtf1, and Leo1 were recently reported to associate with the 19S cap of the proteasome in the absence of ATP (53). However, we did not identify any proteasome components in the mass spectrometry analysis of the Paf1 complex. In addition, when we used antibodies specific to 19S proteasome components Sug1 and Rpt1 to probe TAP-tagged Cdc73 fractions, no proteasome components were found associated with the Paf1 complex (Fig. 6). The reported association of the Paf1 complex with the proteasome may therefore be an artifact of the affinity isolation used in these earlier studies. In particular, the nonspecific association of Rtf1 with many affinity matrices noted above (Fig. 5) may have led to these observations. Using antibodies directed against components of several other high-molecular-mass yeast complexes, we have found no evidence for the presence of Spt6, Bur2, Snf12, or Caf1 in the purified Paf1 complex (data not shown).

**Genetic interactions between *PAF1*, *RTF1*, and *LEO1*.** We have previously reported that although *PAF1* is not an essential gene, its loss results in many phenotypic changes, including slow growth; increased sensitivity to low and high temperature, cell wall-damaging agents, and hydroxyurea; and abnormal morphology relative to wild-type cells (4, 48; Betz et al., submitted). Deletion of *CTR9* results in an identical spectrum of phenotypes (22; Betz et al., submitted). Loss of *CDC73* results in much more modest phenotypic changes that are in general a subset of those seen for *paf1Δ* (4, 47; Betz et al., submitted). Combining a *PAF1* deletion with deletions of *CDC73* or *CTR9* does not result in any phenotypic enhancement (22, 47), consistent with the fact that they function in the same cellular processes. If Rtf1 and Leo1 are also involved in the same cellular process, then combining these mutations with mutations in other Paf1 complex components should not result in an enhanced phenotype (14). We therefore constructed *leo1Δ* and *rtf1Δ* mutants and used these to create double mutants by mating to an appropriate isogenic *paf1Δ* strain. The diploids were sporulated, and tetrads were dissected and tested for genotype and phenotype.

A point mutation in *RTF1* was originally characterized as a suppressor of a mutation in TATA-binding protein (TBP) (48). Deletion of *RTF1* also suppresses the same allele of TBP, and both the point mutation and the deletion result in alterations in transcriptional start sites. We found that *rtf1Δ* strains grew a little more slowly than wild type at 30°C in rich media (Fig. 7A) (doubling times, 97 versus 80 min) and were slightly temperature sensitive at 36°C (Fig. 7B). However, in contrast with a recent report (8), we observed that loss of Rtf1 in our yeast genetic background did not result in sensitivity to 6-azauracil (6AU) (data not shown). When we analyzed several *rtf1Δ paf1Δ* double mutants, we found that loss of Rtf1 dramatically suppressed many *paf1Δ* phenotypes. An *rtf1Δ paf1Δ* double mutant grew significantly faster than *paf1Δ* in rich media at 30°C (Fig. 7A) (doubling times of 145 versus 175 min), although more slowly than *rtf1Δ*. In addition, even though *rtf1Δ* alone is slightly temperature sensitive, the mutation suppressed the temperature-sensitive phenotype of *paf1Δ* (Fig. 7B). Loss of Rtf1 also restored growth of *paf1Δ* on caffeine and
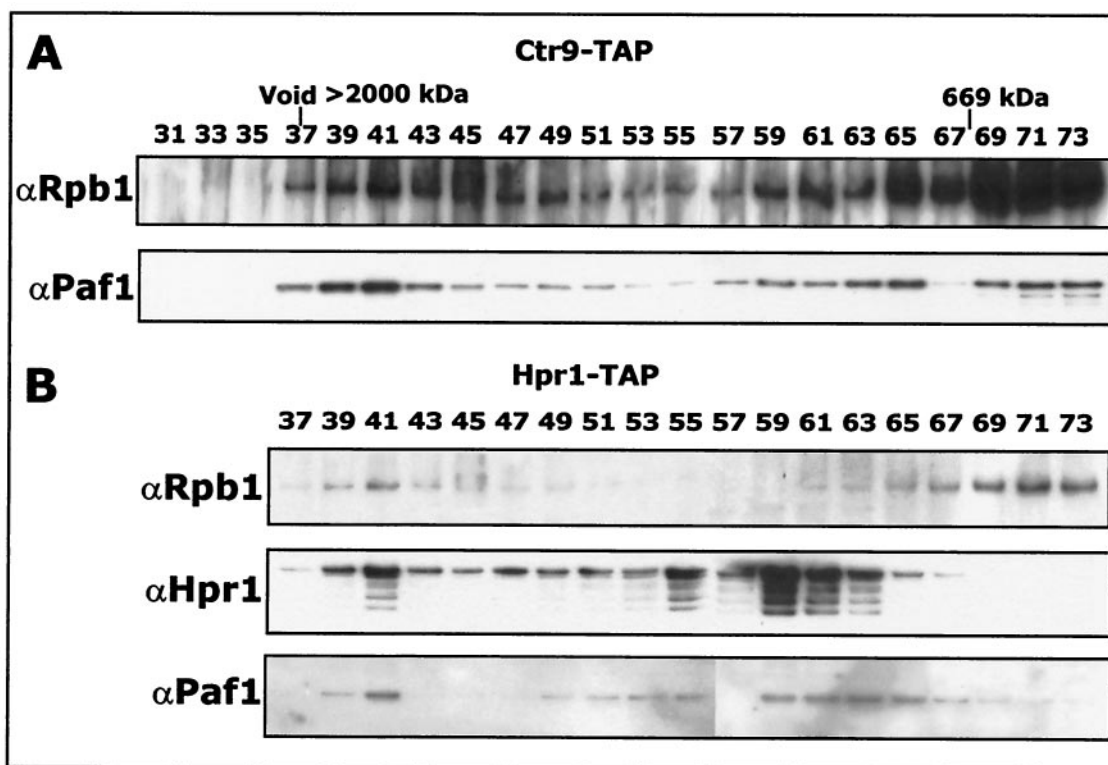
FIG. 4. Both Ctr9 and Hpr1 associate with RNA Pol II and Paf1. (A) Ctr9-associated proteins were purified from a TAP-tagged Ctr9 strain (YJJ1329). Samples were fractionated on a Superose 6 gel filtration column and immunoblotted with the indicated antibodies. (B) Hpr1-associated proteins were purified from a TAP-tagged Hpr1 strain (YJJ1334). Samples were fractionated on a gel filtration column and immunoblotted with the indicated antibodies.

hydroxyurea (Fig. 7B). We also found that *rtf1Δ* suppressed many of these phenotypes when combined with *ctr9Δ* (data not shown). As previously reported (30), we found that *leo1Δ* strains did not demonstrate any obvious phenotype and grew essentially as well as wild type (Fig. 7C). However, we found that a *leo1Δ paf1Δ* double mutant was less temperature sensitive and slightly more resistant to hydroxyurea than a *paf1Δ* strain (Fig. 7C). Therefore, removal of either Rtf1 or Leo1 can suppress some of the pleiotropic defects caused by loss of Paf1.

**Loss of Rtf1 restores *CLN1* expression in *paf1Δ*.** Loss of Paf1 results in changes in expression patterns of many yeast genes (4, 48; Betz et al., submitted). Since loss of Rtf1 does not completely suppress the *paf1Δ* phenotype, we would not expect that all transcription would be restored to normal levels. However, since the double mutant grows significantly faster than the *paf1Δ* strain, we analyzed the level of expression of the $G_1$ cyclin *CLN1* in these mutants. Koch and coworkers found that Paf1 and Ctr9 are required for full expression of *CLN2*, another $G_1$ cyclin (29), and we have found that Paf1 is required for normal expression of many other cell cycle-regulated genes, including *CLN1* (Betz et al., submitted). We found that in *paf1Δ*, *CLN1* mRNA was reduced about threefold, whereas in an *rtf1Δ paf1Δ* strain *CLN1* expression was restored to wild-type levels (Fig. 8). We have observed a similar restoration of mRNA levels for *RNR1* and *CYC1* in *rtf1Δ paf1Δ* relative to *paf1Δ* (data not shown; E. Amiott, personal communication). The restoration of *RNR1* transcripts provides a potential mo-

lecular explanation for the suppression of hydroxyurea sensitivity seen in Fig. 7B. The fact that *rtf1Δ*, and to a lesser extent *leo1Δ*, can suppress the transcriptional defects caused by loss of Paf1 is consistent with the idea that a partially defective Paf1 complex may block transcription and that removal of additional complex components may relieve the block.

**DISCUSSION**

Yeast cells contain at least two complex forms of RNA Pol II. Ten to fifteen percent of Pol II is complexed with the Srb-mediator factors, and this form of Pol II is clearly important for initiation of transcription of most, if not all, yeast genes and for response to many activators and repressors (reviewed in reference 34). In this work we have established that Paf1, Cdc73, and Hpr1 are associated with only about 2% of the cellular Pol II in a high-molecular-mass (~1.7-MDa) complex, distinct from the Srb-mediator. Although the Paf1 complex contains the general initiation factors TFIIB and TFIIF (Fig. 2) (47) and is found to be associated with the nonphosphorylated, initiating form of Pol II (57), it has not been established that it functions as an initiation complex. Loss of Paf1 complex genes causes alterations in the expression of only a small subset of yeast genes (4, 47; Chang et al., unpublished). Are these alterations due to changes in initiation or to changes in another transcriptional step? In these studies we have used affinity purification and mass spectrometry to identify three additional
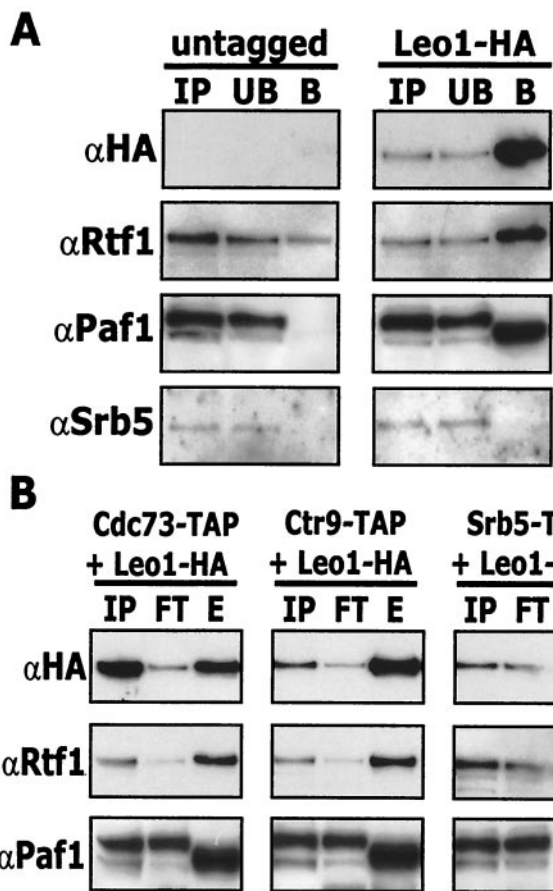
FIG. 5. Leo1-HA interacts with the Paf1 complex but not with the Srb-Med complex. (A) Coimmunoprecipitation of Leo1-HA with Paf1 and Rtf1. Whole-cell extracts prepared from the wild type (YJJ662) or a Leo1-HA strain (YJJ1330) were subjected to immunoprecipitation with an anti-HA (12CA5) antibody as described in Materials and Methods. Immunoprecipitates were analyzed by Western blotting using the indicated antibodies. IP, input; UB, unbound fraction; B, bound fraction. (B) TAP tag purification of Leo1-HA. Whole-cell extracts prepared from strains containing both a TAP tag and a Leo1-HA tag (YJJ1309, YJJ1331, and YJJ1332) were incubated with IgG beads as described in Materials and Methods. After washing, proteins were eluted from the beads with the addition of 25 U of TEV protease. Eluates were TCA precipitated and analyzed by Western blotting with the indicated antibodies. IP, input; FT, flowthrough; E, eluate.
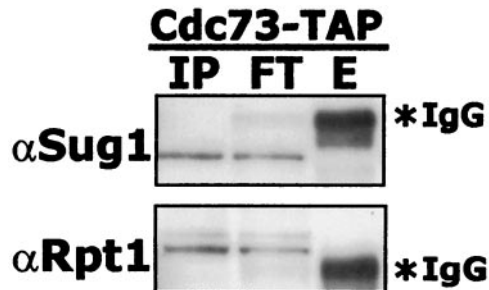


FIG. 6. Cdc73-TAP does not associate with components of the proteasome. Extracts from a Cdc73-TAP strain were affinity purified using rabbit IgG agarose beads. Bound proteins were eluted from the resin by adding TEV protease. Eluates were resolved by SDS-PAGE and immunoblotted with antibodies specific to the proteasome. IP, input; FT, flowthrough; E, eluate.

shared by $paf1\Delta$. Loss of Paf1 does, however, lead to an increase in recombination (4), which may be related to the loss of chromosome stability seen for $ctr9\Delta$. Like $ctr9\Delta$, $paf1\Delta$ strains have a severe defect in the expression of the $G_1$ cyclins $CLN1$ and $CLN2$. In addition Paf1 is required for full expression of many other cell cycle-regulated genes, including $HO$ and $RNR1$ (Porter et al., submitted). Paf1 is also critical for signaling from the protein kinase C–mitogen-activated protein kinase cascade to target genes required for maintenance of cell integrity (4). The requirement for Paf1 and Ctr9 in the expression of a significant subset of yeast genes is true both for the target genes in their chromosomal locations and for a variety of promoter-reporter constructs (4, 10, 22). In fact, $CTR9$ was originally identified as a factor required for $G_1$ cyclin expression in a screen using cell cycle-regulated promoter elements fused to a reporter gene (22). Therefore, Paf1 and Ctr9 act through promoter elements to effect normal transcript levels and to respond to signals during the cell cycle and in response to cell damage.

**Rtf1, important for both start site selection and elongation, is also part of the Paf1 complex.** $RTF1$ was originally identified in a genetic screen for mutations that would suppress a defective form of TBP (50). The $spt15-122$ allele of TBP has an altered specificity for TATA box recognition, which results in changes in transcriptional start sites (2). The $rtf1-1$ allele suppresses the phenotypes caused by the $spt15-122$ mutation and further alters start site selection but does not restore initiation sites to their wild-type positions (50). However, complete deletion of $RTF1$ also suppresses the $spt15-122$ defects and restores wild-type start site selection (50). It is interesting that mutations in Ccr4, also found in the Paf1 complex (4), also have extensive genetic interactions with TBP and the $spt15-122$ allele (3). Based on these studies it is clear that Rtf1, presumably as part of the Paf1 complex, plays an important role at an early stage of transcription.

In addition to its role in initiation, Rtf1 was recently reported to also have extensive genetic interactions with transcriptional elongation factors (8). In particular, $rtf1\Delta$ is synthetically lethal in combination with mutations in $CTK1$, a Pol II CTD kinase (8); $FCP1$, a CTD phosphatase (8); and $POB3$, a chromatin remodeling factor that plays roles in both initiation and elongation (11). In addition, $RTF1$ genetically inter-

components of the Paf1 complex: Ctr9, Rtf1, and Leo1. These identifications suggest new and intriguing connections to both transcriptional initiation and elongation.

**Ctr9, required for $G_1$ cyclin expression, is part of the Paf1 complex.** $CTR9$, also known as $CDP1$, has been isolated in two genetic screens, one looking for genetic interactions with a centromere binding factor (10) and the second searching for genes required for $G_1$ cyclin transcription (22). Loss of Ctr9 results in chromosome instability (10); reduction in the expression of $G_1$ cyclins (21); slow growth; and sensitivity to extremes of temperature, hydroxyurea, and many cell wall-damaging agents, including caffeine and SDS (21; Betz et al., submitted). Except for the chromosome instability phenotype, which has not been tested, all other known phenotypes of $ctr9\Delta$ are
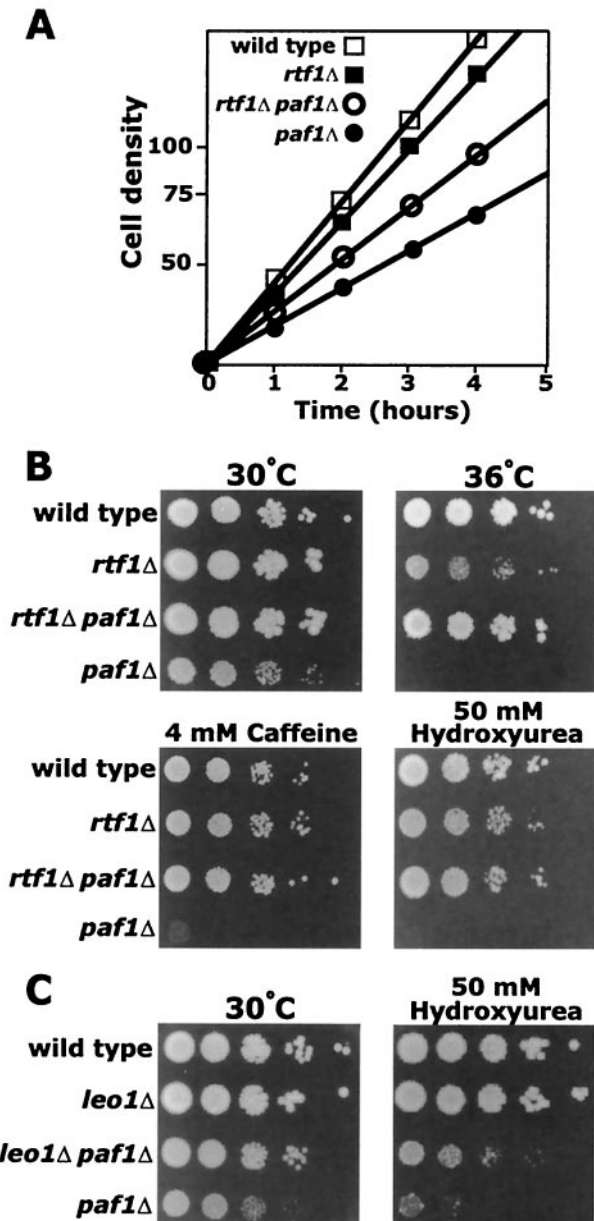
FIG. 7. A deletion of the *RTF1* gene suppresses the defects caused by disruption of the *PAF1* gene. (A) Growth curve of indicated deletion strains grown in YPD at 30°C. (B) Cultures of YJJ662 (wild type), YJJ1303 (*rtf1Δ::kan$^r$*), YJJ1326 (*rtf1Δ::kan$^r$ paf1Δ::HIS3*), and YJJ577 (*paf1Δ::HIS3*) cells (approximately 10$^7$/ml) were spotted in 10-fold serial dilutions on YPD medium and grown at 30 and 36°C and on YPD containing 4 mM caffeine and 50 mM hydroxyurea and grown at 30°C. (C) Cultures of YJJ662 (wild type), YJJ1336 (*leo1Δ*), YJJ1361 (*leo1Δ paf1Δ*), and YJJ577 (*paf1Δ*) were grown on the indicated media as described in panel B. Photographs of plates were taken after 4 days of incubation.

acts with other elongation factors, including TFIIS (*PPR2*) and *SPT5* (8). The interaction with *SPT5* is of particular interest since we detected peptides from Spt5 in our mass spectrometry analysis of TAP-tagged Cdc73, and an anti-Spt5 antibody detects a protein of the correct size for Spt5 in the high-molecular-mass Paf1 complex (Fig. 2B). In addition, Paf1, Cdc73,
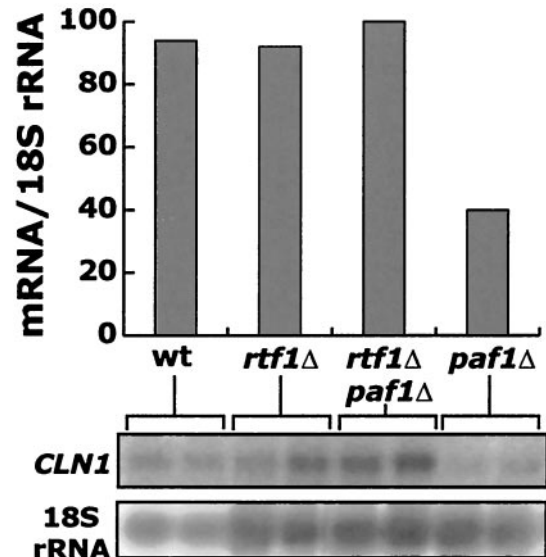


FIG. 8. *CLN1* message is restored to wild-type levels in an *rtf1Δ paf1Δ* double mutant strain. Duplicate cultures of the indicated deletion strains were grown to a density of $1.3 \times 10^7$ cells/ml, total RNA was harvested, and 15 μg of RNA per lane was fractionated. mRNA levels were detected using radiolabeled probes to *CLN1* and normalized to 18S rRNA as described in Materials and Methods. The bars in the upper panel represent the average of the duplicate samples shown in the lower panel.

and Rtf1 have been identified as Spt5-interacting factors (G. Hartzog and K. Arndt, personal communication).

Like those in *RTF1*, mutations in *SPT5* result in changes in transcription start sites and also have effects on elongation (16, 51), and Spt5 interacts with both the initiating and elongating forms of Pol II (26). Spt5 and Spt4, found together in a complex, are the yeast homologues of the human DSIF transcriptional elongation factor identified biochemically by Hartzog et al. (16). The DSIF elongation factor confers sensitivity to the drug DRB (5,6-dichloro-1-β-D-ribofuranosyl benzidazole) in vitro and is thought to play a negative role in transcription elongation in vivo (55). DRB inhibits the pTEFb kinase that phosphorylates the Pol II CTD and is required for an early stage of elongation in vitro (31). Some of the evidence that Spt5 and Rtf1 affect elongation comes from phenotypic analysis of mutants, specifically that mutation of either gene results in sensitivity to 6AU (8, 16). However, the connection between 6AU sensitivity and transcriptional elongation defects is complex. Mutation of the elongation factor TFIIS (*PPR2*) or the Rpb1 (1), Rpb2 (42) or Rpb9 (17) subunits of Pol II results in sensitivity to 6AU. In vitro, the 6AU-sensitive mutant of Rpb2 clearly has defects in elongation. However, in vivo the major effect of the various 6AU-sensitive mutants seems to be reduced expression of the *PUR5* gene encoding IMP dehydrogenase (17, 46). Since the reduction in *PUR5* expression can be transferred using a reporter construct fused to the promoter and 5′-untranslated region of the *PUR5* gene (46), it is not yet clear what stage of transcription is affected by the 6AU-sensitive mutations in vivo.

Is the Paf1 complex involved in transcriptional elongation? Although *paf1Δ* strains are slow growing and sensitive to a

variety of compounds, they are not sensitive to 6AU (Betz et al., submitted) although an isogenic *ppr2* strain is strongly inhibited. In addition, *rtf1Δ* strains are not 6AU sensitive in our genetic background (C. Mueller, unpublished). Neither *cdc73Δ* nor *hpr1Δ* strains are 6AU sensitive, although *ccr4Δ* is very sensitive to the drug (9; Betz et al., submitted) and *leo1Δ* is slightly sensitive (C. Mueller, unpublished). As described above, sensitivity to 6AU neither proves nor disproves a protein's role in elongation. However, the many genetic connections between *RTF1* and elongation factors (8) and the fact that the homologue of Spt5, DSIF, clearly is important for elongation in vitro are compelling evidence for a link.

The Paf1 complex is not the only factor that may straddle the line between initiation and elongation; many transcription factors seem to function at multiple stages of transcription. The GTF TFIIF is clearly required for initiation, promoter escape, and elongation (44, 59), and TFIIH's roles in both initiation and promoter escape can be traced to its action at different regions of downstream promoter DNA (49). Mutations in the Rpb9 subunit of Pol II reveal effects on both start site selection (12) and elongation (17). In addition, the Pob3-Spt16 chromatin-remodeling complex, which has genetic links to *RTF1* (8), also appears to be important for both initiation and elongation of transcription (11). Since initiation, promoter clearance, and entry into elongation are clearly a multistep process (39), perhaps it is not surprising that so many factors seem be involved in this critical transition. However, a central role in elongation for the Paf1 complex is not consistent with the fact that loss of Paf1 or other complex components affects the expression of only a small subset of genes. In addition, our observation that loss of both Paf1 and Rtf1 returns cells to a nearly wild-type phenotype does not support the concept that the Paf1 complex might be required for elongation of all yeast genes. Instead, our results fit a model wherein the Paf1 complex functions at a small subset of promoters to enhance expression in response to signaling pathways (i.e., protein kinase C, cell cycle). Partial defects in the complex, such as loss of Paf1 or Ctr9, could result in a defective complex that blocks transcription. Removal of a second component, such as Rtf1, may release the blocked complex and allow the return to nearly normal transcription patterns, perhaps through the action of the Srb-mediator form of Pol II. Testing this model will require the identification of the primary gene targets of the Paf1 complex.

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