The Spt4p Subunit of Yeast DSIF Stimulates Association of the Paf1 Complex with Elongating RNA Polymerase II

Hongfang Qiu, Cuihua Hu, Chi-Ming Wong, and Alan G. Hinnebusch*

Laboratory of Gene Regulation and Development, National Institute of Child Health and Human Development, Bethesda, Maryland 20892

Received 8 December 2005/Accepted 12 January 2006

The Paf1 complex (Paf1C) interacts with RNA polymerase II (Pol II) and promotes histone methylation of transcribed coding sequences, but the mechanism of Paf1C recruitment is unknown. We show that Paf1C is not recruited directly by the activator Gcn4p but is dependent on preinitiation complex assembly and Ser5 carboxy-terminal domain phosphorylation for optimal association with *ARG1* coding sequences. Importantly, Spt4p is required for Paf1C occupancy at *ARG1* (and other genes) and for Paf1C association with Ser5-phosphorylated Pol II in cell extracts, whereas Spt4p-Pol II association is independent of Paf1C. Since *spt4Δ* does not reduce levels of Pol II at *ARG1*, Ser5 phosphorylation, or Paf1C expression, it appears that Spt4p (or its partner in DSIF, Spt5p) provides a platform on Pol II for recruiting Paf1C following Ser5 phosphorylation and promoter clearance. *spt4Δ* reduces trimethylation of Lys4 on histone H3, demonstrating a new role for yeast DSIF in promoting a Paf1C-dependent function in elongation.

Transcription by RNA polymerase II (Pol II) is a multistep process that includes preinitiation complex (PIC) assembly, open complex formation, initiation, promoter clearance, elongation, and termination. Phosphorylation of Ser5 in the Pol II carboxy-terminal domain (CTD) by the kinase subunit of TFIIF (Kin28p) stimulates promoter clearance and the transition from initiation to elongation. Ser5 phosphorylation also mediates cotranscriptional capping of mRNA by enhancing the recruitment and activity of the capping enzyme (reviewed in reference 42). Phosphorylation of Ser2 in the CTD by Ctk1p is restricted to elongating Pol II, and there is evidence that Ser2 phosphorylation increases, while Ser5 phosphorylation decreases, as Pol II moves from the promoter (13). Consistent with this, Ser2 phosphorylation stimulates recruitment of 3'-end processing factors (1, 10).

CTD phosphorylation is also required for methylation of histones in the coding sequences. Set1p complex (COMPASS) methylates Lys4 in histone H3 (H3-K4) near the 5' ends of transcribed genes (27, 37). Set1p associates with Ser5-phosphorylated Pol II and is recruited to the 5' ends of transcribed genes dependent on Kin28p (27). Set2p methylates H3-K36 throughout the open reading frame (ORF) (16, 52), and CTD Ser2 phosphorylation stimulates recruitment of Set2p (16, 18, 52). Interactions of Set1p and Set2p with phosphorylated Pol II and the attendant methylation events also depend on Paf1 complex (Paf1C) (14, 16, 27, 51). Paf1C further stimulates Set1p function by promoting H2B ubiquitylation by Rad6p/Bre1p complex (7, 26, 49, 51), a prerequisite for H3-K4 methylation (4, 44).

Paf1C was first identified in yeast as a group of proteins associated with unphosphorylated Pol II (47) and consists of five core subunits: Paf1p, Cdc73p, Rtf1p, Leo1p, and Ctr9p (12, 15, 24, 39, 40, 43). Paf1C forms a stoichiometric complex

with Pol II (39), and deletion of Rtf1p or Cdc73p dissociates the remaining Paf1C subunits from chromatin (25). Unlike the Pol II-associated mediator, which is restricted to promoters, Paf1C is often detected at equivalent or higher levels in coding regions (10, 15, 25, 31, 41). It is unclear whether the apparent association of Paf1C with promoters represents an interaction with the PIC or with Pol II in the early stages of elongation.

Paf1C mutants show reduced expression of diverse mRNAs (29, 32, 45), but the impaired Paf1C-dependent step(s) are generally unknown. Genetic data suggest that Paf1C has functions overlapping that of elongation factors Spt4p/Spt5p (DSIF), Ppr2p (TFIIS), and Spt16p/Pob3p (FACT) (3, 43), and *paf1Δ* and *cdc73Δ* extracts show transcription elongation defects (36). However, essentially wild-type (WT) occupancy of Pol II in the *CLN1* ORF was observed in *cdc73Δ* and *rtf1Δ* mutants (25), and these mutations did not reduce the rate or processivity of elongation during transcription of a Gal4p-induced gene (23). Moreover, recent findings suggest that Paf1C is critically required for 3'-end processing during termination. Thus, *rtf1Δ* and *paf1Δ* mutants show defects in recruitment of cleavage/polyadenylation factor and exhibit shorter poly(A) tails (25), and *paf1Δ* can reduce mRNA stability by allowing aberrant utilization of downstream poly(A) sites (29).

The mechanism of Paf1C recruitment to transcribed genes is not well understood. Based on the original method of purification, it was surmised that Paf1C interacts with Pol II independently of CTD phosphorylation (47). Consistent with this, recruitment of Paf1C to transcribing Pol II in vivo is independent of Ser2 phosphorylation by Ctk1p (1). However, the role of Kin28p and Ser5 phosphorylation in Paf1C recruitment has not been examined. The copurification of Paf1C with initiation factors TFIIB and TFIIF (39) and elongation factors Spt5p (43) and Pob3p/Spt16p (15, 43) could indicate that Paf1C is tethered to Pol II indirectly by one of these factors. While this work was in progress, it was shown that cells lacking Bur2p, the cyclin partner of cyclin-dependent protein kinase Bur1p, exhibit decreased recruitment of Paf1C to Pol II in the *PMAl*

* Corresponding author. Mailing address: NIH, Building 6A, Room B1A-13, Bethesda, MD 20892. Phone: (301) 496-4480. Fax: (301) 496-6828. E-mail: ahinnebusch@nih.gov.

TABLE 1. Yeast strains used in this study

Name	Parent	Relevant genotype ^b	Reference
Myc-tagged strains			
HQY389	BY4741 ^a	<i>PAF1-myc₁₃::HIS3*</i>	This work
HQY397	249 ^a	<i>PAF1-myc₁₃::HIS3* gcn4Δ::kanMX4</i>	This work
HQY855	HQY700	<i>PAF1-myc₁₃::HIS3* arg1-ΔTATA</i>	This work
HQY889	5326 ^a	<i>PAF1-myc₁₃::HIS3* cdc73Δ::kanMX4</i>	This work
HQY890	4611 ^a	<i>PAF1-myc₁₃::HIS3* rtf1Δ::kanMX4</i>	This work
HQY891	508 ^a	<i>PAF1-myc₁₃::HIS3* mft1Δ::kanMX4</i>	This work
HQY892	2861 ^a	<i>PAF1-myc₁₃::HIS3* thp2Δ::kanMX4</i>	This work
HQY893	1191 ^a	<i>PAF1-myc₁₃::HIS3* tex1Δ::kanMX4</i>	This work
HQY903	3385 ^a	<i>PAF1-myc₁₃::HIS3* isw1Δ::kanMX4</i>	This work
HQY904	6160 ^a	<i>PAF1-myc₁₃::HIS3* chd1Δ::kanMX4</i>	This work
HQY905	6986 ^a	<i>PAF1-myc₁₃::HIS3* spt4Δ::kanMX4</i>	This work
HQY906	4411 ^a	<i>PAF1-myc₁₃::HIS3* dst1Δ::kanMX4</i>	This work
HQY907	4425 ^a	<i>PAF1-myc₁₃::HIS3* rad6Δ::kanMX4</i>	This work
HQY908	3771 ^a	<i>PAF1-myc₁₃::HIS3* bre1Δ::kanMX4</i>	This work
HQY913	1257 ^a	<i>PAF1-myc₁₃::HIS3* set2Δ::kanMX4</i>	This work
HQY914	7028 ^a	<i>PAF1-myc₁₃::HIS3* ctk1Δ::kanMX4</i>	This work
HQY915	4276 ^a	<i>PAF1-myc₁₃::HIS3* dot1Δ::kanMX4</i>	This work
HQY948	2379 ^a	<i>PAF1-myc₁₃::HIS3* leo1Δ::kanMX4</i>	This work
HQY963	HQY957	<i>PAF1-myc₁₃::HIS3* kin28Δ::kanMX4[LEU2 KIN28-HA]</i>	This work
HQY964	HQY958	<i>PAF1-myc₁₃::HIS3* kin28Δ::kanMX4[LEU2 kin28-HA-ts16]</i>	This work
HQY965	HQY959	<i>PAF1-myc₁₃::HIS3* kin28Δ::kanMX4[LEU2 KIN28-HA] gcn4Δ::kanMX4</i>	This work
HQY997	BY4705	<i>PAF1-myc₁₃::HIS3*</i>	This work
HQY998	SHY508	<i>PAF1-myc₁₃::HIS3* kin28-L83G</i>	This work
HQY1001	4175 ^a	<i>PAF1-myc₁₃::HIS3* bur2Δ::kanMX4</i>	This work
HQY993	BY4741 ^a	<i>PAF1-3HA::kanMX6</i>	This work
HQY994	BY4741 ^a	<i>SPT4-myc₇::HIS3* PAF1-3HA::kanMX6</i>	This work
HQY974	BY4741 ^a	<i>SPT4-myc₇::HIS3*</i>	This work
HQY975	249 ^a	<i>SPT4-myc₁₃::HIS3* gcn4Δ::kanMX4</i>	This work
HQY980	4611 ^a	<i>SPT4-myc₇::HIS3* rtf1Δ::kanMX4</i>	This work
HQY983	5326 ^a	<i>SPT4-myc₇::HIS3* cdc73Δ::kanMX4</i>	This work
HQY981	HQY974	<i>SPT4-myc₇::HIS3* arg1-ΔTATA</i>	This work
HQY987	HQY957	<i>SPT4-myc₇::HIS3* kin28Δ::kanMX4[LEU2 KIN28-HA]</i>	This work
HQY988	HQY958	<i>SPT4-myc₇::HIS3* kin28Δ::kanMX4[LEU2 kin28-HA-ts16]</i>	This work
HQY989	HQY959	<i>SPT4-myc₇::HIS3* kin28Δ::kanMX4[LEU2 KIN28-HA]</i>	This work
HQY403	BY4741 ^a	<i>RPB3-myc₁₀::HIS3*</i>	34
HQY422	BY4741 ^a	<i>RPB3-myc₁₀::HIS3* gcn4Δ::hisG</i>	34
HQY670	5326 ^a	<i>RPB3-myc₁₃::HIS3* cdc73Δ::kanMX4</i>	This work
HQY928	4611 ^a	<i>RPB3-myc₁₃::HIS3* rtf1Δ::kanMX4</i>	This work
Untagged strains			
BY4741 ^a			Research Genetics
249 ^a		<i>gcn4Δ::kanMX4</i>	Research Genetics
HQY700	BY4741 ^a	<i>arg1-ΔTATA</i>	This work
HQY957	BY4741 ^a	<i>kin28Δ::kanMX4[LEU2 KIN28-HA]</i>	This work
HQY958	BY4741 ^a	<i>kin28Δ::kanMX4[LEU2 kin28-HA-ts16]</i>	This work
HQY959	249 ^a	<i>kin28Δ::kanMX4[LEU2 KIN28-HA] gcn4Δ::kanMX4</i>	This work
BY4705		<i>MATa ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0</i>	21
SHY508	BY4705	<i>kin28-L83G</i>	21

^a Purchased from Research Genetics.^b *HIS3** designates the *HIS3* allele from *Saccharomyces kluyveri*.

ORF and reduced H2B-K123 ubiquitylation of bulk histones (17, 50). The defect in ubiquitylation seems to stem from impaired Paf1C recruitment and loss of Rad6p phosphorylation on Ser120 by Bur1p/Bur2p (50). However, it is not understood how Bur1p/Bur2p kinase activity promotes Paf1C association with Pol II.

In this report, we show that deletion of the Spt4p subunit of elongation factor DSIF impairs Paf1C recruitment by activator Gcn4p without reducing Pol II occupancy in the *ARG1* ORF, consistent with a direct role for Spt4p in recruiting Paf1C to elongating Pol II. Supporting this idea, we found that Paf1C is preferentially associated with Pol II phosphorylated on Ser5 in cell extracts in a manner dependent on Spt4p and that inactivation of Kin28p impairs both Paf1C recruitment to *ARG1* and Paf1C-Spt4p association in cell extracts. Since Spt4p is recruited to the *ARG1* ORF independently of Paf1C, we propose that DSIF provides a platform on elongating Pol II for Paf1C recruitment, thereby enhancing Paf1C functions during transcription elongation.

MATERIALS AND METHODS

Yeast strains and plasmids. All yeast strains used in this work are listed in Table 1. The wild-type parent strain BY4741 and deletion derivatives thereof were described previously (48) and purchased from Research Genetics. The presence of all reported deletion alleles was confirmed by PCR amplification or complementation of mutant phenotypes by plasmid-borne wild-type genes. Myc-

tagged strains were constructed as described previously (45), as were strains carrying *arg1-ΔTATA* (34). To construct *kin28Δ::kanMX* strains harboring episomal *KIN28-HA* or *kin28-HA-ts16*, the parent strains were first transformed with *URA3* plasmid pHQ1429 carrying *KIN28-HA*. The resulting strains were transformed with a PCR fragment containing *kin28Δ::kanMX4*, synthesized using DNA from the heterozygous *kin28Δ::kanMX/KIN28* diploid as a template, and selected on G418 medium. Deletion of chromosomal *KIN28* was indicated by the conferred sensitivity to 5-fluoro-orotic acid and confirmed by PCR analysis. pHQ1429 in the confirmed *kin28Δ::kanMX* strain was replaced by *LEU2* plasmids carrying *KIN28-HA* (pHQ1430) or *kin28-HA-ts16* (pHQ1431) by plasmid shuffling.

Plasmids pHQ1429 and pHQ1430 were created by inserting the HindIII-BamHI fragment containing *KIN28-HA* from pGK13 (11) between the corresponding sites of YCplac33 and YCplac111, respectively. pHQ1431 was created by inserting the EcoRI fragment containing *kin28-HA-ts16* from pGK33 (11) into YCplac111. pHQ1437 carries the *SPT4* ORF plus 329 bp and 298 bp of 5' and 3' flanking sequences, respectively, synthesized by PCR using genomic DNA as the template and cloned between the EcoRI and SalI sites of YCplac33.

Biochemical methods. The chromatin immunoprecipitation (ChIP) experiments were conducted as described previously (45) using the primers and antibodies described below, except that sulfometuron induction was carried out for 30 min, the time of maximal Gcn4p binding at *ARG1* (5). The *ARG1* upstream activation sequence (UAS) and TATA primer pairs used for ChIP analysis were described previously (34, 45). The following additional primer pairs were used to amplify the indicated *ARG1* sequences: (i) 5' ORF, 5' TGGCTTATCTGGTG GTTTAG 3' (nucleotide positions +23 to +43) and 5' ATCCACACAAAC GAACITGCA 3' (+186 to +166); (ii) middle ORF, 5' ACATTCTTACG AG GCAGGTA 3' (+557 to +577) and 5' CTTGTGTCGGTGTAGGTCA 3' (+720 to +701); (iii) 3' ORF, 5' TTCTGGGCAGATCTACAA AGA 3' (+1091 to +1111) and 5' AAGTCAACT CTCACCTTTGG 3' (+1258 to +1238); (iv) down1, 5' CTTCTGAGAACAGACATTG 3' (+1489 to +1509) and 5' TTCCTTAT CAGTCAATTGAGG 3' (+1643 to +1623); (v) down2, 5' GTTTATTCCTCGT TGTGAGGT 3' (+1880 to +1900) and 5' TACCAATATCAATTAGCTCA TTG 3' (+2040 to +2018). The primer pairs used to amplify *PMAI* and *PYK1* sequences are as follows: (i) *PMAI* TATA, 5' CGATTATATAAAAAGGCCAAATAT 3' (−341 to −318) and 5' TATCAACG AGGTTGATAGAAAAA 3' (−171 to −193); (ii) *PMAI* 5' ORF, 5' CGACGACGAAGACAGTG ATA 3' (+168 to +187) and 5' ATTCTTTTCGTCAGCCATTG 3' (+331 to +310); (iii) *PYK1* TATA, 5' CATCAAAAACGATATTCGT TGG 3' (−235 to −215) and 5' AAC CAAAGGATGAA AAAGAATG 3' (−80 to −101); (iv) *PYK1* 5' ORF, 5' ACAAGTCTGTCTATTGACAACG 3' (+179 to +199) and 5' CAT CGG TGG TGA AGA TCA TTT 3' (+343 to +323). The primer pair used for an internal control amplifies a nontranscribed intergenic sequence from chromosome V: 5' CCGATTGTGAGATTCTTCCT 3' and 5' GAACAAGGTTACAAATCC TGA T 3'.

Northern analysis was carried out as described previously (34). Western analysis of whole-cell extracts (WCEs) was conducted using WCEs prepared by trichloroacetic acid precipitation (35), and coimmunoprecipitation assays were conducted as described previously (53), using the antibodies described below.

Antibodies. The following antibodies were employed: monoclonal anti-Myc and anti-hemagglutinin (HA) (Roche), anti-Rpb3p (Neoclone), anti-phospho-Ser5 (H14; Covance), anti-Rpb1p (8WG16; Covance) and anti-histone H3 (05-499; Upstate); polyclonal anti-Gcd6p (2), anti-Spt4 and anti-Spt5 (gifts from G. Hartzog), anti-Rtf1p (gift from K. Arndt), anti-trimethyl-histone H3 (Lys4) (abcam, ab8580), and anti-dimethyl-histone H3 (Lys79) (07-366; Upstate).

RESULTS

The Paf1 complex is recruited by Gcn4p to the *ARG1* coding region, dependent on transcription initiation. We showed previously that deletion of *PAF1* produced Gcn[−] phenotypes, indicating impaired transcriptional activation by Gcn4p under conditions of amino acid limitation where Gcn4p synthesis is induced. Using ChIP, we found that Gcn4p recruits a low level of Myc-tagged Paf1p to the promoter of its target gene, *ARG1* (45). Here we found that Gcn4p recruits Myc-Paf1 at a much higher level to the ORF region of *ARG1*. Induction of Gcn4p by starvation for isoleucine and valine produced ~6-fold higher levels of Myc-Paf1p cross-linked to the 5' and 3' ends of the *ARG1* ORF than in isogenic *PAF1-myc* cells lacking Gcn4p

(Fig. 1A and B). The ratio of Myc-Paf1p occupancy in *GCN4* versus *gcn4Δ* cells is plotted in Fig. 1C for different locations at *ARG1*. The results show that Gcn4p-dependent Paf1p binding occurred at much lower levels in the UAS and TATA regions and immediately downstream of *ARG1* compared to that in the ORF sequences (Fig. 1C). The distribution of Paf1p across *ARG1* is similar to that described for other yeast genes (9, 10). As expected, Gcn4p showed high-level binding to the UAS and TATA regions but was detected in the ORF at levels only slightly above the nonspecific background levels of *gcn4Δ* cells (Fig. 1E). (Because the UAS and TATA elements are separated by <200 bp and the sheared chromatin fragments are ~500 bp, most fragments containing the TATA element also contain the UAS, likely accounting for the apparent binding of Gcn4p to the TATA region.)

To determine whether Paf1C recruitment by Gcn4p is mediated by Pol II, we examined the effect of impairing PIC formation on Paf1p occupancy. In agreement with previous results, deletion of the *ARG1* TATA element lowered Pol II (i.e., Rpb3p) occupancy in the promoter and ORF regions (Fig. 1D) and produced commensurate reductions in the steady-state level of *ARG1* mRNA without affecting levels of mRNAs induced from other Gcn4p target genes (Fig. 1F). The *ΔTATA* mutation also decreased the size of *ARG1* transcripts, most likely reflecting aberrant initiation downstream of the normal start site (Fig. 1F). Importantly, the *ΔTATA* mutation greatly reduced Paf1p occupancy at *ARG1* (Fig. 1C), suggesting that Paf1C recruitment is dependent on PIC assembly and transcription. The small amount of shorter *ARG1* transcripts produced in the *ΔTATA* strain (Fig. 1F) probably explains the residual occupancy of Pol II and Paf1p in the *ARG1* ORF in the *ΔTATA* mutant (Fig. 1C and D).

The ratio of Paf1p to Pol II occupancy is lower in the TATA region and downstream from the ORF (Down1) than in the 5' and 3' ORF sequences at *ARG1* (cf. Fig. 1C and D and also WT results in Fig. 2A and B). The relatively low Paf1p occupancy downstream of the ORF is consistent with the finding that Paf1C is released from Pol II at the polyadenylation site (10). The comparatively low Paf1p occupancy in the TATA region suggests that Paf1C associates with Pol II only after the transition from PIC to elongation complex, as discussed further below.

To confirm that Paf1p is recruited in the context of intact Paf1C, we took advantage of the fact that deletions of Paf1C subunits Cdc73p and Rtf1p dissociate Paf1p from the *TEF1* gene in vivo (25). Consistent with this, binding of Paf1p at *ARG1* was greatly reduced in *cdc73Δ* and *rtf1Δ* cells but unaffected by deletion of *Leo1p* (Fig. 2A). Pol II occupancy was unaffected in the *cdc73Δ* and *rtf1Δ* strains (Fig. 2B), suggesting that high-level recruitment of Paf1p is not required for a WT level of elongating Pol II in the *ARG1* ORF (see also Fig. 6B). These last results fit with our finding that *cdc73Δ* and *rtf1Δ* mutants do not exhibit Gcn[−] phenotypes (45) and with previous data indicating that *cdc73Δ* and *rtf1Δ* did not reduce Pol II occupancy at *CLN1*, *TEL1*, or *TEF1* (25) nor decrease the rate or processivity of elongation during transcription of a Gal4p-induced gene (23).

Evidence that Paf1C interacts primarily with elongating Pol II. Although we detected a low level of Paf1p binding at the TATA region (Fig. 1C and 2A), it was possible that this signal

FIG. 1. Induction of Gcn4p increases Paf1p occupancy in the transcribed regions of *ARG1*. (A) Diagram depicting the *ARG1* ORF, TATA element (open box), and Gcn4p binding sites in the UAS (gray boxes). Dashed lines depict the intergenic region between *ARG1* and the adjacent gene, *YOL057w*. Names and positions (relative to the start codon) of fragments PCR amplified in ChIP analyses are given below. (B-E) ChIP analysis of Myc-Paf1p, Rpb3, and Gcn4p binding at *ARG1* following induction of Gcn4p. *PAF1-myc* strains HQY397 (*gcn4Δ*), HQY389 (WT), and HQY855 (*ΔTATA*) were cultured in synthetic complete medium lacking Ile and Val and treated with sulfometuron for 30 min to induce Gcn4p and then were subjected to ChIP analysis with anti-Myc (panel C), anti-Rpb3p (panel D), or anti-Gcn4p (panel E) antibodies. DNA was extracted from the immunoprecipitates (IP) and input chromatin (Inp) samples and subjected to PCR in the presence of [³³P]dATP to amplify the radiolabeled fragments described in panel A and a control (C) fragment from an intergenic region on chromosome V. PCR products were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography, with representative results shown in panel B. PCR products were quantified with a phosphorimager, and the ratios of *ARG1* to control signals in IP samples were normalized for the corresponding ratios for Inp samples. The resulting values measured for the *GCN4* strains were normalized to the corresponding values for *gcn4Δ* cells to yield the percent IP (*GCN4/gcn4Δ*) values plotted in histograms. (F) Northern analysis of *gcn4Δ* (249), WT (BY4741), and *ΔTATA* (HQY700) strains cultured under the conditions described above, probing for transcripts of Gcn4p target genes *ARG1*, *ARG4*, and *SNZ1* and for Pol III transcript *SCR1* analyzed as a loading control.

FIG. 2. Recruitment of Paf1p, but not Pol II, by Gcn4p requires the Paf1C subunits Cdc73p and Rtf1p. *PAF1-myc* (panel A) and *RPB3-myc* (panel B) strains containing no mutations (WT) or the indicated deletions were subjected to ChIP analysis using anti-Myc antibodies as described in the legend to Fig. 1. *PAF1-myc gcn4Δ* strain HQY397 and *RPB3-myc gcn4Δ* strain HQY422 were analyzed in each experiment as a negative control. Down1, region downstream of the ORF, as shown in Fig. 1A.

derives from chromatin fragments that contain both promoter and 5'-ORF sequences and that Paf1C associates with Pol II only after it clears the promoter. This would explain the relatively low Paf1p:Rpb3p ratio in the TATA region versus the ORF sequences noted above. To explore this possibility, we examined the effect on Paf1p recruitment of impairing the kinase Kin28p that phosphorylates Ser5 of the CTD and stimulates promoter clearance.

We first measured the occupancy of Rpb1p hyperphosphorylated on Ser5 (Ser5P) across the *ARG1* gene and found that Ser5P occupancy in WT cells was highest in the TATA region and declined from 5' to 3' across the ORF (Fig. 3A), in accordance with previous findings (13). In *kin28-ts16* cells at 37°C, by contrast, nearly background levels of Ser5P were found at all locations tested at *ARG1* (Fig. 3A). Western analysis of WCEs showed that the cellular level of Ser5P was reduced but not abolished in *kin28-ts16* cells at 37°C and that the level of Kin28p itself was decreased by the *ts16* mutation (Fig. 3B). We observed reduced Pol II occupancy in the 5' and 3' regions of the *ARG1* ORF in *kin28-ts16* cells at 37°C but no reduction in Pol II occupancy of the TATA region (Fig. 3C), consistent with a defect in promoter clearance. As expected from the WT level of Pol II binding to the promoter, there was no reduction in Gcn4p occupancy of the UAS in the *kin28-ts16* mutant (Fig. 3D). Together, the results in Fig. 3A to D indicate that inactivation of the *kin28-ts16* product had the expected effects at *ARG1* of reducing the level of Ser5P with attendant reductions in promoter clearance by Pol II. The fact that oc-

cupancy in the ORF was reduced less for Rpb3p than for Ser5P by *kin28-ts16* suggests that promoter clearance at *ARG1* can be achieved, albeit inefficiently, by Pol II molecules hypophosphorylated on Ser5. Similar results were reported previously for this (27) and other *kin28* alleles (13, 21). The presence of hypophosphorylated Pol II in the *ARG1* ORF in *kin28-ts16* cells could be attributed to the residual Ser5 phosphorylation (Fig. 3B) produced by either the *kin28-ts16* product or Srb10p (21) that is below the detection limit of the ChIP assay or to lack of an absolute requirement for Ser5 phosphorylation in promoter clearance (22, 38).

Importantly, *kin28-ts16* at 37°C reduced to nearly background levels Paf1p occupancy at both TATA and ORF regions of *ARG1* (Fig. 3E). The reductions in Paf1p occupancy produced by *kin28-ts16* (Fig. 3E) were more extensive than the corresponding reductions in total Pol II occupancy (Fig. 3C), leading to a reduced Paf1p:Rpb3p ratio in *kin28-ts16* versus WT cells at all three locations tested (Fig. 3G). This comparison suggests that Paf1C binds poorly to elongating Pol II molecules that escaped the promoter but are hypophosphorylated on Ser5. We reached the same conclusion after examining the *kin28-as* strain containing a mutant form of Kin28p that can be inactivated by the inhibitor NA-PP1 (21). As shown in the left panel of Fig. 3F, treatment of *kin28-as* cells with NA-PP1 reduced the Gcn4p-dependent occupancy of Ser5P in the *ARG1* promoter and 5' ORF sequences by ~70%. Whereas inhibition of the *kin28-as* product decreased Rpb3p occupancy of these sequences by only ~20% (middle panel), it decreased

FIG. 3. Optimal Paf1p recruitment to *ARG1* requires Kin28p-dependent phosphorylation of the Pol II CTD on Ser5. (A-E) Isogenic *PAF1-myc* strains HQY963 (*KIN28* [WT]), HQY964 (*kin28-ts16*), and HQY965 (*gcn4Δ*) were grown at 25°C to an optical density at 600 of ~0.6, transferred to 37°C for 30 min, and treated with sulfometuron for an additional 30 min at 37°C. (A) ChIP analysis was conducted as described in the legend to Fig. 1 using H14 antibodies against phosphorylated Ser5. (B) Western analysis of WCEs from the strains in panel A using H14 antibodies and antibodies against Rpb3p, Myc (to detect Myc-Paf1p), HA (to detect HA-Kin28p), and Gcd6p. (C-E) ChIP analysis was conducted as described in the legend to Fig. 1 for the strains in panel A using Rpb3p antibodies (panel C), Gcn4p antibodies (panel D), or Myc antibodies (panel E). (F) ChIP analysis was conducted on *PAF1-myc* strains HQY397 (*gcn4Δ*), HQY997 (WT), and HQY998 (*kin28-as*), which were cultured in synthetic complete medium lacking Ile and Val and treated with NA-PP1 at 12 μM for 12 min and then with sulfometuron for 30 min. (G) The ratio percent immunoprecipitation (GCN4/gcn4Δ) values for Myc-Paf1p in panel E were normalized to those for Rpb3p in panel C.

Myc-Paf1p binding to the TATA and 5' ORF sequences by ~50%. The fact that Paf1p occupancy was not reduced as much as Ser5P occupancy may indicate that Paf1C can still associate with Pol II molecules phosphorylated on a fraction of the CTD which might persist in NA-PP1-inhibited *kin28-as* cells.

We obtained independent evidence that Paf1C is associated

primarily with elongating Pol II phosphorylated on Ser5 by coimmunoprecipitation analysis of Pol II-Paf1C complexes in WCEs. Myc-Paf1p was immunoprecipitated with anti-Myc antibodies, and immune complexes were probed with H14 antibodies specific for Ser5P (employed above in ChIP analysis) or with 8WG16 antibodies that react primarily with hypophosphorylated Rpb1p. (Although it appears that 8WG16 antibod-

FIG. 4. Paf1p preferentially coimmunoprecipitates with Ser5-phosphorylated Pol II dependent on Cdc73p and Spt4p. (A) WCEs from *PAF1-myc* strains HQY389 (WT), HQY889 (*cdc73Δ*), and HQY905 (*spt4Δ*) were immunoprecipitated (IP) with antibodies against Myc, and immune complexes were subjected to Western analysis using antibodies against Myc, Ser5P (H14), hypophosphorylated Rpb1p (8WG16), Spt5p, and Spt4p. (B) WCEs of *SPT4* strain HQY993 and *SPT4-myc* strain HQY994, both containing *PAF1-HA*, were immunoprecipitated with Myc antibodies and analyzed as in panel A except using HA antibodies to detect HA-Paf1p. I, 5% of input WCE; P, 50% of immunoprecipitated pellet; S, 5% of supernatant. (C) WCEs from *SPT4-myc* strains HQY987 (WT) and HQY988 (*kin28-ts*) were immunoprecipitated with Myc antibodies and subjected to Western analysis using antibodies against Myc, Spt5p, and Rtf1p.

ies recognize Rpb1p that is Ser5 phosphorylated in a subset of heptapeptide repeats of the CTD, this species has been observed only in *ctk1Δ* cells lacking Ser2P (28).) As shown in Fig. 4, a much larger proportion of hyperphosphorylated versus hypophosphorylated Rpb1p coimmunoprecipitated with Myc-Paf1p (Fig. 4A, lanes 1 and 2), suggesting that Paf1C is preferentially associated with Ser5P. The association of Myc-

Paf1p with Ser5P is specific, because it was nearly abolished by deletion of the Paf1C subunit Cdc73p (lanes 4 to 6).

Recruitment of Paf1p and Pol II-Paf1C interaction requires elongation factor Spt4p. Our finding that Paf1C is preferentially associated with Ser5P but shows low occupancy of the TATA region where Ser5P occupancy peaks suggests that another factor associated exclusively with elongating Pol II is required for Paf1C recruitment. Hence, we measured Gcn4p-dependent recruitment of Paf1p in isogenic *PAF1-myc* strains carrying deletions of known Pol II-associated elongation factors (42). These include (i) Set2p and Dot1p, involved in histone H3 methylation; (ii) Rad6p and Bre1p, required for histone H2B ubiquitylation and H3 methylation; (iii) nucleosome-remodeling factors Chd1p and Isw1p; (iv) subunits of the THO complex (Mft1p, Thp2p, and Tex1p); (v) the Ser2 CTD kinase Ctk1p; (vi) Spt4p, a subunit of yeast DSIF; and (vii) Dst1p/TFIIS. Of the 12 deletion mutants examined, only *spt4Δ* produced a strong reduction in Paf1p occupancy (Fig. 5A and B). This defect was complemented by plasmid-borne WT *SPT4* (see Fig. 5C, left panel), which also complements the known (6) 6-azauracil sensitivity of this *spt4Δ* strain (data not shown). Importantly, *spt4Δ* does not reduce the steady-state level of Myc-Paf1p (Fig. 5D), nor does it decrease the amount of Rtf1p that coimmunoprecipitates with Myc-Paf1p (Fig. 5F). Thus, Spt4p is important for recruitment of Paf1C to *ARG1*, not to maintain the cellular level of intact Paf1C.

It was possible that *spt4Δ* impairs Paf1C recruitment indirectly by reducing Pol II occupancy at *ARG1*. However, we found that *spt4Δ* did not reduce Rpb3p occupancy at any position in the *ARG1* ORF (Fig. 5C, right panel). It was shown recently that *spt4Δ* decreases the processivity of elongating Pol II on a Gal4p-induced gene in vivo; however, a substantial reduction in Pol II occupancy was evident only at a distance of 8 kb from the promoter (23). Thus, a detectable decrease in Pol II occupancy in *spt4Δ* cells would not be predicted for the ~1.25-kb *ARG1* ORF, as we observed. We found that *spt4Δ* also does not reduce the cellular level of Ser5P (Fig. 5D) nor the amount of Ser5P associated with the *ARG1* ORF (Fig. 5E), ruling out an indirect role for Spt4p in Paf1C recruitment at the level of Ser5 phosphorylation. We conclude that Spt4p is required for association of Paf1C with elongating Pol II phosphorylated at WT levels on Ser5.

We obtained evidence that Spt4p plays a general role in promoting Pol II-Paf1C interaction by showing that coimmunoprecipitation of Ser5P with Myc-Paf1p was greatly reduced by *spt4Δ* (Fig. 4A, cf. lanes 1 to 3 and 7 to 9 in second row). Spt4p is likely to function directly in linking Paf1C to elongating Pol II because Spt4p is associated with both of these factors in vivo. Thus, we found that Spt4p and its partner, Spt5p, both coimmunoprecipitated from WCEs with Myc-Paf1p in a manner reduced by disruption of Paf1C by *cdc73Δ* (Fig. 4A, lanes 4 to 6). In addition, both HA-tagged Paf1p and Ser5P coimmunoprecipitated with Myc-Spt4p (Fig. 4B). These findings agree with previous results indicating that Spt5p is physically associated in vivo with Pol II (6) and Paf1C (43). Importantly, we found that coimmunoprecipitation of Spt5p with Myc-Paf1p was impaired by *spt4Δ* (Fig. 4A, cf. lanes 1 to 3 and 7 to 9), suggesting that Spt4p enhances interaction of Spt5p with Paf1C. Finally, by ChIP experiments we found that the low-level Paf1p occupancy in the TATA region and the high-level Paf1p occupancy in the ORF at both *PMA1* and *PYK1* were

FIG. 5. Deletion of *SPT4* impairs Paf1p association with *ARG1*, *PMAl*, and *PYK1* without decreasing cellular levels of Paf1C or Ser5P. (A-B) *PAF1-myc* strains containing the indicated deletions were subjected to ChIP analysis as described in Fig. 1, using anti-Myc antibodies. *PAF1-myc gcn4Δ* strain HQY397 was analyzed as a negative control. (C) *PAF1-myc* strains HQY389 (WT), HQY905 (*spt4Δ*) containing an empty vector, and HQY905 containing *SPT4* plasmid pHQ1437 (*spt4Δ*+pSPT4) were subjected to ChIP analysis as described in the legend to Fig. 1 using Myc antibodies (left panel) or Rpb3p antibodies (right panel). HQY397 was analyzed as a negative control. (D) Western analysis of WCEs from strains HQY389 (WT) and HQY905 (*spt4Δ*) conducted as described in the legend to Fig. 3B. (E) *PAF1-myc* strains HQY389 (WT), HQY905 (*spt4Δ*), and HQY397 (*gcn4Δ*) were subjected to ChIP analysis using H14 antibodies. (F) WCEs of strains BY4741 (*PAF1*), HQY389 (WT), and HQY905 (*spt4Δ*) were immunoprecipitated with Myc antibodies, and immune complexes were probed with antibodies against Myc or Rtf1p. Inp, adjacent lanes contain 1.25%, 2.5%, and 5% of input WCEs; P, 50% of pellet; S, 5% of supernatant. (G) ChIP analysis of Myc-Paf1p occupancy at the promoter and 5' ORF of *PMAl* and *PYK1* measured as described in the legend to Fig. 1, using Myc antibodies and strains described in the legend to panel E, except that the results were not normalized to those observed with *gcn4Δ* cells.

strongly diminished by *spt4Δ* (Fig. 5G). Note that *gcn4Δ* had no effect on the Paf1p occupancy at *PMAl* and *PYK1* because these are not Gcn4p target genes. These results support a global role for Spt4p in Paf1C recruitment to the ORF that is not limited to activator Gcn4p or amino acid starvation conditions.

Recruitment of Spt4p to the *ARG1* ORF does not require Paf1C. Our finding above that a much larger proportion of Ser5P versus unphosphorylated Rpb1p coimmunoprecipitated with Myc-Spt4p (Fig. 4B) is consistent with the idea that Spt4p is specifically associated with elongating Pol II. Supporting this interpretation, Myc-Spt4p cross-linked at higher levels to the

FIG. 5—Continued.

coding region of *ARG1* (both 5' and 3' ORF sequences) than to the TATA region (Fig. 6A, WT columns), as described above for Paf1p (Fig. 1C and 2A). In contrast to Paf1p, however, Spt4p maintains high-level occupancy in the region downstream of the *ARG1* ORF (Fig. 6A, WT, Down1), as observed for Pol II itself (Fig. 6B). These findings fit with previous results on several other yeast genes (10). We found that recruitment of Spt4p to the *ARG1* ORF is strongly impaired by deletion of the TATA box (Fig. 6A), just as observed for Pol II (Rpb3p) (Fig. 6B). In addition, Spt4p occupancy at *ARG1* was reduced by *kin28-ts16* (Fig. 6D) but to a much smaller degree than was Ser5P (Fig. 6C). In fact, Spt4p occupancy was reduced to the same extent observed for Pol II (Rpb3p), so that the Spt4p:Rpb3p ratio at *ARG1* was virtually unchanged by inactivation of *kin28-ts16* (Fig. 6E). Thus, it appears that Spt4p can interact with elongating Pol II that is hypophosphorylated on Ser5 of Rpb1p, a conclusion reached previously for Spt5p from coimmunoprecipitation experiments (19). Note that Spt4p differs from Paf1p, which showed little association with the *ARG1* ORF in the *kin28-ts16* mutant (Fig. 3E), suggesting

that Paf1C is more highly dependent than Spt4p on Ser5 phosphorylation for association with elongating Pol II.

The ability of Spt4p, but not Paf1p, to interact with hypophosphorylated Pol II at *ARG1*, plus the fact that Spt4p but not Paf1p is present at high levels downstream of the *ARG1* ORF, implies that Spt4p association with Pol II is independent of Paf1C. Mueller et al. reported that Spt5p occupancy in the *CLN1* ORF was reduced by ~50% in *paf1Δ* and *rtf1Δ* mutants (25). However, we found that Spt4p occupancy in the *ARG1* ORF was unaffected by disruption of Paf1C in the *rtf1Δ* and *cdc73Δ* mutants (Fig. 6A). Thus, while strong Paf1C recruitment to the *ARG1* ORF requires Kin28p and Spt4p, recruitment of Spt4p to the *ARG1* ORF is independent of intact Paf1C and is reduced by impairing Kin28p only to the extent that Pol II occupancy in the ORF is diminished. The simplest explanation for our findings is that Spt4p (likely in association with Spt5p) interacts with elongating Pol II and mediates recruitment of Paf1C in a manner stimulated by CTD phosphorylation (Fig. 6F). A final piece of evidence supporting a stimulatory role for Ser5 phosphorylation in Paf1C recruitment is provided by the data in Fig. 4C showing that inactivation of

FIG. 6. Spt4p recruitment to *ARG1* is independent of Paf1C and Ser5 CTD phosphorylation. (A-B) *SPT4-myc* strains containing deletions of *RTF1*, *CDC73*, or the *ARG1* TATA were subjected to ChIP analysis using Myc (A) or Rpb3p (B) antibodies, with strain HQY975 (*gcn4Δ*) analyzed as a negative control. (C and D) *SPT4-myc* strains containing *KIN28* (WT) (HQY987) or *kin28-ts16* (HQY988) were subjected to ChIP analysis using H14 (C) or Myc (D) antibodies, analyzing HQY989 (*gcn4Δ*) as negative control. (E) The ratio percent immunoprecipitation (*Gcn4/gcn4Δ*) values for Myc-Spt4p in panel D were normalized to the ratio percent immunoprecipitation (*Gcn4/gcn4Δ*) values for Rpb3p in Fig. 3C. (F) Hypothetical model summarizing the role of Spt4p/Spt5p in bridging interaction between Paf1C and elongating Pol II phosphorylated on Ser5. Paf1C is shown interacting directly with Pol II based on the observed Cdc73p-Pol II interaction (39). Spt4p/Spt5p are shown bridging Paf1C and Pol II based on our findings on the role of Spt4p in promoting Paf1C-Pol II interaction. Paf1C is shown interacting with the Ser5-phosphorylated Pol II to reflect our finding of preferential association of Paf1p with Ser5-hyperphosphorylated versus hypophosphorylated Rpb1p. Paf1C may interact directly with the phosphorylated CTD (30), but this is conjectural, as is the suggestion that Spt4p and Spt5p each interact directly with both Paf1C and Pol II. Bur1p/Bur2p is shown stimulating the association of Paf1C with Spt4p/Spt5p and Ser5-phosphorylated Pol II based on our findings shown in Fig. 7A and previously published results (17).

FIG. 7. Deletion of *SPT4* reduces global histone H3-K4 methylation. (A) Deletion of *BUR2* impairs Paf1p association with *ARG1* without affecting Pol II occupancy. *PAF1-myc* strains HQY389 (WT) and HQY1001 (*bur2Δ*) were subjected to ChIP analysis as described in the legend to Fig. 1 using Myc (left panel) or Rpb3 (right panel) antibodies, with HQY397 (*gcn4Δ*) analyzed as a negative control. (B) Bur2p promotes association of Paf1C with Spt4p, Spt5p, and Ser5-phosphorylated Pol II. WCEs from *PAF1-myc* strains HQY389 (WT) and HQY1001 (*bur2Δ*) were immunoprecipitated with Myc antibodies, and immune complexes were probed with Myc antibodies, H14 antibodies, 8WG16 antibodies, and antibodies against Spt5p and Spt4p. (C) *spt4Δ* reduces H3-K4 trimethylation at *ARG1*. *PAF1-myc* strains HQY389 (WT) and HQY905 (*spt4Δ*) were subjected to ChIP analysis as described in the legend to Fig. 1 using antibodies against H3-K4-Me3. The results were not normalized to those observed in *gcn4Δ* cells. (D) *spt4Δ* reduces H3-K4-Me3 in bulk histones. Increasing amounts of WCEs prepared from independent cultures of HQY389 (WT) and HQY905 (*spt4Δ*) were subjected to Western analysis using antibodies against histone H3-K4-Me3, H3-K79-Me2, and total H3. (E) Densities of bands in lanes 2, 5, 8, and 11 of panel C were calculated with a scanner and NIH image software (version 1.63), and the values for H3-K4-Me3 and H3-K79-Me2 in the duplicate cultures were averaged and normalized to the corresponding averages for total H3. IP, immunoprecipitation.

kin28-ts16 eliminates coimmunoprecipitation of the Paf1C subunit Rtf1p with Myc-Spt4p without affecting interaction of Myc-Spt4p with Spt5p.

Deletion of *BUR2* reduces association of Spt4p, Spt5p, and Pol II with Paf1p. It was reported recently that deletion of *BUR2*, encoding the cyclin partner of cyclin-dependent protein kinase Bur1p, impaired Paf1C occupancy of the *PMA1* ORF without reducing Pol II occupancy of this gene (17). We made a similar observation at the *ARG1* gene, where *bur2Δ* nearly eliminated Paf1C recruitment by Gcn4p but had little impact on Pol II occupancy (Fig. 7A, compare right and left panels). Given our findings on the importance of Spt4p in Paf1C recruitment, we asked whether *BUR2* deletion would reduce association of Spt4p with Paf1C. As shown in Fig. 7B, *bur2Δ* essentially eliminated coimmunoprecipitation of Ser5P with Myc-Paf1p, providing additional evidence that Bur2p is required for Paf1C recruitment by Pol II (17). Interestingly, *bur2Δ* also greatly reduced association of Spt4p with Myc-Paf1p and substantially diminished Spt5p-

Paf1p interaction (Fig. 7B). Thus, Bur2p may promote Paf1C-Pol II association at least partly by enhancing interaction of Spt4p/Spt5p with Paf1C (Fig. 6F).

Deletion of *SPT4* reduces histone H3-K4 trimethylation. Mutants lacking Rtf1p or Paf1p are defective in transcription-coupled histone H3 methylation, including trimethylation of Lys4 (H3-K4-Me3) by Set1p and dimethylation of Lys79 (H3-K79) by Dot1p (14, 27). We obtained two pieces of evidence that disrupting Paf1C-Pol II interaction by *spt4Δ* is sufficient to reduce H3-K4-Me3 at *ARG1* and many other loci. First, ChIP analysis using antibodies specific for H3-K4-Me3 revealed a significant reduction in this modification at the 5' end of *ARG1* in *spt4Δ* cells (Fig. 7C). Next, we quantified the levels of H3-K4-Me3 in bulk histones by Western analysis. Interestingly, *spt4Δ* lowers the ratio of H3-K4-Me3 to total H3 by ~70% but does not significantly reduce the corresponding ratio for H3-K79-Me2 (Fig. 7D and E). A selective reduction in H3-K4-Me3 was unexpected; however, Larabee et al. (17) observed a similar phenotype in *bur2Δ* cells.

Our findings suggest that the reduction in Paf1C-Pol II association in *spt4Δ* cells is sufficient to reduce H3-K4-Me3 formation but not to impair dimethylation of H3-K79.

DISCUSSION

Our results provide strong evidence that Paf1C is associated primarily with elongating, hyperphosphorylated Pol II in a manner dependent on elongation factor Spt4p, revealing a new role for DSIF as an enhancer of Paf1C function during transcription elongation. ChIP experiments showed that Paf1p has a much higher occupancy in the ORF than the promoter region of *ARG1*, and coimmunoprecipitation analysis showed that Paf1p is more highly associated with hyperphosphorylated than with hypophosphorylated Pol II. Consistent with this, Paf1p occupancy was more strongly impaired than was Pol II occupancy by *kin28* mutations that reduced or eliminated detectable Ser5 phosphorylation at *ARG1*. These findings suggest that Paf1C is associated primarily with hyperphosphorylated Pol II engaged in elongation. Our finding that Ser5 phosphorylation is greatest in the promoter whereas Paf1C shows relatively higher occupancy in the ORF implied the involvement of another factor in Paf1C recruitment that is associated exclusively with elongating Pol II. This led to our discovery that Spt4p is critically required for Paf1C recruitment to Pol II. Thus, *spt4Δ* eliminated detectable Paf1p occupancy without reducing Pol II occupancy at *ARG1*, and it greatly reduced coimmunoprecipitation of Paf1p with Ser5P without affecting the levels of Ser5 phosphorylation or Paf1C abundance. Reducing Ser5 phosphorylation by a *kin28* mutation did not appear to impair association of Spt4p with elongating Pol II at *ARG1*, nor did the disruption of Paf1C by deleting Rtf1p or Cdc73p. These results indicate that Spt4p, probably in conjunction with Spt5p, provides a platform on elongating Pol II that enables efficient recruitment of Paf1C. We also found that inactivation of the *kin28-ts16* product disrupted the association of Paf1C with Spt4p, supporting a role for Ser5 phosphorylation in stabilizing Paf1C interaction with the elongating Pol II-Spt4p/Spt5p complex (Fig. 6F). Finally, *spt4Δ* reduced the level of H3-K4 trimethylation, indicating that Spt4p enhances transcription-coupled methylation of H3-K4 by the Set1p complex. These findings reveal a new function for yeast DSIF in stimulating a Paf1C-dependent histone modification during transcription elongation.

The preferential association of Paf1C with Pol II phosphorylated on Ser5 versus hypophosphorylated Pol II was unexpected considering that Paf1C was originally isolated by its association with unphosphorylated Pol II (47). Our findings do not contradict these previous results but rather suggest that Paf1C is more highly associated with elongating, hyperphosphorylated Pol II than with the hypophosphorylated enzyme unbound to chromatin or residing in PICs. Interestingly, Paf1C subunits were purified recently by their affinity for CTD repeats doubly phosphorylated on Ser5 and Ser2 (30). Thus, Paf1C may interact directly with the phosphorylated CTD or with another phospho-CTD-interacting factor, in addition to Spt4p/Spt5p.

It could be argued that the preferential association of Paf1C with Ser5-phosphorylated Pol II is inconsistent with the uniform distribution of Paf1C across the ORF observed here and

elsewhere (10), given the apparent reduction in Ser5P from 5' to 3' across the ORF (13) (Fig. 3A). However, Ser5P is detected at the 3' ends of ORFs in WT cells at higher levels than in the promoters and 5' ORF sequences of *kin28* mutants (13) (Fig. 3A), indicating that a certain level of Ser5 phosphorylation persists throughout the ORF. Indeed, there is evidence that the CTD phosphorylated on Ser2 is also phosphorylated on Ser5 (8). It is also uncertain whether Ser5 phosphorylation, as opposed to accessibility of the epitope recognized by the H14 antibody, diminishes across the ORF (30).

Our demonstration that Spt4p is required for efficient recruitment of Paf1C to elongating Pol II is consistent with previous results indicating genetic interactions between Spt4p/Spt5p and Paf1C subunits and the physical association of these factors in the same Pol II complexes (3, 43). Likewise, there are genetic and physical interactions linking Spt4p/Spt5p to elongating Pol II and other elongation factors (6, 19, 20, 23, 46). On the basis of such interactions, the requirement for Rtf1p and Cdc73p to tether other Paf1C subunits to chromatin, and the evidence for direct binding of Cdc73p to Pol II (39), Mueller et al. speculated recently that Paf1C-Pol II association would depend on direct Rtf1p-Spt5p and Cdc73p-Pol II interactions (25). To our knowledge, there is no evidence for direct interaction between Rtf1p and Spt5p; however, a role for Spt5p in stabilizing Paf1C-Pol II interaction would not be surprising, since Spt4p and Spt5p are associated with one another in the yeast equivalent of DSIF. In addition, we observed residual Paf1p association with Pol II and Spt5p in *spt4Δ* cells (Fig. 4A; also data not shown), consistent with a direct role for Spt5p in Paf1C recruitment. We consider it likely that Spt4p also interacts with a Paf1C subunit, a Pol II subunit, or both, although Spt4p might only facilitate interactions of Spt5p with Paf1C and Pol II. Considerable effort will be required to identify all of the contacts between Spt4p/Spt5p, Paf1C subunits, and Pol II subunits and evaluate their relative importance for optimal recruitment of Paf1C to elongating Pol II in vivo.

Do any other elongation factors make critical contributions to Paf1C recruitment? Our results in Fig. 5 eliminate this possibility for TFIIS and the THO complex and also rule out important contributions from H2B ubiquitylation by Bre1p/Rad6p, histone H3 methylation, and the activities of Chd1p and Isw1p. The essential elongation factor Spt6 interacts genetically with Paf1C subunits and, like Paf1C, can be coimmunoprecipitated with Spt5p (20, 43). While there are indications that Spt6p promotes Paf1C association with elongating Pol II, the reduced Paf1C occupancy at the *GAL1,10* genes observed in an *spt6* mutant might result from decreased levels of Paf1C rather than a recruitment defect. In addition, no reduction in global levels of H3-K4 trimethylation was observed in this *spt6* mutant (9). There is also genetic and physical evidence (15, 43) that Paf1C interacts with the elongation factor FACT, comprised in yeast of the essential proteins Spt16p/Pob3p (42), so that Spt16p or Pob3p could also contribute to Paf1C recruitment.

Apart from our work implicating Spt4p, only the Bur1p/Bur2p kinase complex has been shown clearly to be required for Paf1C recruitment to elongating Pol II (17), a finding that we have confirmed here. It is interesting that *bur1* mutants have phenotypes in common with *spt4* mutants (33), given that they share a defect in Paf1C recruitment. Moreover, *spt4Δ* resembles *bur2Δ* in reducing H3-K4 trimethylation without

affecting H3-K79 methylation (17). It was shown recently that Bur1p/Bur2p kinase stimulates H2B ubiquitylation by phosphorylation of Rad6p (50), consistent with the requirement for Bur1p/Bur2p in H3-K4 trimethylation. However, phosphorylation of Rad6p cannot account for the role of Bur1p/Bur2p in Paf1C recruitment, because we found that Rad6p is dispensable for WT Paf1C recruitment at *ARG1*. Given our finding that *bur2Δ* reduces association of Paf1p with Spt4p and Spt5p, it is possible that Bur1p/Bur2p stimulates the interaction of Paf1C with Spt4p/Spt5p as one way of promoting Paf1C-Pol II interaction (Fig. 6F).

ACKNOWLEDGMENTS

We thank Grant Hartzog for anti-Spt4p and anti-Spt5p antibodies and Karen Arndt for anti-Rtf1p antibodies. We thank Steve Hahn for the *kin28-as* strain, Kevan Shokat for NA-PP1, Mark Solomon for the *kin28-ts16* plasmids, and Fred Winston for *SPT4* plasmids. We also thank Chhabi Govind and Dan Ginsburg for critical reading of the manuscript.

This research was supported by the Intramural Research Program of the NICHD, NIH.

REFERENCES

- Ahn, S. H., M. Kim, and S. Buratowski. 2004. Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. *Mol. Cell* 13:67–76.
- Cigan, A. M., M. Foiani, E. M. Hannig, and A. G. Hinnebusch. 1991. Complex formation by positive and negative translational regulators of *GCN4*. *Mol. Cell. Biol.* 11:3217–3228.
- Costa, P. J., and K. M. Arndt. 2000. Synthetic lethal interactions suggest a role for the *Saccharomyces cerevisiae* Rtf1 protein in transcription elongation. *Genetics* 156:535–547.
- Dover, J., J. Schneider, M. A. Tawiah-Boateng, A. Wood, K. Dean, M. Johnston, and A. Shilatifard. 2002. Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. *J. Biol. Chem.* 277:28368–28371.
- Govind, C. K., S. Yoon, H. Qiu, S. Govind, and A. G. Hinnebusch. 2005. Simultaneous recruitment of coactivators by Gcn4p stimulates multiple steps of transcription in vivo. *Mol. Cell. Biol.* 25:5626–5638.
- Hartzog, G. A., T. Wada, H. Handa, and F. Winston. 1998. Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in *Saccharomyces cerevisiae*. *Genes Dev.* 12:357–369.
- Hwang, W. W., S. Venkatasubrahmanyam, A. G. Ianculescu, A. Tong, C. Boone, and H. D. Madhani. 2003. A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. *Mol. Cell* 11:261–266.
- Jones, J. C., H. P. Phatnani, T. A. Haystead, J. A. MacDonald, S. M. Alam, and A. L. Greenleaf. 2004. C-terminal repeat domain kinase I phosphorylates Ser2 and Ser5 of RNA polymerase II C-terminal domain repeats. *J. Biol. Chem.* 279:24957–24964.
- Kaplan, C. D., M. J. Holland, and F. Winston. 2005. Interaction between transcription elongation factors and mRNA 3'-end formation at the *Saccharomyces cerevisiae* GAL10-GAL7 locus. *J. Biol. Chem.* 280:913–922.
- Kim, M., S. H. Ahn, N. J. Krogan, J. F. Greenblatt, and S. Buratowski. 2004. Transitions in RNA polymerase II elongation complexes at the 3' ends of genes. *EMBO J.* 23:354–364.
- Kimmelman, J., P. Kaldis, C. J. Hengartner, G. M. Laff, S. S. Koh, R. A. Young, and M. J. Solomon. 1999. Activating phosphorylation of the Kin28p subunit of yeast TFIIF by Cak1p. *Mol. Cell. Biol.* 19:4774–4787.
- Koch, C., P. Wollmann, M. Dahl, and F. Lottspeich. 1999. A role for Ctr9p and Paf1p in the regulation G1 cyclin expression in yeast. *Nucleic Acids Res.* 27:2126–2134.
- Komarnitsky, P., E. J. Cho, and S. Buratowski. 2000. Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. *Genes Dev.* 14:2452–2460.
- Krogan, N. J., J. Dover, A. Wood, J. Schneider, J. Heidt, M. A. Boateng, K. Dean, O. W. Ryan, A. Golshani, M. Johnston, J. F. Greenblatt, and A. Shilatifard. 2003. The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. *Mol. Cell* 11:721–729.
- Krogan, N. J., M. Kim, S. H. Ahn, G. Zhong, M. S. Kobor, G. Cagney, A. Emili, A. Shilatifard, S. Buratowski, and J. F. Greenblatt. 2002. RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: a targeted proteomics approach. *Mol. Cell. Biol.* 22:6979–6992.
- Krogan, N. J., M. Kim, A. Tong, A. Golshani, G. Cagney, V. Canadien, D. P. Richards, B. K. Beattie, A. Emili, C. Boone, A. Shilatifard, S. Buratowski, and J. Greenblatt. 2003. Methylation of histone H3 by Set2 in *Saccharomyces cerevisiae* is linked to transcriptional elongation by RNA polymerase II. *Mol. Cell. Biol.* 23:4207–4218.
- Larabee, R. N., N. J. Krogan, T. Xiao, Y. Shibata, T. R. Hughes, J. F. Greenblatt, and B. D. Strahl. 2005. BUR kinase selectively regulates H3 K4 trimethylation and H2B ubiquitylation through recruitment of the PAF elongation complex. *Curr. Biol.* 15:1487–1493.
- Li, B., L. Howe, S. Anderson, J. R. Yates III, and J. L. Workman. 2003. The Set2 histone methyltransferase functions through the phosphorylated carboxyl-terminal domain of RNA polymerase II. *J. Biol. Chem.* 278:8897–8903.
- Lindstrom, D. L., and G. A. Hartzog. 2001. Genetic interactions of Spt4-Spt5 and TFIIS with the RNA polymerase II CTD and CTD modifying enzymes in *Saccharomyces cerevisiae*. *Genetics* 159:487–497.
- Lindstrom, D. L., S. L. Squazzo, N. Muster, T. A. Burckin, K. C. Wachter, C. A. Emigh, J. A. McCleery, J. R. Yates III, and G. A. Hartzog. 2003. Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. *Mol. Cell. Biol.* 23:1368–1378.
- Liu, Y., C. Kung, J. Fishburn, A. Z. Ansari, K. M. Shokat, and S. Hahn. 2004. Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. *Mol. Cell. Biol.* 24:1721–1735.
- Makela, T. P., J. D. Parvin, J. Kim, L. J. Huber, P. A. Sharp, and R. A. Weinberg. 1995. A kinase-deficient transcription factor TFIIF is functional in basal and activated transcription. *Proc. Natl. Acad. Sci. USA* 92:5174–5178.
- Mason, P. B., and K. Struhl. 2005. Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. *Mol. Cell* 17:831–840.
- Mueller, C. L., and J. A. Jaehning. 2002. Ctr9, Rtf1, and Leo1 are components of the Paf1/RNA polymerase II complex. *Mol. Cell. Biol.* 22:1971–1980.
- Mueller, C. L., S. E. Porter, M. G. Hoffman, and J. A. Jaehning. 2004. The Paf1 complex has functions independent of actively transcribing RNA polymerase II. *Mol. Cell* 14:447–456.
- Ng, H. H., S. Dole, and K. Struhl. 2003. The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. *J. Biol. Chem.* 278:33625–33628.
- Ng, H. H., F. Robert, R. A. Young, and K. Struhl. 2003. Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol. Cell* 11:709–719.
- Patturajan, M., N. K. Conrad, D. B. Bregman, and J. L. Corden. 1999. Yeast carboxyl-terminal domain kinase I positively and negatively regulates RNA polymerase II carboxyl-terminal domain phosphorylation. *J. Biol. Chem.* 274:27823–27828.
- Penheiter, K. L., T. M. Washburn, S. E. Porter, M. G. Hoffman, and J. A. Jaehning. 2005. A posttranscriptional role for the yeast Paf1-RNA polymerase II complex is revealed by identification of primary targets. *Mol. Cell* 20:213–223.
- Phatnani, H. P., J. C. Jones, and A. L. Greenleaf. 2004. Expanding the functional repertoire of CTD kinase I and RNA polymerase II: novel phosphoCTD-associated proteins in the yeast proteome. *Biochemistry* 43:15702–15719.
- Pokholok, D. K., N. M. Hannett, and R. A. Young. 2002. Exchange of RNA polymerase II initiation and elongation factors during gene expression in vivo. *Mol. Cell* 9:799–809.
- Porter, S. E., T. M. Washburn, M. Chang, and J. A. Jaehning. 2002. The yeast paf1-RNA polymerase II complex is required for full expression of a subset of cell cycle-regulated genes. *Eukaryot. Cell* 1:830–842.
- Prelich, G., and F. Winston. 1993. Mutations that suppress the deletion of an upstream activating sequence in yeast: involvement of a protein kinase and histone H3 in repressing transcription in vivo. *Genetics* 135:665–676.
- Qiu, H., C. Hu, S. Yoon, K. Natarajan, M. Swanson, and A. G. Hinnebusch. 2004. An array of coactivators is required for optimal recruitment of TBP and RNA polymerase II by promoter-bound Gcn4p. *Mol. Cell. Biol.* 24:4104–4117.
- Reid, G. A., and G. Schatz. 1982. Import of proteins into mitochondria. *J. Biol. Chem.* 257:13062–13067.
- Rondon, A. G., M. Gallardo, M. Garcia-Rubio, and A. Aguilera. 2004. Molecular evidence indicating that the yeast PAF complex is required for transcription elongation. *EMBO Rep.* 5:47–53.
- Santos-Rosa, H., R. Schneider, A. J. Bannister, J. Sherri, B. E. Bernstein, N. C. Emre, S. L. Schreiber, J. Mellor, and T. Kouzarides. 2002. Active genes are tri-methylated at K4 of histone H3. *Nature* 419:407–411.
- Serizawa, H., J. W. Conaway, and R. C. Conaway. 1993. Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription. *Nature* 363:371–374.
- Shi, X., M. Chang, A. J. Wolf, C. H. Chang, A. A. Frazer-Abel, P. A. Wade, Z. F. Burton, and J. A. Jaehning. 1997. Cdc73p and Paf1p are found in a novel RNA polymerase II-containing complex distinct from the Srbp-containing holoenzyme. *Mol. Cell. Biol.* 17:1160–1169.
- Shi, X., A. Finkelstein, A. J. Wolf, P. A. Wade, Z. F. Burton, and J. A. Jaehning. 1996. Paf1p, an RNA polymerase II-associated factor in *Saccha-*

- romyces cerevisiae*, may have both positive and negative roles in transcription. *Mol. Cell. Biol.* **16**:669–676.
41. Simic, R., D. L. Lindstrom, H. G. Tran, K. L. Roinick, P. J. Costa, A. D. Johnson, G. A. Hartzog, and K. M. Arndt. 2003. Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. *EMBO J.* **22**:1846–1856.
 42. Sims, R. J., III, R. Belotserkovskaya, and D. Reinberg. 2004. Elongation by RNA polymerase II: the short and long of it. *Genes Dev.* **18**:2437–2468.
 43. Squazzo, S. L., P. J. Costa, D. L. Lindstrom, K. E. Kumer, R. Simic, J. L. Jennings, A. J. Link, K. M. Arndt, and G. A. Hartzog. 2002. The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. *EMBO J.* **21**:1764–1774.
 44. Sun, Z. W., and C. D. Allis. 2002. Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* **418**:104–108.
 45. Swanson, M. J., H. Qiu, L. Sumibcay, A. Krueger, S.-J. Kim, K. Natarajan, S. Yoon, and A. G. Hinnebusch. 2003. A multiplicity of coactivators is required by Gcn4p at individual promoters in vivo. *Mol. Cell. Biol.* **23**:2800–2820.
 46. Wada, T., T. Takagi, Y. Yamaguchi, A. Ferdous, T. Imai, S. Hirose, S. Sugimoto, K. Yano, G. A. Hartzog, F. Winston, S. Buratowski, and H. Handa. 1998. DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs. *Genes Dev.* **12**:343–356.
 47. Wade, P. A., W. Werel, R. C. Fentzke, N. E. Thompson, J. F. Leykam, R. R. Burgess, J. A. Jaehning, and Z. F. Burton. 1996. A novel collection of accessory factors associated with yeast RNA polymerase II. *Protein Expr. Purif.* **8**:85–90.
 48. Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J. D. Boeke, H. Bussey, A. M. Chu, C. Connelly, K. Davis, F. Dietrich, S. W. Dow, M. E. Bakkoury, F. Foury, S. H. Friend, E. Gentalen, G. Giaever, J. H. J. Hegemann, T. Jones, M. Laub, H. Liao, N. Liebundguth, D. J. Lockhart, A. Lucau-Danila, M. Lussier, N. M'Rabet, P. Menard, M. Mittmann, C. Pai, C. Rebischung, J. L. Revuelta, L. Riles, C. J. Roberts, P. Ross-McDonald, B. Scherens, M. Snyder, S. Sookhai-Mahadeo, R. K. Storms, S. Veronneau, M. Voet, G. Volckaert, T. R. Ward, R. Wysocki, G. S. Yen, K. Yu, K. Zimmermann, P. Philippsen, M. Johnston, and R. W. Davis. 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**:901–906.
 49. Wood, A., N. J. Krogan, J. Dover, J. Schneider, J. Heidt, M. A. Boateng, K. Dean, A. Golshani, Y. Zhang, J. F. Greenblatt, M. Johnston, and A. Shilatifard. 2003. Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. *Mol. Cell* **11**:267–274.
 50. Wood, A., J. Schneider, J. Dover, M. Johnston, and A. Shilatifard. 2005. The Bur1/Bur2 complex is required for histone H2B monoubiquitination by Rad6/Bre1 and histone methylation by COMPASS. *Mol. Cell* **20**:589–599.
 51. Wood, A., J. Schneider, J. Dover, M. Johnston, and A. Shilatifard. 2003. The Paf1 complex is essential for histone monoubiquitination by the Rad6/Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. *J. Biol. Chem.* **278**:34739–34742.
 52. Xiao, T., H. Hall, K. O. Kizer, Y. Shibata, M. C. Hall, C. H. Borchers, and B. D. Strahl. 2003. Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. *Genes Dev.* **17**:654–663.
 53. Zhang, F., L. Sumibcay, A. G. Hinnebusch, and M. J. Swanson. 2004. A triad of subunits from the Gal11/tail domain of Srb mediator is an in vivo target of transcriptional activator Gcn4p. *Mol. Cell. Biol.* **24**:6871–6886.