A Complex Containing RNA Polymerase II, Paf1p, Cdc73p, Hpr1p, and Ccr4p Plays a Role in Protein Kinase C Signaling

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Received 19 August 1998/Returned for modification 2 October 1998/Accepted 27 October 1998

Yeast contains at least two complex forms of RNA polymerase II (Pol II), one including the Srbps and a second biochemically distinct form defined by the presence of Paf1p and Cdc73p (X. Shi et al., *Mol. Cell. Biol.* 17:1160–1169, 1997). In this work we demonstrate that Ccr4p and Hpr1p are components of the Paf1p-Cdc73p-Pol II complex. We have found many synthetic genetic interactions between factors within the Paf1p-Cdc73p complex, including the lethality of *paf1Δ ccr4Δ*, *paf1Δ hpr1Δ*, *ccr4Δ hpr1Δ*, and *ccr4Δ gal11Δ* double mutants. In addition, *paf1Δ* and *ccr4Δ* are lethal in combination with *srb5Δ*, indicating that the factors within and between the two RNA polymerase II complexes have overlapping essential functions. We have used differential display to identify several genes whose expression is affected by mutations in components of the Paf1p-Cdc73p-Pol II complex. Additionally, as previously observed for *hpr1Δ*, deleting *PAF1* or *CDC73* leads to elevated recombination between direct repeats. The *paf1Δ* and *ccr4Δ* mutations, as well as *gal11Δ*, demonstrate sensitivity to cell wall-damaging agents, rescue of the temperature-sensitive phenotype by sorbitol, and reduced expression of genes involved in cell wall biosynthesis. This unusual combination of effects on recombination and cell wall integrity has also been observed for mutations in genes in the Pkc1p-Mpk1p kinase cascade. Consistent with a role for this novel form of RNA polymerase II in the Pkc1p-Mpk1p signaling pathway, we find that *paf1Δ mpk1Δ* and *paf1Δ pkc1Δ* double mutants do not demonstrate an enhanced phenotype relative to the single mutants. Our observation that the Mpk1p kinase is fully active in a *paf1Δ* strain indicates that the Paf1p-Cdc73p complex may function downstream of the Pkc1p-Mpk1p cascade to regulate the expression of a subset of yeast genes.

Initiation of eukaryotic mRNA synthesis in vitro requires RNA polymerase II (Pol II) and the general transcription factors (GTFs), including TBP, TFIIB, TFIIE, TFIIIF, and TFIIH (reviewed in references 46 and 49). Regulation of transcription, however, requires additional cofactors to mediate the communication between DNA-binding activators or repressors, and Pol II and the GTFs. These cofactors include the TAFs associated with TBP to form TFIID (reviewed in references 17 and 54) and the mediator proteins, including the Srbps, associated with Pol II to form the “holoenzyme” (reviewed in reference 30). Recent work from several laboratories has shown that multiple forms of these complexes exist in different cell types and under different growth conditions (reviewed in reference 6). The different cofactor complexes therefore contribute to the complex regulatory patterns of eukaryotic genes.

We have reported the isolation and characterization of a novel form of Pol II in yeast distinct from the Srbp-containing “holoenzyme” (51, 58). This Pol II complex contains the GTFs TFIIB and TFIIIF but lacks TBP and TFIIH (51). The Gal11p-Sin4p-Rgr1p subcomplex is found in both forms of Pol II (35, 51). The products of the *CDC73* and *PAF1* genes are present in the novel form of Pol II but are not found in the Srbp-containing holoenzyme. Cdc73p and Paf1p are localized in the

nucleus, and deletion of either gene causes pleiotropic phenotypes, including temperature sensitivity and slow growth (52). In contrast to the requirement for some of the Srbps for transcription of most yeast genes (55), the expression of only a few genes is affected by mutations in *PAF1* and *CDC73* (51, 52).

Why does yeast contain at least two complex initiating forms of Pol II? In this work we have begun to answer this question by identifying two additional proteins found uniquely in the Paf1p-Cdc73p complex but not in the Srbp-containing form of Pol II. These proteins, Ccr4p and Hpr1p, have both been implicated in the transcription of subsets of yeast genes (11, 63). Both factors have also been demonstrated to have genetic interactions with components of the transcription machinery (16, 38). By analyzing the phenotypes of single and multiple mutants of these Pol II-associated proteins, we have found that the complex appears to play a role both in recombination and in the expression of genes involved in cell wall biosynthesis. A feature that links these two seemingly disparate properties is a known connection to the protein kinase C–mitogen-activated protein (MAP) kinase signaling pathway (23, 25).

In yeast the protein kinase C homologue encoded by the *PKC1* gene has been shown to play an essential role in maintenance of cell integrity. A mutation in *PKC1* leads to cell lysis, which can be rescued by the osmotic stabilizer sorbitol (32, 47). Pkc1p is activated in response to alterations in the cell membrane caused by heat shock, hypoosmotic shock, or α -factor treatment (27, 62). Activated Pkc1p initiates the sequential activation of its downstream MAP kinase cascade, including Bck1p, Mkk1p-Mkk2p, and Mpk1p (Slp2p) (reviewed in reference 33). The status of the membrane may be signaled to

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
YJJ453	<i>MATα leu2Δ1/leu2Δ1 his3Δ200/his3Δ200 ura3-52/ura3-52</i>	56
YJJ564	<i>MATα leu2Δ1 his3Δ200 ura3-52 gal11Δ::LEU2</i>	52
YJJ577	<i>MATα leu2Δ1 his3Δ200 ura3-52 paf1Δ::HIS3</i>	52
YJJ662	<i>MATα leu2Δ1 his3Δ200 ura3-52</i>	52
YJJ664	<i>MATα leu2Δ1 his3Δ200 ura3-52 paf1Δ::HIS3</i>	52
YJJ665	<i>MATα leu2Δ1 his3Δ200 ura3-52 cdc73Δ::HIS3</i>	52
YJJ681	<i>MATα leu2Δ1 his3Δ200 ura3-52 cdc73Δ::HIS3</i>	X. Shi
YJJ691	<i>MATα leu2Δ1 his3Δ200 ura3-52 cdc73Δ::HIS3 (pEGST-CDC73)</i>	52
YJJ693	<i>MATα leu2Δ1 his3Δ200 ura3-52 cdc73Δ::HIS3 (pEGST)</i>	52
YJJ755	<i>MATα bar1 his6 his7 leu2 ura3 pep4 prb1 trp1</i>	R. Sclafani
YJJ756	<i>MATα bar1 his6 his7 leu2 ura3 pep4 prb1 trp1 paf1Δ::TRP1</i>	This work
YJJ832	<i>MATα leu2Δ1 his3Δ200 ura3-52 sin4::LEU2</i>	This work
YJJ854	<i>MATα leu2Δ1 his3Δ200 ura3-52 trg2Δ::HIS3 (pEGST-TFG2)</i>	52
YJJ855	<i>MATα leu2Δ1 his3Δ200 ura3-52 (pEGST)</i>	52
YJJ875	<i>MATα leu2Δ1 his3Δ200 ura3-52 srb5Δ::URA3</i>	This work
YJJ879	<i>MATα leu2Δ1 his3Δ200 ura3-52 ccr4Δ::URA3</i>	This work
YJJ898	<i>MATα leu2Δ1 his3Δ200 ura3-52 hpr1Δ::HIS3</i>	This work
YJJ899	<i>MATα leu2Δ1 his3Δ200 ura3-52 hpr1Δ::HIS3</i>	This work
YJJ932	<i>MATα leu2Δ1 his3Δ200 ura3-52 ccr4Δ::URA3</i>	This work
YJJ935	<i>MATα leu2Δ1 his3Δ200 ura3-52 srb5Δ::URA3</i>	This work
YJJ956	<i>MATα leu2Δ1 his3Δ200 ura3-52 srb5Δ::HIS3</i>	This work
YJJ952	<i>MATα leu2Δ1 his3Δ200 ura3-52 hpr1Δ::HIS3 (pEGST-HPR1)</i>	This work
YJJ954	<i>MATα leu2Δ1 his3Δ200 ura3-52 hpr1Δ::HIS3 (pEGST)</i>	This work
YJJ998	<i>MATα/MATα leu2Δ1/leu2Δ1 his3Δ200/his3Δ200 ura3-52/ura3-52 PAF1/paf1Δ::HIS3</i>	This work
	<i>MPK1/mpk1Δ::URA3</i>	
YJJ1027	<i>MATα his3Δ1 leu2 trp1 ura3 can1 cyh2 gal1</i>	R. Sclafani
YJJ1036	<i>MATα/MATα bar1 his3Δ1 his6 his7 leu2/leu2 ura3/ura3 pep4 prb1 trp1/trp1 can1 cyh2 gal1</i>	This work
HKY870-12A	<i>MATα leu2-k::ADE2-URA3::leu2-k ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100</i>	15
HFY998-2C	<i>MATα leu2-k::ADE2-URA3::leu2-k ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 hpr1::HIS3</i>	15
HFY2074	<i>MATα leu2-k::ADE2-URA3::leu2-k ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 paf1::HIS3</i>	15
HFY2059-1A	<i>MATα leu2-k::ADE2-URA3::leu2-k ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 cdc73::HIS3</i>	15
HFY2085	<i>MATα leu2-k::ADE2-URA3::leu2-k ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 ccr4::HIS3</i>	15
HFY2162	<i>MATα leu2-k::ADE2-URA3::leu2-k ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 sin4::TRP1</i>	15
HFY2069	<i>MATα leu2-k::ADE2-URA3::leu2-k ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 srb5::HIS3</i>	15

Pkc1p by the putative transmembrane protein Slg1p (18) through a step requiring Rho1p, a small GTP-binding protein (45). Activation of Pkc1p is critically important for cell cycle progression (34), where it plays a role in bud emergence in response to signals from Cdc28p (18, 43). Pkc1p is also sensitive to the mating pathway of yeast via direct communication with the Ste20p-activated MAP kinase cascade (5). In both bud emergence and the mating pathway, activation of Pkc1p and the MAP kinase cascade controls increased synthesis of gene products required for the newly made cell walls (25).

Pkc1p also plays a less-well-characterized role in recombination in yeast. Huang and Symington (22, 23) found that mutations in *PKC1* led to increased rates of mitotic recombination but that, unlike the case for expression of the cell wall biosynthetic genes, a direct connection to the MAP kinase cascade was not evident. Our discovery of a Pol II complex containing factors that affect both the expression of cell wall biosynthetic genes and frequency of recombination therefore may begin to resolve these two very different functions of Pkc1p. Our results are consistent with the possibility that the Pol II complex containing Cdc73p, Paf1p, Ccr4p, and Hpr1p functions, at least in part, to transmit signals from the *PKC1* kinase cascade to target genes involved in recombination and cell wall integrity.

MATERIALS AND METHODS

Strains and media. The *Saccharomyces cerevisiae* strains used in this study are shown in Table 1. Strains YJJ564, YJJ577, YJJ662, YJJ664, YJJ665, YJJ681, YJJ691, YJJ693, YJJ832, YJJ854, YJJ875, YJJ879, YJJ898, YJJ899, YJJ932, YJJ935, YJJ952, YJJ954, YJJ956, and YJJ1027 were all derived from the ho-

mozygous diploid YJJ453 and are therefore isogenic. YJJ998 was created by mating YJJ577 with YJJ662 and then disrupting a single copy of the *MPK1* gene in the diploid by using the construct described below. YJJ1027 and YJJ756 are isogenic with YJJ755. The HKY strains used for the recombination analyses are isogenic (16). Yeast strains were grown in YPD or synthetic medium prepared according to standard methods (19).

Protein-protein interaction assays. Strains containing glutathione *S*-transferase (GST)-tagged forms of Tfg2p and Cdc73p have been previously described (51). *hpr1Δ* strains containing the pGEST vector or a GST-Hpr1p construct were created as follows. The *HPR1* coding region was amplified by PCR with primer 1 (5'-CGCGGATCCATGTCTAATACCGAGGAATTG-3') and primer 2 (5'-GCGGGATCCTTATTCATATCTTGGGTAGATG-3'). Both primers are flanked by *Bam*HI sites. The PCR product was digested with *Bam*HI and ligated into pJJ560 (pGEST vector) to make an in-frame *GST-HPR1* fusion under the control of a *GAL* promoter. The pGEST vector and the pGEST-Hpr1p construct were transformed into strain YJJ899 to create strains YJJ954 and YJJ952, respectively. The presence of the GST-Hpr1p construct, but not the vector alone, corrected the slow growth and temperature-sensitive (ts) defect of the *hpr1Δ* strain. The expression of GST-HPR1 in the *hpr1Δ* strain was confirmed by Western blot with antibody directed against Hpr1p. Transcriptionally active whole-cell extracts were prepared from yeast strains YJJ691, YJJ693, YJJ854, YJJ855, YJJ952, and YJJ954 as described previously (61). The protein concentration of the whole-cell extracts were measured by using reagents from Bio-Rad. Equal amounts of the extracts were mixed with glutathione-agarose beads in SK(20) (20 mM HEPES [pH 7.9], 20% glycerol, 10 mM MgSO₄, 10 mM EGTA, 5 mM dithiothreitol [DTT], 20 mM potassium acetate, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, 0.4 μg of bestatin per ml, 0.35 μg of pepstatin A per ml), incubated at 4°C for 2 to 4 h, and washed once with SK(20) and several times with SK(200) (30 mM HEPES [pH 7.9], 10% glycerol, 1 mM EDTA, 1 mM DTT, 200 mM potassium acetate, and protease inhibitors as specified above). The glutathione-agarose beads were spun down briefly and eluted with the sample buffer. The samples were resolved on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel (20). Western blot analysis was performed as described by using either alkaline phosphatase or enhanced chemiluminescence (ECL) for detection (20). The sources of the antibodies used were as follows: anti-Paf1p (52), anti-Cdc73p (51), anti-Hpr1p- from M. Christman,

anti-Ccr4p (42), anti-Gal11p from T. Fukasawa, anti-Srb5p from R. Young, and anti-TFIIS and anti-TBP (57).

Differential display and yeast mRNA analysis. Total yeast RNA was isolated as described previously (13). Differential display analysis has been described (51). RNA samples for Northern blots were quantitated by measuring the optical density at 260 nm, equal amounts of RNA were run on 1% agarose-formaldehyde gels, and RNA blots were prepared according to standard methods (50). [α - 32 P]dATP-labeled probes were prepared by random priming (50). An 18S rRNA oligonucleotide probe was labeled with T4 kinase and [γ - 32 P]ATP (50). Northern blots were quantitated by PhosphorImager analysis.

Double deletion and tetrad analysis. Appropriate deletion strains were crossed to obtain the desired diploid strains, which were sporulated, and at least 30 tetrads for each diploid were dissected (19). The genotypes of the individual spores were determined by analysis of the different markers used to replace the deleted genes. In some cases, PCR was used to confirm the genotype of the spores.

Construction of deletion strains. *SRB5* coding and flanking regions were amplified by using primer 1 (5'-TGCAGCAGCTAAACCTCCAC-3') and primer 2 (5'-GACGATGACGAAGAGCTAC-3'). The PCR product was ligated into pGEM-T (Promega) vector. Primer 3 (5'-ACGCAAGCTTTCTTCTTAAATATGGAATAC-3') and primer 4 (5'-GACGAAGCTTATAATCATTGGCACCTGG-3') were used to amplify the resulting plasmid. The PCR product was then cut with *Hind*III and ligated with the 1.2-kb *Hind*III *URA3* fragment from YEp24 (4) to give pJJ995. pJJ995 was cut with *Hind*III, blunt ended with Klenow, and ligated with the *Bam*HI/*Xho*I-cut and Klenow-treated 1.4-kb *HIS3* fragment from YIp1 (53) to give pJJ1072. pJJ995 or pJJ1072 was cut with *Spe*I/*Sac*II and used to transform YJJ453. The *MPK1* deletion construct was made as follows. *MPK1* coding and flanking regions were amplified with primer 1 (5'-ATGGCTGATAAGATAGAGAGG-3') and primer 2 (5'-AGGAATTCAAGAGGCGATAAC-3'). The PCR product was ligated into the PCRII (Invitrogen) vector. The resulting plasmid was partially digested with *Hind*III, and the 4.2-kb fragment was ligated with the 1.2-kb *Hind*III *URA3* fragment from YEp24 (4) to give pJJ1143. pJJ1143 was cut with *Eco*RI and used to transform YJJ453. The *CCR4* deletion construct was as described earlier (42). The *SIN4* deletion construct was derived from M1381 containing *sin4Δ::LEU2* (from D. Stillman) which is similar to the *sin4Δ::TRP1* disruption described by Jiang and Stillman (26). The *HPR1* deletion construct *hpr1Δ::HIS3* was as described earlier (2). The deletion constructs described above were transformed into diploid strain YJJ453. The *paf1Δ::TRP1* disruptor was made as follows. Primer P1 (5'-CTTAGCACAACTGAATTCGAAAGG-3') and primer P4 (5'-ATACGAATGATGTTAATGGAGACTCCAGGATTGTCGACT-3') were used to PCR amplify the *paf1Δ::HIS3* construct from genomic DNA (YJJ664). The PCR product was subcloned into the pGEMT vector (Promega) to give pJJ902. pJJ902 was cut with *Xho*I/*Bam*HI to release the 1.2-kb *HIS3* fragment. The vector fragment was gel purified and ligated with the 900-bp *Sal*I/*Bgl*II *TRP1* fragment, which resulted in pJJ904. pJJ904 was linearized with *Spe*I/*Sac*II and used to transform yeast strain YJJ755 to obtain *paf1Δ* strain YJJ756. The *pkc1Δ::LEU2* disruptor was made as follows. The *PKC1* coding and flanking sequences were amplified from yeast genomic DNA (YJJ755) by PCR with primer 1 (5'-AACTGCAGCATGAGTTTTCAC AATTGTAG-3') and primer 2 (5'-AACTGCAGTCATGGCATGACCTTTTC TTC-3').

The PCR product was cut with *Pst*I, gel purified, and ligated into pJJ998 (a modified pGEM-T vector) at the *Pst*I sites. The resulting plasmid pJJ1193 was cut with *Stu*I to release a 1.3-kb internal *PKC1* fragment, and the vector fragment was gel purified and ligated to the 2.2-kb *Hpa*I *LEU2* fragment from YEp13. The resulting plasmid pJJ1217 was linearized with *Pst*I prior to transforming a diploid yeast strain from a cross between YJJ756 and YJJ1027 to obtain the heterozygous diploid YJJ1036. Southern blotting and PCR analysis were used to confirm the gene replacements. Sporulation and tetrad dissection were used to obtain haploid deletion strains (19).

Enzymatic assays. *CYC1* and *FKS1* promoter-*lacZ* fusion reporter plasmids used in β -galactosidase assays were as previously described (25, 28). Yeast cells transformed with the plasmids (19) were grown in Ura-Casamino Acid media supplemented with 4% glucose. Extract preparation and β -galactosidase assays were performed as described elsewhere (44). Mpk1p kinase assays with hemagglutinin (HA)-tagged Mpk1p to phosphorylate MBP were as described by Zarzov et al. (62).

Determination of recombination rates. Recombination rates were calculated according to the median method of Lea and Coulson (31) as described previously (1).

RESULTS

Ccr4p and Hpr1p are in the Paf1p-Cdc73p-Pol II complex.

Paf1p and Cdc73p were originally identified as proteins associated with a transcriptionally active form of Pol II immobilized by an antibody directed against the nonphosphorylated form of the C-terminal domain (CTD [58]). The proteins bound to Pol II and eluted with moderate salt included TFIIB, the subunits of TFIIF, TFIIS, Paf1p, Cdc73p, Gal11p, and

Sin4p, as well as approximately 13 other polypeptides, but did not include TBP, TFIIB, the Srpbs or the Swip-Snp factors (58). To confirm that these Pol II-associated proteins defined a form of Pol II distinct from the Srp-containing holoenzyme, we created strains bearing GST-tagged forms of Cdc73p and Paf1p functionally replacing the *CDC73* and *PAF1* genes in yeast cells and analyzed the composition of the complexes isolated by using glutathione-agarose chromatography (51). These experiments confirmed that Cdc73p and Paf1p are found in a distinct complex with Pol II, TFIIB, TFIIF, Gal11p, and Sin4p. Elongation factor TFIIS apparently defines at least one additional form of Pol II, since it was not in any of the GST-tagged complexes nor is it present in the Srp-containing holoenzyme (51).

Like Cdc73p and Paf1p, Ccr4p and Hpr1p have both been reported to affect the expression of subsets of yeast genes (11, 63, 37, 38), to be required for growth at high temperature (42, 14), and to be associated with large complexes of proteins distinct from the Srp-containing holoenzyme (63, 38). We therefore tested the possibility that Ccr4p and Hpr1p were also present in the GST-tagged Cdc73p-Pol II complex. As shown in Fig. 1A, both Ccr4p and Hpr1p are found in the GST-Cdc73p complex (Fig. 1A, lane 8) and in a complex isolated via a GST tag on the Tfg2p subunit of TFIIF (Fig. 1A, lane 4); neither protein is found in the GST alone control lanes (Fig. 1A, lanes 2 and 6). In addition, we have found that a GST-tagged form of Paf1p colocalizes with Hpr1p and Ccr4p (data not shown) and that a GST-tagged Hpr1p construct copurifies with Pol II, Cdc73p, Paf1p, and Ccr4p (Fig. 1B, lane 4, and data not shown).

We also looked for Hpr1p and Ccr4p in the original collection of affinity-isolated proteins associated with Pol II used to identify Paf1p and Cdc73p (58). As shown in Fig. 1C, both proteins are present in the fraction affinity purified with the anti-CTD antibody (lane 3) but not in a control fraction (lane 2). The association of Ccr4p and Hpr1p with the Paf1p-Cdc73p-Pol II complex is quite stable, since it is resistant to washing with 0.5 M potassium acetate and 0.5 M ammonium sulfate (data not shown). Therefore, analyzing complexes isolated by two very different purification strategies—GST-tagged complexes and an antibody affinity isolated Pol II complex—we find that Paf1p, Cdc73p, Hpr1p, and Ccr4p are found together in a stable complex with Pol II, TFIIB, TFIIF, and the Gal11p and Sin4p subcomplex. Although we cannot entirely rule out that we are looking at multiple complexes by these techniques, the fact that Paf1p, Cdc73p, and Hpr1p each colocalizes with each other and with Ccr4p is consistent with these factors being present in one Pol II-associated complex.

We have shown that Cdc73p can associate directly with purified RNA Pol II (51), but we do not have any additional information about the assembly of this complex of proteins. We therefore analyzed the effect of mutating the *CDC73* and *PAF1* genes on the abundance of the other factors in the complex. In particular, we were interested in the possibility that the elimination of these proteins might destabilize other complex components. We found that the *cdc73Δ* and *paf1Δ* mutations did not reduce the abundance of transcripts from the *GAL11*, *CCR4*, *HPR1*, or *SRB5* genes. Mutations in *PAF1* and *CDC73* also had little or no effect on each other's transcript abundance (data not shown). However, as shown in Fig. 2, the *cdc73Δ* mutation but not the *paf1Δ* mutation appears to significantly reduce the abundance of Gal11p and Ccr4p and, to a lesser extent, Paf1p and Hpr1p (Fig. 2, lane 2) when compared to either the isogenic wild-type strain (Fig. 2, lane 1), to the deletion strain complemented by the GST-tagged

FIG. 1. Hpr1p and Ccr4p are present in the Paf1p-Cdc73p-Pol II complex. Proteins separated on SDS polyacrylamide gels were transferred and probed with antibodies directed against Hpr1p and Ccr4p as indicated. ECL was used for antibody detection. (A) Transcription-competent whole-cell extracts (WCE) were isolated and used as a source to purify GST-tagged Tfg2p, Cdc73p, and associated proteins by glutathione agarose chromatography as described in Materials and Methods. Lanes 1, 3, 5, and 7 (labeled IP) contain the input WCE from the indicated strains; lanes 2, 4, 6, and 8 (labeled B) contain the proteins bound to the glutathione agarose beads. Lanes 1 and 2 (labeled WT-GST) are from wild-type (YJJ662) cells transformed with the GST vector alone; lanes 3 and 4 (labeled GST-TFG2) are from the *tfg2Δ* mutant complemented with GST-Tfg2p (YJJ854); lanes 5 and 6 (labeled *cdc73Δ*-GST) are from *cdc73Δ* (YJJ665) mutant cells transformed with the GST vector; and lanes 7 and 8 (labeled GST-CDC73) are from the *cdc73Δ* mutant complemented by GST-Cdc73p (YJJ691). (B) Transcription-competent WCE were isolated and used as a source to purify GST-tagged Hpr1p and associated proteins by glutathione agarose chromatography as described in Materials and Methods. Lanes labeled IP and B are as described in panel A. Lanes 1 and 2 (labeled *hpr1Δ*-GST) are from an *hpr1Δ* strain transformed with the GST vector alone (YJJ954); lanes 3 and 4 (labeled GST-HPR1) are from an *hpr1Δ* strain transformed with GST-Hpr1p (YJJ952). (C) Fractions from antibody affinity chromatography performed as described in Wade et al. (58). Lanes: WCE, 40 μ g of protein from a transcription competent WCE; α - σ^{70} , 10 μ l of the salt-eluted fraction from a control column containing antibody directed against the σ^{70} subunit of *E. coli* RNA polymerase; α -CTD, 10 μ l of the salt-eluted fraction from a column containing antibody directed against the C-terminal domain of the largest subunit of RNA Pol II.

form of Cdc73p (Fig. 2, lane 3), or to either of the *paf1Δ* strains (Fig. 2, lanes 4 and 5).

Protein levels of factors not found in the Paf1p-Cdc73p-Pol II complex, including TBP, TFIIS, and Srb5p, remain un-

changed in the *cdc73Δ* strain (Fig. 2, compare lane 2 with lanes 1, 3, 4, and 5). Since the *cdc73Δ* mutation did not reduce mRNA abundance for any of the genes analyzed, these results suggest that Cdc73p may play a role in the assembly or stabilization of the Pol II complex. The reduced abundance of Gal11p, Ccr4p, Hpr1p, and Paf1p in the *cdc73Δ* strain further supports the idea that these proteins are present in the same complex.

Identification of transcripts affected by mutations in Paf1p-Cdc73p-Pol II complex components. Mutations in *PAF1* and *CDC73* affect the transcript abundance of only a very few yeast genes (51, 52). In general, mutation of *PAF1* affects the expression of more transcripts than mutation of *CDC73*, a finding consistent with its more severe phenotype. For example, we have previously shown that inducible *GAL* gene expression measured in the chromosomal *GAL7+10* genes and in a *GAL* promoter-reporter construct is reduced five- to eightfold in *paf1Δ* but is relatively unaffected by *cdc73Δ* (52). We have also analyzed the inducible expression of the *GAL1,7+10* genes in isogenic *ccr4Δ* and *hpr1Δ* strains. We found that *GAL* gene induction in a *ccr4Δ* strain was actually enhanced in both rate and extent, while there was little or no effect on *GAL* gene expression in an *hpr1Δ* strain (data not shown). Therefore, although these proteins are clearly found together in a complex, their effects on gene expression are not equivalent.

We have previously reported the use of differential display (36) to identify genes whose expression is differentially affected by mutations in *PAF1*, *CDC73*, and *GAL11* (51). We have extended this analysis to the identification of genes differentially expressed in *ccr4Δ*, *hpr1Δ*, and *srb5Δ* strains. A partial

FIG. 2. A mutation in the *CDC73* gene affects the abundance of Ccr4p, Hpr1p, Gal11p, and Paf1p. The abundance of the indicated proteins was analyzed as described in Materials and Methods in different transcription-competent WCEs. Antibodies were detected with alkaline phosphatase. Lanes 1, 2, and 4 show WCEs from wild-type (WT; YJJ662), *cdc73Δ* (YJJ665), and *paf1Δ* (YJJ664) cells, respectively, transformed with the GST vector alone. Lanes 3 and 5 show *cdc73Δ* and *paf1Δ* mutant strains complemented by GST-Cdc73p (YJJ691) and GST-Paf1p (YJJ676), respectively. The arrowhead above Paf1p points to the position of the GST-Paf1p protein seen in lane 5. Proteins absent from the Paf1p-Cdc73p-Pol II complex, including Srb5p, TFIIS, and TBP, are used as loading controls.

FIG. 3. Transcripts identified by differential display are differentially expressed in isogenic *paf1Δ*, *cdc73Δ*, *gal11Δ*, *srb5Δ*, *ccr4Δ*, and *hpr1Δ* mutant strains. Differential display was performed as described in Materials and Methods. DNA encoding the differentially expressed transcripts was cloned and sequenced to identify the yeast gene. RNA was isolated from the indicated isogenic strains and probed for transcripts from each gene as described in Materials and Methods. Abundance was determined with a PhosphorImager and was normalized to the signal for 18S rRNA. The data is presented relative to a transcript abundance in wild type set as 1, which is shown as a dashed line in each panel. The results shown represent the averages and standard deviations from six to nine separate RNA isolations. The yeast strains used for RNA isolation were *paf1Δ*-YJJ664, *cdc73Δ*-YJJ665, *gal11Δ*-YJJ664, *srb5Δ*-YJJ875, *ccr4Δ*-YJJ879, and *hpr1Δ*-YJJ898.

collection of the genes identified is shown in Fig. 3. Although some of the effects shown in Fig. 3 appear to be subtle (less than twofold), note that the data represent the averages of six to nine independent repetitions of the RNA analyses. Therefore, as indicated by the use of error bars, the differences shown are in many cases significant relative to the levels in the wild-type strain. These studies have identified some known genes, including *CYC1*, *RSP7B*, *PRS1*, and *PFK26*, and some open reading frames of unknown function. As described above for the inducible *GAL1,7+10* genes, the effects on transcript abundance are not the same for mutations in different genes of the complex. The most common pattern of expression observed is that transcripts are reduced in abundance two- to fourfold in *paf1Δ* (note *CYC1*, *RSP7B*, *PRS1*, *YBR265*, and *YNR067c*), and there are several examples where transcript

TABLE 2. Expression of promoter-reporter constructs is reduced in the *paf1Δ* strain^a

Strain	β-Galactosidase activity (U/mg) with promoter:	
	<i>CYC1</i>	<i>FKS1</i>
Wild type	24,000 ± 1,500	20,000 ± 3,200
<i>paf1Δ</i>	1,900 ± 370	2,600 ± 150

^a Extracts from wild-type (YJJ662) and *paf1Δ* mutant (YJJ664) strains bearing the pF712-380 *pFKS1-lacZ* reporter plasmid (25) or the AJ-1 *CYC1-lacZ* reporter plasmid (28) were prepared and assayed as described in Materials and Methods. β-Galactosidase activity (± the standard error of the mean) is expressed in units per milligram of protein, where 1 U is defined as the $A_{420} \times 10^3$ per minute. Values are derived from replicate assays of at least three different transformants.

abundance is significantly increased in *hpr1Δ* (note *PRS1*, *PFK26*, and *YLR346c*). However, as previously reported, in some cases transcript abundance increases in *paf1Δ* (note the fourfold increase of *YLR346c*). In most of the examples shown the *cdc73Δ* and *srb5Δ* mutations have little effect on transcript abundance, although expression of *YNR067c* is reduced in these backgrounds. As previously observed by Liu et al. (38), *ccr4Δ* leads to both positive (*YLR346c*) and negative (*CYC1*) effects on transcription.

Although we cannot rule out that some of the changes in transcript abundance may be due to secondary effects, we can confirm, as previously demonstrated for the *paf1Δ* reduction of *GAL7+10* transcription (52), that the effects are at the level of initiation of transcription by using a promoter fusion-reporter construct as shown in Table 2. Using the *CYC1* promoter-regulatory region fused to β-galactosidase, we have confirmed the reduction in expression seen by the RNA analysis shown in Fig. 3. Liu et al. (38) have reported that *ccr4Δ* reduces expression of a *CYC1-lacZ* reporter construct about eightfold, which is somewhat greater than the fivefold reduction in *CYC1* mRNA levels seen in Fig. 3. The effect of *paf1Δ* is also more dramatic with the reporter construct, with expression reduced over 12-fold compared to the 4-fold reduction seen in the RNA analysis. In the *srb5Δ* strain we saw no differences in *CYC1-lacZ* expression relative to the wild-type strain (data not shown), a result consistent with the results presented in Fig. 3. It is therefore clear that the reduced abundance of *CYC1* mRNA seen in the *ccr4Δ* and *paf1Δ* strains in Fig. 3 is due to effects on the promoter-driven initiation of transcription.

Genetic interactions between *PAF1*, *CDC73*, *CCR4*, and *HPR1*. We have previously reported that a *paf1Δ cdc73Δ* double mutant is viable and does not show an enhanced phenotype (51), while a *paf1Δ gal11Δ* double mutant demonstrates a dramatically enhanced slow-growth phenotype (52). These data are consistent with the idea that Paf1p and Gal11p have at least partially overlapping essential functions in the yeast cell, with Paf1p and Cdc73p participating in the same pathway such that a double mutant is no more deleterious than either single mutation. To determine the genetic interactions between *PAF1*, *CDC73*, and the newly identified components of the Pol II complex, *CCR4* and *HPR1*, we created double mutants in an isogenic background as described in Materials and Methods. Many of the double-mutant analyses were repeated in other genetic backgrounds with identical results. We also evaluated the effects of combining mutations in these genes with mutations in other holoenzyme components, including *gal11Δ*, *sin4Δ*, and *srb5Δ*. Based on the results of 30 or more tetrads for each doubly mutant diploid, we found extensive evidence for genetic interactions, as summarized in Fig. 4. We found that combining a *paf1Δ* mutation with either *ccr4Δ* or *hpr1Δ* results

	<i>cdc73Δ</i>	<i>ccr4Δ</i>	<i>hpr1Δ</i>	<i>gal11Δ</i>	<i>sin4Δ</i>	<i>srb5Δ</i>
<i>paf1Δ</i>	ts*	Lethal	Lethal	Slow Growth*	ts	Lethal
<i>cdc73Δ</i>		Lethal	ts at 30°	ts*	ts	Slow Growth
<i>ccr4Δ</i>			Lethal	Lethal	Slow Growth	Lethal
<i>hpr1Δ</i>				ts	ts	Slow Growth
<i>gal11Δ</i>					ts	Slow Growth
<i>sin4Δ</i>						Lethal

FIG. 4. Synthetic genetic interactions between pairwise combinations of factors in RNA Pol II complexes. Tetrad analysis of heterozygous diploids obtained by sporulating crosses between isogenic single deletion mutants and tetrad dissection. At least 30 tetrads were dissected for each diploid analyzed. All of the single deletion mutants are ts at 38°C. The growth phenotypes of the double-deletion mutants are shown in the figure, with shaded areas highlighting significant synthetic interactions (solid box, synthetic lethality; gray box, synthetic enhancement). The genotypes of inviable spores were deduced by the markers used to delete the genes. "Slow growth" means that the double mutants grew significantly more slowly than either of the parents. The "ts at 30°" means that the permissive temperature for the double mutant is reduced to 22°C. The "ts" in an unshaded box indicates that the phenotype of the double mutant was not significantly worse than either of the parent strains. The asterisks refer to previously published results (51, 52) included for completeness. The strains used in the crosses were *paf1Δ*-YJJ664 or -YJJ577; *cdc73Δ*-YJJ665 or YJJ681; *gal11Δ*-YJJ564; *sin4Δ*-YJJ832 *srb5Δ*-YJJ956, -YJJ935, or -YJJ875; *ccr4Δ*-YJJ932 or -YJJ879 and *hpr1Δ*-YJJ898 or -YJJ899.

in lethality. Deletion of *CCR4* is also lethal in combination with *cdc73Δ*, *gal11Δ*, or *hpr1Δ* and has an enhanced phenotype with *sin4Δ*. In each case the nonviable spores did germinate and formed microcolonies. The combination of *cdc73Δ* and *hpr1Δ* results in an enhanced ts phenotype in which the permissive temperature is reduced from 30 to 22°C. These results, like our earlier analysis of the *paf1Δgal11Δ* double mutant (51), support the idea that the components of this novel Pol II complex have redundant essential functions for the yeast cell.

In addition to the genetic interactions between factors within the Paf1p-Cdc73p-Pol II complex, we also found dramatic genetic interactions between mutations in *SRB5* and mutations in *PAF1*, *CDC73*, *CCR4*, *HPR1*, *GAL11*, and *SIN4*. As described above, most of the double mutants were also created and analyzed in other genetic backgrounds with identical results. The *srb5Δ* mutation is lethal in combination with *paf1Δ*, *ccr4Δ*, or *sin4Δ* and has an enhanced slow-growth phenotype in combination with *cdc73Δ*, *hpr1Δ*, or *gal11Δ*. This suggests that there are redundant essential functions both within and between the two Pol II complexes.

The Paf1p-Cdc73p-Pol II complex is involved in recombination. The differential display and genetic analyses described above suggested that factors within the Paf1p-Cdc73p-Pol II complex have some similar functions. Aguilera and Klein (1) reported that the *hpr1* mutation leads to increased levels of recombination between direct repeats. We therefore asked if mutations in the other factors in the Paf1p-Cdc73p-Pol II complex demonstrated a similar phenotype. As shown in Table 3, the recombination rates in the *paf1Δ* and *cdc73Δ* strains increased 82- and 45-fold, respectively, relative to the wild type, while a 700-fold increase was observed in the *hpr1Δ* strain. In contrast, recombination rates were essentially unchanged relative to wild type in *ccr4Δ*, *sin4Δ*, and *srb5Δ* strains. This result strongly supports the idea that defects in some components of a Pol II complex, specifically the Paf1p-Cdc73p-Pol II complex, lead to elevated levels of recombination. Whether the complex is directly involved in recombination or the formation of a recombinogenic substrate, or indirectly affecting the expression of genes required for this process is discussed further below.

Cdc73p, Paf1p, and Ccr4p are required for the integrity of the cell wall. Both *paf1Δ* and *ccr4Δ* show enlarged cell morphology and use glycerol as a carbon source very poorly (52, 10). In addition, *ccr4Δ* is hypersensitive to staurosporine and to 8 mM caffeine and 0.04% SDS (21, 37, 38), an indication of weakened cell walls (8, 39). We therefore tested the possibility that other components of the Paf1p-Cdc73p-Pol II complex might also have cell wall defects. As shown in Fig. 5, both *paf1Δ* and *ccr4Δ* are hypersensitive to growth on 8 mM caffeine (Fig. 5B) and somewhat sensitive to growth on 20 μg of calcofluor per ml (Fig. 5D), another assay for cell wall integrity (39, 48). These mutant strains and the *gal11Δ* strain are inhibited by growth on 0.02% SDS (Fig. 5C). A plasmid expressing *PAF1* can correct the caffeine and calcofluor sensitivity phenotypes of *paf1Δ*, confirming that the phenotypes are caused by deletion of the *PAF1* gene (data not shown). In contrast, isogenic *cdc73Δ*, *srb5Δ*, *hpr1Δ*, and *sin4Δ* strains are no more sensitive to these cell wall-damaging agents than is the wild-type strain (Fig. 5).

TABLE 3. Mutations in *HPR1*, *PAF1*, and *CDC73* lead to elevated rates of recombination between direct repeats

Genotype ^a	Recombination rate (10 ⁻⁶) ^b	Fold increase
Wild type	3.8 ± 1.8	1
<i>hpr1Δ</i>	2,700 ± 1,400	700
<i>paf1Δ</i>	320 ± 50	82
<i>cdc73Δ</i>	170 ± 40	45
<i>ccr4Δ</i>	4.1 ± 1.9	1
<i>sin4Δ</i>	3.0 ± 1.7	1
<i>srb5Δ</i>	9.2 ± 4.8	2

^a Strains used in this table are wild type (HKY870-12A), *hpr1Δ* (HFY988-2C), *paf1Δ* (HFY2074), *cdc73Δ* (HFY2059-1A), *ccr4Δ* (HFY2085), *sin4Δ* (HFY2162), and *srb5Δ* (HFY2069).

^b Rates were calculated as described in Materials and Methods from 5-FOA resistance frequencies of strains carrying the duplication *leu2-k::ADE2-URA3::leu2-k*. Each rate was calculated from three independent fluctuation tests on three strains of the same genotype and is expressed as the mean rate ± the standard deviation of the three determinations.

FIG. 5. Mutations in *PAF1*, *CCR4*, and *GAL11* lead to increased sensitivity to cell wall-damaging agents. Isogenic wild type (WT; YJJ662) and *paf1* Δ (YJJ664), *cdc73* Δ (YJJ665), *gal11* Δ (YJJ564), *srb5* Δ (YJJ875), *ccr4* Δ (YJJ879), *sin4* Δ (YJJ832), and *hpr1* Δ (YJJ898) were grown on YPD or YPD plus the indicated additions. The cells were allowed to grow at 30°C for 3 to 4 days.

Defects in the yeast cell wall can be compensated for by osmotic stabilizers such as sorbitol. It has previously been shown that the caffeine and temperature sensitivity of *ccr4* Δ can be corrected by the addition of 1 M sorbitol to the medium (37, 38). We confirmed this observation in the genetic background used in this work and found that the ts phenotype of *gal11* Δ can also be corrected by sorbitol (Fig. 6A). Although *cdc73* Δ and *sin4* Δ are not obviously sensitive to the cell wall-damaging agents shown in Fig. 5, they are at least partially rescued at high temperature by sorbitol (Fig. 6A), indicating that these mutations do cause some cell wall defects. Although *paf1* Δ cannot be rescued by sorbitol at 38°C (Fig. 6A), it does show partial rescue at 35.5°C (Fig. 6B). We have also found that inclusion of sorbitol in liquid culture medium significantly reduces the doubling time of *paf1* Δ strains at 30°C (data not shown). Sorbitol is not sufficient to rescue *srb5* Δ and *hpr1* Δ , a finding consistent with the fact that they are not sensitive to cell wall-damaging agents. These results demonstrate that at least part of the growth defect in the *paf1* Δ , *ccr4* Δ , and possibly the *cdc73* Δ strains is due to defective cell wall formation.

Expression of cell wall biosynthetic genes is affected by *paf1* Δ , *cdc73* Δ , and *ccr4* Δ . The two phenotypes described above, i.e., increases in recombination between direct repeats and defects in cell wall integrity, have both been observed for mutations in yeast protein kinase C (*PKC1*) (23, 47). Although the direct connection between recombination and *PKC1* is not yet clear, Igual et al. (25) have provided a partial explanation for the cell wall defects by measuring a reduction in expression of several key genes in cell wall biosynthesis (*FKS1*, *MNN1*, *GAS1*, *KRE6*, and *VAN2*) in *pkc1* and *mpk1* mutant strains. We therefore analyzed the effects of mutations in the Paf1p-Cdc73p-Pol II complex on the expression of the *FKS1*, *MNN1*, *GAS1*, *KRE6*, and *VAN2* genes as shown in Fig. 7. A representative example of some of the RNA analyses are shown in Fig. 7A, and a quantitation of three independent sets of sam-

FIG. 6. The ts phenotype of *paf1* Δ , *cdc73* Δ , *ccr4* Δ , and *gal11* Δ can be corrected by the cell wall-stabilizing agent sorbitol. (A) Isogenic wild type (WT; YJJ662), *paf1* Δ (YJJ664), *cdc73* Δ (YJJ665), *gal11* Δ (YJJ564), *srb5* Δ (YJJ875), *ccr4* Δ (YJJ879), *sin4* Δ (YJJ832), and *hpr1* Δ (YJJ898) strains were grown on YPD or YPD plus 1 M sorbitol at 38°C for 4 days. (B) Isogenic wild-type (WT; YJJ662) and *paf1* Δ (YJJ664) strains were grown on YPD or YPD plus 1 M sorbitol at 35.5°C for 2.5 days.

ples are shown in Fig. 7B. We found that the *paf1* Δ mutation causes a two- to threefold decrease in the expression of several of these genes at the permissive temperature, and a four- to fivefold reduction in *FKS1* expression at 38°C. For comparison, Igual et al. (25) reported that mutations in *PKC1* and in *MPK1* (*SLT2*), the terminal MAP kinase in the *PKC1* signaling cascade, reduced *FKS1* mRNA abundance from 1.5- to 2-fold at 30°C and from 3- to 4-fold at 38°C. In addition, they found that *MNN1* and *GAS1* expression was reduced two- to threefold, while there was little effect on the abundance of the *KRE6* and *VAN2* mRNAs (25).

FKS1 expression is not significantly reduced in the *ccr4* Δ strain, a finding consistent with the observation of Liu et al. (38) that expression from an *FKS1* promoter-reporter construct is relatively unaffected by a mutation in *CCR4*. However, expression of *VAN2* and *KRE6* is reduced in the *ccr4* Δ strain and expression of *FKS1*, *GAS1*, and *VAN2* is reduced in the *gal11* Δ strain. Although the *cdc73* Δ mutation has little effect on most of the cell wall genes assayed, it does cause an almost twofold reduction in *FKS1* expression, which may help to explain the rescue by sorbitol described above. The *hpr1* Δ and *srb5* Δ strains show little effect on expression of any of these genes at 30°C, a finding that is consistent with their resistance to cell wall-damaging agents. We also confirmed that the effect of the *paf1* Δ mutation was at the level of initiation of transcription using an *FKS1*-promoter-*lacZ* reporter construct and measuring β -galactosidase activity in the mutant strain. As

FIG. 7. Expression of genes involved in cell wall biosynthesis is reduced in *paflΔ ccr4Δ* and *gal11Δ* mutants. (A) Total yeast RNA was prepared from isogenic mutant strains and probed for the indicated genes as described in Materials and Methods. (B) The results shown are based on three or more independently isolated sets of RNA. The RNA abundance was normalized to 18S rRNA, and the wild-type value is set as 1, which is shown as a dashed line in each panel. The RNA in the panel labeled *FKS1* at 38°C was isolated from cells shifted from 30 to 38°C and incubated for 5 h. The yeast strains used for RNA isolation were wild type-YJJ662, *paflΔ*-YJJ664, *cdc73Δ*-YJJ665, *gal11Δ*-YJJ564, *srb5Δ*-YJJ875, *ccr4Δ*-YJJ879, and *hpr1Δ*-YJJ898.

FIG. 8. Interactions between *PAF1* and *MPK1*. (A) A strain heterozygous for *paflΔ* and *mpk1Δ* (YJJ998) was sporulated, and tetrads were dissected. The figure shows 6 of 30 tetrads, all of which showed similar results. The genotype of the spores was determined from the markers associated with the deletions. wt, Wild type; m, *mpk1Δ::URA3*; p, *paflΔ::HIS3*; mp, *mpk1ΔpaflΔ*. (B) Cell extracts were prepared from wild type (WT; YJJ755) and *paflΔ* (YJJ756) strains containing a 3HA-tagged form of Mpk1p and grown at the indicated temperatures. The tagged Mpk1p was isolated and used to phosphorylate the MAP kinase substrate MBP as described by Zarzov et al. (62). The data represent the phosphorylation of MBP normalized to the amount of HA-tagged Mpk1p in each extract.

shown in Table 2, we observed an almost eightfold reduction in activity from the reporter construct in the *paflΔ* strain relative to the wild type, which is very similar to the effect observed for a mutation in *PKC1* (25). These results are consistent with the theory that the *Pafl1p-Cdc73p-Pol II* complex is involved in the *Pkc1p*-dependent pathway affecting cell wall biosynthesis.

The *Pafl1p-Cdc73p-Pol II* complex functions in the *Pkc1p-Mpk1p* MAP kinase cascade. If the *Pafl1p-Cdc73p-Pol II* complex functions in the same pathway as the *Pkc1p*-MAP kinase cascade, then combining a mutation in *PKC1* or *MPK1* with a *PAF1* mutation should not have a more deleterious effect on cell wall integrity than either single mutation. The phenotype of mutations in *MPK1* are less severe and more variable in different strains than is the phenotype of a mutation in *PKC1* (43). We therefore created *paflΔ mpk1Δ* double mutants in two different genetic backgrounds which varied in the extent of the effect of the *mpk1Δ* mutation. Spores dissected from both *mpk1Δ-MPK1 paflΔ-PAF1* heterozygous diploids were all viable (Fig. 8A). The double-mutant spores were slow growing, ts and caffeine sensitive, and indistinguishable from the *paflΔ* parent. We also created *paflΔ pkc1Δ* double mutants and again found that all of the doubly mutant spores were viable and dependent on sorbitol like the *pkc1Δ* parent (data not shown). The *paflΔ pkc1Δ* double mutant did grow somewhat more slowly than either of the single mutations. However, when we analyzed the abundance of *FKS1* mRNA in the *paflΔ*

mpk1Δ and *paf1Δ pkc1Δ* double mutants we found no additional reduction in expression relative to the single mutants (data not shown). We conclude from these results that the similar effects of the *PAF1* gene and the *PKC1* and *MPK1* genes on cell wall biosynthesis are due to their participation in the same regulatory pathway.

Does the Paf1p-Cdc73p-Pol II complex function up- or downstream of the MAP kinase cascade? To position the Pol II complex in this pathway, we asked if the *paf1Δ* phenotype could be corrected by overexpression of Pkc1p or Mpk1p. We found that the addition of neither single- nor high-copy plasmid forms of *PKC1* nor high-copy forms of *MPK1* would correct the phenotype of a mutation in *PAF1* (data not shown), which is consistent with the possibility that the Paf1p-Cdc73p-Pol II complex functions downstream of the *PKC1-MPK1* cascade. If the Paf1p-Cdc73p-Pol II complex does function downstream of the kinase cascade, then the terminal kinase, Mpk1p, should be fully active in *paf1Δ* and *cdc73Δ* strains under conditions which activate Pkc1p (heat shock). We confirmed this supposition by measuring the activity of a tagged form of Mpk1p isolated from *paf1Δ* and *cdc73Δ* strains. In both cases, Mpk1p was as active in the mutant strains as in the wild-type strain (Fig. 8B and data not shown). Paf1p and Cdc73p therefore appear to function downstream of the activation of the kinase cascade, presumably at the level of transcription of the genes affected by Pkc1p and Mpk1p.

DISCUSSION

A large and growing collection of proteins has been identified as mediators or coactivators critical for communicating signals from transcriptional activators and repressors to RNA Pol II (reviewed in reference 6). In this work we have extended the description of a unique collection of proteins associated with Pol II, each of which appears to function as a transcriptional mediator. The first components of this complex to be identified, Paf1p and Cdc73p, are not encoded by essential genes but are required for the normal expression of a subset of yeast genes (51, 52). In this work we have shown that Ccr4p and Hpr1p, also encoded by nonessential genes, are also components of this Pol II complex, where they play a role in the expression of subsets of yeast genes. Although mutations in *PAF1*, *CDC73*, *HPR1*, and *CCR4* lead to complex changes in expression patterns and, in some cases, to very different phenotypes, we have established that many of the functions of the Pol II complex are consistent with a specific role in the protein kinase C signaling cascade. In particular we have found that Paf1p, Ccr4p, and possibly Cdc73p play a role in the expression of genes required for cell wall biosynthesis and that Paf1p, Cdc73p, and Hpr1p are required for normal levels of recombination between direct repeats. As outlined in the introduction, similar effects on both the expression of cell wall biosynthetic genes and on recombination have been associated with defects in *PKC1* (see Fig. 9).

Paf1p, Cdc73p, Hpr1p, and Ccr4p are found together in a complex with Pol II. We have shown that Hpr1p and Ccr4p are present in the previously described Paf1p-Cdc73p-Pol II complex. Two very different isolation procedures, affinity isolation of a transcriptionally active form of RNA Pol II and isolation of GST-tagged forms of Cdc73p, Paf1p, Hpr1p, and Tfg2p have established that these four proteins exist in a complex with RNA Pol II, TFIIB, TFIIF, and the Gal11p-Rgr1p-Sin4p subcomplex. None of these proteins is present in the Srbp-containing form of Pol II (38, 51, 63), which further supports our earlier evidence that the complex is biochemically distinct from the mediator-containing "holoenzyme." We cannot cur-

rently rule out the possibility that more than one complex containing subsets of these proteins exists in yeast cells, but the fact that Hpr1p and Ccr4p colocalize with tagged forms of both Cdc73p and Paf1p, and the fact that Ccr4p, Paf1p, and Cdc73p are all found in a tagged Hpr1p complex is consistent with this being a single complex. Does this complex, like the Srbp-containing form of Pol II (29), have homologues in other eukaryotes? Currently, there are no examples of homologues of Paf1p, Cdc73p, or Hpr1p, although homologues of Ccr4p can be found in the database. Since the Paf1p-Cdc73p form of Pol II is present in low amounts in yeast cells (51), the failure to have observed homologues in other systems may be due to low abundance of the gene products or a restricted distribution of the factors.

Although a major fraction of Paf1p and Cdc73p appears to be in the Pol II-associated complex (51), a smaller fraction of Hpr1p colocalizes with Paf1p and Cdc73p (Fig. 1), and Ccr4p is clearly not exclusively in this Pol II complex. There is evidence that Ccr4p is present in at least one other distinct complex in the yeast cell associated with Caf1p, Not1p, Not2p, and Not3p (21, 38). We have found little or no Caf1p, Not1p, Not2p, or Not3p in the Paf1p-Cdc73p-Pol II complex or in the original collection of affinity-isolated proteins associated with Pol II (data not shown), so the Pol II complex described in this work is clearly distinct from the Not complex. Consistent with the evidence that Ccr4p is present in more than one complex, it also appears to play multiple roles in the expression of genes, based on the fact that many of its properties are quite different than those of Paf1p. For example, despite some overlap, the spectrum of transcripts affected by the two genes is quite different (Fig. 3 and 7 and data not shown). In addition, a mutation in *CCR4* causes sensitivity to staurosporine (21) and to 6-azauracil (data not shown); neither of these phenotypes is observed in *paf1* or *cdc73* mutant strains; *hpr1* mutants are slightly sensitive to 6-azauracil but not to staurosporine (7; data not shown). Finally, a mutation in *CCR4* leads to subtle defects in the cell cycle (37), while we have not observed any cell cycle effects of mutations in *PAF1* or *CDC73*.

Phenotypes of *paf1*, *cdc73*, *hpr1*, and *ccr4* mutations are consistent with a role in Pkc1p signaling. Although the phenotypes of mutations in the different Pol II-associated factors are not identical, there are some shared patterns, including the facts that mutations in *PAF1* and *CCR4* cause sensitivity to caffeine, calcofluor, and SDS (Fig. 5); the ts phenotype of mutations in *CDC73* and *CCR4*, and to a lesser extent, *PAF1* can be rescued by sorbitol (Fig. 6); and mutations in *HPR1*, *PAF1*, and *CDC73* all result in elevated rates of recombination (Table 3).

The mechanism for the sensitivity to the cell wall-damaging agents can be directly explained, at least in part, by the effects of the mutations on the expression of certain genes involved in cell wall biosynthesis. For example, mutations in *FKS1* and *KRE6* are hypersensitive to calcofluor (8, 39); a *PAF1* mutation reduces the abundance of both mRNAs, and a *CCR4* mutation reduces the abundance of *KRE6* mRNA (Fig. 7). In addition, a mutation in *GAS1*, whose mRNA abundance is reduced by *paf1*, causes sensitivity to SDS (39). The complete explanation of the altered patterns of expression leading to the sensitivity to cell wall-damaging agents is, however, far more complex than that shown in Fig. 7. Lussier et al. (39) recently reported the identification of 82 yeast genes whose products are important for cell wall integrity. It is interesting that the genetic screen used in this analysis did not identify *PAF1* or *CCR4*, confirming the authors' conclusion that there are many additional genes yet to be identified. Knowledge of the expression patterns of many or all of these genes will be required to

understand the defects in the various pathways. It is, however, useful to note that the patterns that we observe for the cell wall biosynthetic genes shown in Fig. 7 are very similar to the alterations in mRNA abundance observed by Igual et al. (25) for mutations in *PCK1* and *MPK1*, indicating that much of the effect of the mutations in the kinase-encoding genes can be explained by the properties of the Pol II-associated factors.

Connections between transcription and recombination. To establish that the effects of the mutations in the Pol II-associated factors were at the level of transcriptional initiation, we used promoter-reporter constructs to confirm the results of the RNA analysis described above. Although the results of the β -galactosidase reporter assays agreed with the mRNA analyses, the effects were more dramatic in the reporter assays. For example a *PAF1* mutation reduces the level of *FKS1* mRNA 2.5-fold but reduces the level of expression from the *FKS1* promoter construct nearly 8-fold (Fig. 7 and Table 2). We saw similar effects with *CYC1* mRNA and a *CYC1* reporter construct with 3- to 4-fold and 12-fold reductions, respectively, in a *paf1* background (Fig. 3 and Table 2). This same phenomenon has been observed by Fan and Klein (14) and Chavez and Aguilera (7) for expression of *GAL10* and *GAL1* mRNAs versus expression from promoter-fusion constructs in an *HPR1* mutant background. Chavez and Aguilera (7) have shown that the discrepancy is due to an elongation pause site in the *lacZ* coding region causing reduced abundance of full-length RNA in *hpr1* cells. Does the fact that we see a similar discrepancy for *paf1* indicate that these genes play a direct role in transcriptional elongation? This seems unlikely since we have shown that unlike *GAL1* expression in *hpr1*, the level of *GAL1*, *7+10*, *CYC1*, and *FKS1* mRNAs (plus many other mRNAs shown in Fig. 3 and 7) are significantly reduced in *paf1* and, in some cases, the *ccr4* and *cdc73* backgrounds. It is probable that the additional effects that we see with the reporter constructs are due to secondary effects on elongation through the *lacZ* gene.

Chavez and Aguilera (7) have also speculated that the elongation pause site in *lacZ* is the cause of the increased levels of recombination seen in the constructs used for analyzing changes in resolution of direct repeats. Mutations in *PAF1*, like mutations in *HPR1*, increase recombination and show enhanced effects with *lacZ* reporter constructs relative to results with direct RNA analysis. Therefore, our results are consistent with the idea that transcriptional pauses in the *lacZ* gene correlate with increased recombination. We cannot rule out the possibility that mutated forms of the Paf1p-Cdc73p-Pol II complex are directly involved in a transcriptional pause leading to increased levels of recombination. However, this complex also contains initiation factors TFIIB and TFIIF and was originally identified in association with the nonphosphorylated, initiating form of RNA Pol II (51, 58). In addition, mutations in *PAF1* and *CDC73* do not result in sensitivity to 6-azauracil (data not shown), a phenotype often indicative of defects in transcriptional elongation (3, 7). It is therefore equally likely at this point that the effects of Hpr1p, Paf1p, and Cdc73p on recombination may be indirect through changes in expression of factors required for transcriptional elongation or recombination. Since a mutation in *HPR1* often leads to increased mRNA abundance (see Fig. 3 and 7), perhaps increased expression of a factor responsible for formation of a recombinogenic substrate leads to the dramatic increases in recombination.

The Paf1p-Cdc73p-Pol II complex is downstream of the Pkc1p-MAP kinase cascade. As described above, many of the phenotypes of mutations in the *PKC1-MPK1* pathway are mimicked by mutations in *PAF1*, *CDC73*, *CCR4*, and *HPR1*. The observation that *paf1 mpk1* and *paf1 pkc1* double mutants do not demonstrate an enhanced phenotype (Fig. 8 and data not

FIG. 9. A model for the interactions between the Paf1p-Cdc73p-Pol II transcription complex and the Pkc1p-Mpk1p protein kinase cascade. An explanation of the model is provided in the text.

shown), strongly supports the theory that all of these factors function in the same pathway. Our results do not rule out the possibility that the Pol II complex is integrating inputs from more than one signaling pathway. The fact that many double mutants of factors within the complex (*paf1 ccr4*, *paf1 hpr1*, *cdc73 ccr4*, and *ccr4 hpr1*) are synthetically lethal and cannot be rescued by sorbitol is consistent with this view. Our results are therefore in agreement with the conclusions of Hata et al. (21), who determined that Ccr4p might be functioning in both the Pkc1p and an independent Caf1p(Pop2p) pathway. The existence of distinct complexes and pathways is also consistent with the fact that Caf1p is not present in the Paf1p-Cdc73p-Pol II complex.

Where is the Paf1p-Cdc73p-Pol II complex in the signaling pathway? The following points support the model shown in Fig. 9, placing the Pol II complex downstream of the kinase cascade in a position to directly affect the transcription of target genes. First, we have found that overexpression of Pkc1p or Mpk1p does not suppress a *paf1* defect (data not shown). Second, although the abundance of *PKC1* mRNA is reduced about twofold in *paf1* cells, we have found little or no effect of mutations in the other polymerase-associated factors on the expression of *PKC1* (data not shown), thus indicating that the defect is not due to critically reduced levels of Pkc1p. Third, as shown in Fig. 8B, the terminal MAP kinase, Mpk1p, is fully activated by heat stress in a *paf1* strain, indicating that the *paf1* defect is not due to failure of the kinase cascade to be activated. Finally, although the phenotypes of *paf1* encompass many of the defects seen in a *pkc1* mutant strain, mutations in *HPR1* and *CCR4* each only encompass a subset of the phenotypes, indicating that these factors are more likely to be downstream of Pkc1p.

One difficulty in trying to determine the order of the factors in this pathway is the variable phenotype of mutations in *MPK1*. As demonstrated by Madhani et al. (41), complete deletion of a MAP kinase as done in this study and in many other analyses of the *PKC1-MPK1* pathway can result in inaccurate conclusions about the necessity for the kinase. It is possible that when the kinase protein is absent other related gene products can compensate for the deficiency, whereas when a defective form of the kinase protein is present a neg-

ative phenotype might be observed. Since another potential MAP kinase encoded by the YKL161C gene (24) has also been shown to play a role in cell wall integrity (60), it may be that the role of both kinases will have to be evaluated to arrive at a clear picture of the biochemical signaling pathway. These same issues may help to explain why Huang and Symington (22, 23) found that mutations in *PKC1*, but not in other downstream components of the pathway, lead to increases in recombination. Redundant factors in the cascade may mask the effects on recombination in the deletion strains used for these analyses. Without further clarification of this issue, we have used a separate dashed line in Fig. 9 to indicate the uncertainty in the path of the signal from Pkc1p to the Pol II complex for this activity.

There are two possible models for how the Pol II-associated factors respond to the signals from the kinase cascade. First, Mpk1p may directly phosphorylate one or more of the Pol II-associated factors and alter their activity or ability to assemble into the complex. We have, in fact, observed that Cdc73p is a substrate for Mpk1p in vitro (data not shown). However, we have not detected any direct association of Mpk1p with the polymerase-associated factors. A second possibility, in which the Paf1p-Cdc73p-Pol II complex responds to changes in the phosphorylation state of known targets of Mpk1p, currently seems more likely. Mpk1 is known to associate with and/or phosphorylate several transcriptional regulatory factors, including the SBF complex composed of Swi4p and Swi6p (40), Rlm1p (12, 59, 60), and the HMG-1-like proteins Nhp6Ap and Nhp6Bp (9). There is evidence that each of these factors plays a role in the Pkc1p-Mpk1p pathway leading to maintenance of cell wall integrity. Ultimately, it will be interesting to study the interactions between the Paf1p-Cdc73p-Pol II complex and these regulatory factors to determine the role of the polymerase-associated factors in the expression of Pkc1p-responsive genes.

ACKNOWLEDGMENTS

We thank A. Johnson, L. Johnston, D. Stillman, and M. Snyder for disruption and expression constructs; R. Sclafani for helpful advice and yeast strains; M. Christman for the anti-Hpr1p antibody; R. Young for the anti-Srb5p antibody; T. Fukasawa for the anti-Gal11p antibody; and L. Johnston for the RNA probes.

These studies were supported by NIH grants GM 38101 (J.A.J.), GM 30439 (H.K.), and GM 41215 (C.L.D.).

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