The Paf1 Complex Represses SER3 Transcription in Saccharomyces cerevisiae by Facilitating Intergenic Transcription-Dependent Nucleosome Occupancy of the SER3 Promoter

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Previous studies have shown that repression of the Saccharomyces cerevisiae SER3 gene is dependent on transcription of SRG1 from noncoding DNA initiating within the intergenic region 5’ of SER3 and extending across the SER3 promoter region. By a mechanism dependent on the activities of the Swi/Snf chromatin remodeling factor, the HMG-like factor Spt2, and the Spt6 and Spt16 histone chaperones, SER3 transcription deposits nucleosomes over the SER3 promoter to prevent transcription factors from binding and activating SER3. In this study, we uncover a role for the Paf1 transcription elongation complex in SER3 repression. We find that SER3 repression is primarily dependent on the Paf1 and Ctr9 subunits of this complex, with minor contributions by the Rtf1, Cdc73, and Leol subunits. We show that the Paf1 complex localizes to the SER3 transcribed region under conditions that repress SER3, consistent with it having a direct role in mediating SER3 transcription-dependent repression. Importantly, we show that the defect in SER3 repression in strains lacking Paf1 subunits is not a result of reduced SRG1 transcription or reduced levels of known Paf1 complex-dependent histone modifications. Rather, we find that strains lacking subunits of the Paf1 complex exhibit reduced nucleosome occupancy and reduced recruitment of Spt6 and, to a lesser extent, Spt16 at the SER3 promoter. Taken together, our results suggest that Paf1 and Ctr9 repress SER3 by maintaining SRG1 transcription-dependent nucleosome occupancy.

The packaging of eukaryotic DNA into chromatin presents a major obstacle to transcription initiation by preventing access of transcription factors to promoter DNA and also to transcription elongation by physically hindering the passage of RNA polymerase II (RNA Pol II) (44). Eukaryotic cells rely on the activities of three major classes of factors to alter chromatin architecture during transcription. The first class consists of chromatin remodeling factors that use the energy of ATP hydrolysis to alter the position or occupancy of nucleosomes (16). The second class contains histone-modifying enzymes that add or remove covalent modifications, such as acetyl, methyl, and ubiquitin groups, which can alter the dynamic properties of nucleosomes and influence the binding of additional regulatory proteins (9). The third class is comprised of histone-interacting proteins that function as chaperones to mediate the disassembly and reassembly of nucleosomes during transcription by RNA Pol II (23).

Recent studies have provided evidence that the Saccharomyces cerevisiae SER3 gene is controlled by transcription-dependent chromatin dynamics (27, 85). SER3 encodes an enzyme required for serine biosynthesis that is repressed when serine is abundant and is rapidly activated upon serine depletion (50). SER3 repression is mediated by the serine-dependent transcription of SRG1, initiating within intergenic noncoding DNA (ncDNA) 5’ of SER3 and extending across the SER3 promoter region before terminating near the SER3 translational start site (48, 50, 86). SRG1 intergenic transcription acts in cis to promote nucleosome occupancy of the SER3 promoter, thereby occluding this region from the binding of transcription factors. SRG1 transcription-dependent nucleosome occupancy of the SER3 promoter requires the functions of the Swi/Snf chromatin remodeling complex, an HMG-like protein Spt2, and the Spt6 and Spt16 histone chaperones (27, 49, 85). Swi/Snf is recruited to the SRG1 promoter by the Cha4 activator protein in a serine-dependent manner and is thought to mobilize nucleosomes, allowing RNA Pol II to initiate transcription of SRG1 (49, 50). As a consequence of SRG1 transcription, RNA Pol II traverses the SER3 promoter, where it deposits and maintains nucleosomes over this region by a mechanism that is dependent on Spt6, Spt16, and Spt2 (27, 85).

Another factor that is important for facilitating transcription elongation through chromatin is the Paf1 complex (30). The Paf1 complex is conserved across all eukaryotes and has important roles in embryonic development (2, 84, 90), maintenance of stem cell fate (22), and tumorigenesis (11, 45, 54). In S. cerevisiae, the Paf1 complex is comprised of five subunits, Paf1, Ctr9, Rtf1, Cdc73, and Leol (42, 55, 76, 79) that colocalize with RNA Pol II across transcribed genes exiting near the polyadenylation sites (38, 42, 53, 67). During elongation, the Paf1 complex has been shown to promote histone modifications (15, 41, 59, 94), alter the phosphorylation state of the RNA Pol II carboxy-terminal domain (CTD) (56, 61), and facilitate proper transcription termination (34, 56, 64, 75, 87). Colocalization of the Paf1 complex with RNA Pol II is dependent on the Bur1/Bur2 and Spt4/Spt5 transcription elongation factors (43, 46, 69, 102). Other factors, such as Spt6, FACT (composed of Spt16 and Pob3), and Ccr4-Not, may also con-
One of the primary functions of the Pafl complex is to promote histone modifications associated with active transcription (30). In yeast, the Pafl complex promotes monoubiquitylation of histone H2B at lysine 123 (K123ub) by the ubiquitin conjugase Rad6 and ubiquitin ligase Bre1 (59, 94). Ubiquitylation of H2B is required for subsequent methylation of histone H3 at lysine 4 (K4me) and lysine 79 (K79me) by the Set1 and Dot1 methyltransferases, respectively (41, 59, 60, 74, 82, 94). These modifications are predominantly dependent on the Rtf1 subunit of the Pafl complex, specifically involving a central region of the protein termed HMD for the histone modification domain (87, 91). Furthermore, the Pafl and Ctr9 subunits are required for trimethylation of histone H3 at lysine 36 (K36me3) by the Set2 methyltransferase (14). Together, these modifications control histone acetylation across transcribed genes through the recruitment of histone deacetylases (10, 32, 36, 39, 66, 100).

Other connections of the Pafl complex to chromatin have also been described. In yeast, Pafl and Ctr9 were shown to affect the rate of induction of GAL genes by promoting nucleosome eviction from the GALI-10 promoter during activation (51). The Drosophila melanogaster Pafl complex has been shown to facilitate recruitment of the Sp6 and FACT histone chaperones during transcription (1). These studies established the importance of the Pafl complex in transcription through chromatin, but little is known about how the complex mediates this function.

In this study, we examine the role of the Pafl complex in SRG1 transcription-dependent repression of SER3. We find SER3 repression to be primarily dependent on the Pafl and Ctr9 subunits and provide genetic evidence suggesting that these factors act in a previously described pathway with the Bur1/Bur2 kinase/cyclin and the Spt4/Spt5 transcription elongation complex. Our results indicate that while the Pafl complex colocalizes with SRG1 transcription, its absence does not reduce SRG1 transcript levels, and its role in SER3 repression is largely independent of its ability to orchestrate covalent histone modifications. Rather, we find that Pafl and Ctr9 promote both nucleosome occupancy over actively transcribing SRG1 and normal recruitment of Sp6 and Spt6 to this region. Our results suggest that the Pafl and Ctr9 subunits of the Pafl complex repress SER3 by facilitating SRG1 transcription-dependent nucleosome occupancy of the SER3 promoter, possibly by stabilizing the association of Sp6 and Spt6.

### Table 1. Saccharomyces cerevisiae strains used in this study

| Name  | Genotype |
|-------|----------|
| FY4   | MATa     |
| FY5   | MATa     |
| KY399 | MATTo rfa1Δ100::URA3 leu2Δ1 ura3-52 trp1Δ63 |
| KY716 | MATTo his3Δ200 lys2-1286 ura3-52 sps1-194 |
| KY735 | MATTo his4+9126 lys2-1286 leu2Δ1 ura3-52 arg4-12 spo11-2Δ ura3Δ1::URA3 |
| KY785 | MATa his4+9126 lys2-1286 leu2Δ1(0 or 1) trp1Δ63 CTF9*6::MYC::LEU2 LE01-3::HA::HIS3 SPT5-FLAG |
| KY786 | MATa his4+9126 lys2-1286 leu2Δ1(0 or 1) ura3-52 trp1Δ63 CTF9*6::MYC::LEU2 3×HA-CDCC3::URA3 SPT5-FLAG |
| KY1349 | MATb bar2Δ::URA3 his4+9126 lys2-1286 suc2::UAS (-1900)→390) ura3-52 trp1Δ63 |
| KY1451 | MATTo paf1Δ::KanMX bar2Δ::KanMX |
| KY1700 | MATTo paf1Δ::KanMX |
| KY1703 | MATTo rfa1Δ::KanMX |
| KY1706 | MATTo cdc73Δ::KanMX |
| KY1712 | MATTo rad6Δ::KanMX |
| KY1713 | MATTo bre1Δ::KanMX |
| KY1721 | MATTo 3×HA-PAF1 |
| KY1805 | MATTo leo1Δ::KanMX |
| KY2167 | MATa HTA1-hbh1K123ub (hta2-hbh2)Δ::KanMX ura3Δ0 |
| KY2170 | MATa ctn9Δ::KanMX leu2Δ1 |
| KY2172 | MATa (hta2-hbh2)Δ::KanMX ura3Δ0 |
| KY2082 | MATa ura3-52 lys2-1286 leu2Δ1 trp1Δ63 3×HA-RTF1 |
| YJ759 | MATa ctn9Δ::KanMX leu2Δ1::hisΔ300 lys2Δ0 |
| YJ786 | MATTo paf1Δ::KanMX ura3Δ0 his3Δ200 lys2Δ0 |
| YJ882 | MATa ura3-52 or uraΔΔ lys2Δ0 or lys2-1286 his3Δ200 leu2Δ0 or leu2Δ1 RPB3-3::HA::LEU2 SPT6-FLAG |
| YJ1013 | MATa ura3-52 or uraΔΔ lys2Δ0 or lys2-1286 leu2Δ0 or leu2Δ1 RPB3-3::HA::LEU2 SPT6-FLAG rtf1Δ::KanMX |
| YJ1014 | MATa ura3-52 or uraΔΔ lys2Δ0 or lys2-1286 leu2Δ0 or leu2Δ1 his3Δ200 RPB3-3::HA::LEU2 SPT6-FLAG rtf1Δ::KanMX |
| YJ1016 | MATa ura3-52 or uraΔΔ lys2Δ0 or lys2-1286 leu2Δ0 or leu2Δ1 his3Δ200 RPB3-3::HA::LEU2 SPT6-FLAG ctn9Δ::KanMX |
| YJ1030 | MATa his4+9126 trp1Δ63 ura3-52 or URA3 lys2-1286 leu2Δ1 SPT6-FLAG RPB3-3::HA::LEU2 paf1Δ::URA3 |
| YJ1031 | MATa his4+9126 trp1Δ63 ura3-52 or URA3 lys2-1286 leu2Δ1 SPT6-FLAG RPB3-3::HA::LEU2 paf1Δ::URA3 |
| YJ1087 | MATa ura3Δ0 prs416 |

* FY and KY strains were kindly provided by Fred Winston and Karen Arndt, respectively.
grown in YPD at 30°C to 10^7 cells/ml and separated on a 1% formaldehyde–agarose gel as described previously (3). RNA was transferred to a Gene Screen membrane (Perkin-Elmer) and hybridized with radiolabeled probes generated by random-primed labeling of PCR fragments that were amplified from the following genomic sequences: SRG1 (chromosome V [ChrV], nucleotides 322258 to 322559), SER3 (ChrV, nucleotides 324059 to 324307), and SCR1 (ChrV, nucleotides 441741 to 442266), which was used as a control for RNA loading.

**Chromatin immunoprecipitation (ChIP)**: Cells were grown in YPD at 30°C to a density of 10^7 to 2 × 10^7 cells/ml and then treated with 2% formaldehyde for 20 min. Chromatin was isolated and sonicated as previously described (77) and then incubated with antibodies overnight at 4°C. Anti-FLAG M2 agarose (30 μl; A2220; Sigma) was used to immunoprecipitate Sp6-FLAG. Anti-HA antibody (1 μl; sc-7392; Santa Cruz Biotechnology) was used to immunoprecipitate HA-Paf1, HA-Rtf1, Leol-HA, HAdc73, and Rpb3-HA. Anti-Myc (1 μl; sc-789; Santa Cruz Biotechnology), anti-Spt16 (1 μl; kind gift from T. Formosa, University of Utah), and anti-histone H3 (5 μl; ab1791; Abcam) antibodies were used to immunoprecipitate Ctr9-MYC, Spt16, and histone H3, respectively. Primers and antibody-protein conjugates were isolated by incubating with 30 μl protein-A or protein G-coupled Sepharose beads (GE Healthcare) at 4°C for 2 to 3 h. After purifying DNA through PCR purification columns (Qiagen), the amount of immunoprecipitated IP DNA relative to input DNA was determined by quantitative PCR (qPCR) and then normalized to a control region on chromosome V that lacks open reading frames (no ORF), which has been previously described (40).

**Nucleosome scanning assay**: Nucleosome scanning assays were performed as previously described (3). Briefly, cells were grown in YPD to 2 × 10^7 to 3 × 10^7 cells/ml at 30°C and then treated with 2% formaldehyde followed by 300 mM glycine. A total of 1.2 to 10^7 cells were spheroplasting with Zymolyase 20T (Seikagaku Biobusiness) and divided into six aliquots, which were then incubated with increasing concentrations of micrococcal nuclease (MNase; nuclease S7; Roche). DNA was extracted, treated with RNase A, and subjected to gel electrophoresis and qPCR to determine the extent of MNase digestion. Well-characterized regions of the SCR1 locus. The amount of amplification for each primer pair that amplified ~100-bp fragments that tile the SER3 locus. The amount of amplification for each primer pair (SER3-3 to SER3-41) in the digested sample was made relative to the undigested sample and normalized to the GAL1 NB region.

**qPCR**: Results of nucleosome scanning and ChIP assays were analyzed with an ABI 7300 or StepOnePlus real-time PCR system and SYBR green reagents (Fermentas). Primer sets that amplified SER3 (SER3-1 to SER3-41) and the no-ORF control have been previously described (27). Primer sets specific to PMA1 ORF, ADH1, GAL1 ORF, and CYC1 have also been described previously (26). Quantitation of real-time PCR results was performed using the Pfaffl method (65).

**Western blot analysis**: Whole-cell extracts (WCE) were prepared from cells grown in YPD at 30°C to 10^7 to 2 × 10^7 cells/ml using trichloroacetic acid (TCA) as previously described (17, 101). Equal volumes of WCE were separated by 10% acrylamide SDS-PAGE, transferred to nitrocellulose (Whatman), and immunoblotted with anti-FLAG antibody (F3165; Sigma) or anti-Spt16 antibody (kind gift from T. Formosa, University of Utah). After incubation with anti-mouse (FLAG) or anti-rabbit (Spt16) horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare), the immunoreactive proteins were visualized by enhanced chemiluminescence detection (Perkin-Elmer) using a Kodak image station 440CF. Blots were then stripped and reprobed with anti-glucose-6-phosphate dehydrogenase antibody (A9521; Sigma) as a loading control. Quantitation of Sp6-FLAG and Spt16 protein levels was performed by measuring the signal intensities using the Kodak ID 3.6 software. Sp6-FLAG and Spt16 signals were made relative to the signal from the G6PDH loading control and normalized to wild-type signal, which was set to 1.

**RESULTS**

Spt4, Spt5, and Bur2 are required to repress SER3 transcription through a pathway involving Paf1. Previously, we showed that transcription of SRG1 intergenic ncDNA across the SER3 promoter region strongly interferes with the initiation of SER3 transcription (48, 50). To identify factors involved in SER3 repression by intergenic transcription, we screened the haploid S. cerevisiae gene deletion collection (Open Bio- systems) for gene deletions that derepress a SER3-pro-HIS3 reporter gene (J. A. Pruneski, unpublished data). Two of the genes identified in this screen encode the Spt4 and Bur2 transcription elongation factors. Spt4 partners with the essential factor Spt5 to form the yeast homolog of the mammalian DSIF complex (yDSIF) (89). yDSIF associates with Pol II over actively transcribed genes, has numerous physical and genetic interactions with other transcription factors, and facilitates elongation through chromatin (28, 71, 78, 79). Bur2 acts as a cyclin to activate the essential cyclin-dependent kinase Bur1, forming a partial functional homolog of mammalian P-TEFb (96, 99). The Bur1/2 complex plays a variety of roles in transcription through the phosphorylation of substrates, including the CTD of the RNA Pol II subunit Rpb1 (58, 68), the ubiquitin-conjugating enzyme Rad6 (95), and the C-terminal repeat region of Spt5 (46, 102). Phosphorylation by Bur1/2 activates the Spt5 protein, which promotes the recruitment of the Pafl complex to chromatin (46, 102). To determine if these transcription elongation factors are required for endogenous SER3 repression, we measured SER3 transcript levels in strains lacking functional copies of Spt4, Spt5, Bur2, and the Pafl subunit of the Pafl complex (Fig. 1A). In agreement with the results from our genetic screen, both spt4Δ and bur2Δ mutants...
These experiments revealed strong derepression of each lacked one of the five subunits of the Paf1 complex (Fig. SER3

repression in these mutants cannot be explained solely by re-

levels were not dramatically reduced, indicating that

bur2 or SRG1 transcript levels. Similar to

levels but showed only a modest

increase in

transcript levels, as

cdc73, rtf1, and

strains did not exhibit reduced

levels, as

reo1Δ and rtf1Δ strains, but when multiple experiments were

averaged, there was no difference from wild-type levels (Fig.

2B). We conclude from these data that Paf1 complex-mediated

repression occurs primarily through the activities of the Paf1 and Ctr9 subunits by a mechanism that does not

involve the control of the Paf1 and Ctr9 subunits.

Fig. 2. SER3 repression is primarily dependent on the Paf1 and Ctr9 subunits. (A) Representative Northern analysis of SER3, SRG1, and SCR1 (loading control) RNA levels in wild-type (FY5), paf1Δ (KY1700), ctr9Δ (KY2170), leo1Δ (KY1805), cdc73Δ (KY1706), and rtf1Δ (KY1703) strains. (B) Quantitation of results from a minimum of seven biological replicates. The values shown are the mean SER3 (black) and SRG1 (gray) transcript levels, normalized to the SCR1 loading control and made relative to the wild-type strains. Error bars indicate standard errors of the means, and asterisks indicate statistical significance compared to the wild type (*, P < 0.05; **, P < 0.01). (C) Representative Northern analysis of SER3, SRG1, and SCR1 (loading control) RNA levels. The wild-type strain (YJ1087) was transformed with a control plasmid (pRS416). The paf1Δ strain (YJ786) was transformed with either pRS416 (+PAF1) or pRS316-HA-PAF1 (+PAF1). The ctr9Δ (YJ759) strain was transformed with either pRS416 (~CTR9) or pAP10 (+CTR9). (D) Quantitation of results from a minimum of three biological replicates. The values shown are the mean SER3 (black) and SRG1 (gray) transcript levels, normalized to the SCR1 loading control and made relative to the wild-type strains. Error bars indicate standard errors of the means, and asterisks indicate statistical significance compared to the wild type (*, P < 0.05; **, P < 0.01).

strongly derepressed SER3, with the sp4Δ mutant being slightly more defective in SER3 repression. A temperature-
sensitive mutation of the essential SPT5 gene, sps5-194, not only derepressed SER3 at a nonpermissive temperature (39°C [data not shown]), as had been previously shown (20), but also at a permissive temperature (30°C) (Fig. 1). Strains lacking Paf1 exhibited increased SER3 transcript levels, similar to those observed in the bur2Δ and sps5-194 strains. This result agrees with previous microarray data that showed increased SER3 levels in a paf1Δ strain (64). Consistent with these factors working in the same pathway, a paf1Δ bur2Δ double mutant derepressed SER3 to a level that was equivalent to either paf1Δ or bur2Δ single mutants (Fig. 1). Importantly, SRG1 transcript levels were not dramatically reduced, indicating that SER3 repression in these mutants cannot be explained solely by re-
duced SRG1 transcription. Taken together, these results show that Paf1 is required for SER3 repression, likely involving its recruitment to SRG1 by Bur1/2 and Spt4/5, as has been seen at other transcribed regions (46, 102).

SER3 repression by the Paf1 complex depends primarily on the Paf1 and Ctr9 subunits. To further investigate the requirement for Paf1 complex subunits in SER3 repression, we exam-
ined SER3 and SRG1 transcript levels from mutant strains that each lacked one of the five subunits of the Paf1 complex (Fig. 2). These experiments revealed strong derepression of SER3 (8- to 10-fold) in paf1Δ and ctr9Δ mutants compared to wild-
type strains. Complementation of these strains with plasmid-borne copies of wild-type PAF1 and CTR9, respectively, re-
stored SER3 repression (Fig. 2C and D). More modest defects in SER3 repression (2- to 3-fold increases in SER3 transcript levels) were seen in leo1Δ, cdc73Δ, and rtf1Δ strains (Fig. 2B). Whereas the Paf1 complex has been shown to facilitate transcription elongation (12, 30, 37, 63, 70, 88), it is important to note that the paf1Δ and ctr9Δ strains did not exhibit reduced SRG1 RNA levels. Rather, SRG1 levels were increased 2-fold in these mutants. However, these increases in SRG1 RNA levels were unlikely to impact SER3 levels, as cdc73Δ strains also exhibited higher SRG1 levels but showed only a modest increase in SER3 levels. SRG1 levels showed some variability in leo1Δ and rtf1Δ strains, but when multiple experiments were averaged, there was no difference from wild-type levels (Fig. 2B). We conclude from these data that Paf1 complex-mediated repression of SER3 occurs primarily through the activities of the Paf1 and Ctr9 subunits by a mechanism that does not involve the control of SRG1 transcript levels.

The Paf1 complex localizes to the SER3 promoter when SRG1 is transcribed. We next performed ChIP experiments to determine if the Paf1 complex is physically associated with the SER3 promoter when SER3 is repressed. Cells expressing previ-
ously described epitope-tagged derivatives of Paf1, Rtf1,
Leo1, Cdc73, and Ctr9 (79, 91) were grown in serine-rich medium. Chromatin was isolated from these cells after cross-linking with formaldehyde and then subjected to immunoprecipitation with antibodies that recognize the epitope tags. We detected strong occupancy of all Paf1 complex subunits at SER3 (SER3-22 and SER3-19) and the flanking AIN9 (SER3-41) and SER3 (SER3-1) genes from untagged (FY4), 3×HA-Paf1 (KY1721), 3×HA-Rtf1 (KY2082), Leo1-3×HA (KY785), and 3×HA-Cdc73 (KY786) strains grown in YPD at 30°C. (B) ChIP analysis of Ctr9-Myc from untagged (KY399) and CTR9-6×MYC (KY785) strains grown in YPD at 30°C. The relative occupancies of these factors were calculated from untagged (KY399) and CTR9-6×MYC (KY785) strains grown in YPD at 30°C. Error bars indicate standard errors of the means, and asterisks indicate statistical significance compared to the wild type (*, P < 0.05; **, P < 0.01).

FIG. 3. The Paf1 complex colocalizes with actively transcribed SRG1. (A) ChIP analysis of HA-tagged Paf1 complex subunits at SRG1 (SER3-22 and SER3-19) and the flanking AIN9 (SER3-41) and SER3 (SER3-1) genes from untagged (FY4), 3×HA-Paf1 (KY1721), 3×HA-Rtf1 (KY2082), Leo1-3×HA (KY785), and 3×HA-Cdc73 (KY786) strains grown in YPD at 30°C. (B) ChIP analysis of Ctr9-Myc from untagged (KY399) and CTR9-6×MYC (KY785) strains grown in YPD at 30°C. The relative occupancies of these factors were calculated from untagged (FY4), 3×HA-Paf1 (KY1721), 3×HA-Rtf1 (KY2082), Leo1-3×HA (KY785), and 3×HA-Cdc73 (KY786) strains grown in YPD at 30°C. Error bars indicate standard errors of the means, and asterisks indicate statistical significance compared to the wild type (*, P < 0.05; **, P < 0.01).

SER3 repression is largely independent of histone H2B ubiquitylation. (A) Representative Northern analysis of SER3, SRG1, and SCR1 (loading control) RNA levels in wild-type (FY5), rad6Δ (KY1712), bre1Δ (KY1713), (hta2-htb2)Δ (KY2172), and (hta2-htb2)Δ htb1-K123R (KY2167) strains grown in YPD at 30°C. (B) Quantitation of results from a minimum of three biological replicates. The values shown are the mean SER3 (black) and SRG1 (gray) transcript levels, normalized to the SCR1 loading control and made relative to the wild-type strains. Error bars indicate standard errors of the means, and asterisks indicate statistical significance compared to the wild type (*, P < 0.05; **, P < 0.01).
this modification (59, 87, 91, 94). Taken together, the role of the Paf1 complex in SER3 repression seems to be largely independent of its known roles in regulating histone modifications.

Paf1 and Ctr9 are required for nucleosome occupancy over the SER3 promoter. We recently demonstrated a role for chromatin in the repression of SER3 (27). Under conditions in which SRG1 is transcribed and SER3 is repressed, the SER3 promoter is occupied by randomly positioned nucleosomes that prevent transcription factors from binding to this region. When SRG1 is downregulated, the SER3 promoter region becomes nucleosome free, allowing transcription factors to bind and induce SER3 expression (27). These results support a promoter occlusion model whereby intergenic SRG1 transcription deposits and maintains nucleosomes over the SER3 promoter to mediate repression. To test whether the Paf1 complex contributes to SER3 repression by affecting SRG1 transcription-dependent nucleosome occupancy of the SER3 promoter, we first performed nucleosome scanning assays on mutant strains that each lacked one of the five subunits of the Paf1 complex (Fig. 5A). MNase protection across SER3 was determined by qPCR and normalized to the protection of a well-positioned nucleosome in the GAL1 promoter, whose digestion by MNase was unaffected in these mutants (data not shown). In wild-type cells (black line), we observed a broad peak of protection over the SRG1 transcribed unit that overlaps the SER3 promoter, as we have previously reported (27). Strikingly, the effects of these mutants on MNase protection across the SRG1 transcription unit were consistent with their effects on SER3 expression. For paf1Δ and ctr9Δ strains that exhibit strong SER3 derepression, MNase protection across SRG1 was significantly reduced. For leo1Δ, rtf1Δ, and cdc73Δ mutants, which more modestly derepress SER3, we observed more modest decreases in MNase protection across this region.

We also performed histone H3 ChIP assays on paf1Δ, ctr9Δ, and rtf1Δ strains (Fig. 5B). Consistent with our nucleosome scanning results, we detected reduced histone H3 occupancy specifically over the SRG1 transcribed region in both paf1Δ and ctr9Δ strains, but not rtf1Δ strains, compared to wild-type strains (Fig. 5B, compare amplicons SER3-20 and SER3-22 to SER3-1 and SER3-41). Taken together, our nucleosome scanning and histone H3 ChIP results indicate that the Paf1 complex, primarily through the activities of the Paf1 and Ctr9 subunits, contributes to SER3 repression by promoting SRG1 transcription-dependent nucleosome occupancy across the SER3 promoter.

Spt16 colocalization with RNA Pol II across SRG1 is reduced in a paf1Δ strain. We previously showed that the transcription-dependent nucleosome assembly mediating SER3 repression requires the essential histone chaperones Spt6 and Spt16. Impairment of either factor reduces nucleosome occupancy over the SER3 promoter and strongly derepresses SER3, even though SRG1 transcription is maintained (27). Given the importance of these factors in SER3 repression and that a previous study with Drosophila provided evidence to support a role for the Paf1 complex in recruiting Spt6 and FACT to actively transcribed genes (1), we performed ChIP assays to compare the occupancy of Spt6, Spt16, and the Rpb3 subunit of RNA Pol II at actively transcribed SRG1 between wild-type, paf1Δ, and rtf1Δ strains (Fig. 6). Consistent with our Northern blotting data, we found equivalently high levels of Rpb3 associating with actively transcribed SRG1, which overlaps the SER3 promoter (Fig. 6C, amplicons SER3-19 and SER3-22).
all three strains. In wild-type cells, we detected strong Spt6 and Spt16 occupancy that colocalized with Rpb3 across the SRG1 transcription unit (Fig. 6A and B). Whereas the occupancies of these factors were not significantly affected in an rtf1/H9004 mutant, we observed a 4- to 5-fold reduction in Spt16 occupancy over this region in a paf1/H9004 mutant, while Spt6 occupancy was more moderately reduced (less than 2-fold). Notably, the decreased association of these factors was not due to a reduction in Spt6 or Spt16 expression levels as determined by Western analysis of whole-cell extracts prepared from wild-type and Paf1 complex mutant strains (Fig. 6D and E). Taken together, these data show that Paf1 is required for Spt16 occupancy and, to a lesser extent, Spt6 occupancy over actively transcribed SRG1, which overlaps the SER3 promoter.

Effects of Paf1 complex mutants at other transcribed yeast genes. Since the Paf1 complex colocalizes with Pol II over actively transcribed genes (38, 42, 53, 67), we performed ChIP assays to determine if the occupancy of histone H3, Spt6, and Spt16 at other transcribed genes is dependent on Paf1, as we have observed for SRG1. The Paf1 complex, Spt6, Spt16, and Rpb3 were present at high levels within the open reading frames of the highly transcribed PMA1 and ADH1 genes compared to background levels of association with the lowly transcribed GAL1 and CYC1 genes (Fig. 7A to E). Similar to our results for SRG1, occupancy of Spt6 and Spt16 was reduced over PMA1 and ADH1 in paf1/H9004 mutants but not rtf1/H9004 mutants (Fig. 7C and D). However, in contrast to what we observed at SRG1, Rpb3 occupancy was modestly reduced over PMA1 and
ADH1 in paf1Δ strains (Fig. 7E). Since interactions with RNA Pol II may contribute to the recruitment of Spt6 and Spt16 to actively transcribed genes (21, 52, 81), we recalculated Spt6 and Spt16 occupancies relative to Rpb3 occupancy (Fig. 7G and H). Relative to Rpb3 occupancy, a reduction in Spt16 occupancy in paf1Δ cells remained evident over these two highly transcribed genes, although the difference was less significant and was now similar to what we observed for rtf1Δ cells. Additionally, the reduction in Spt6 occupancy that was observed in paf1Δ cells was no longer evident. Interestingly, we

FIG. 7. Effects of the Paf1 complex mutant on histone H3, Spt6, and Spt16 occupancy at other transcribed genes. (A and B) Relative occupancies of HA-Paf1, HA-Rtf1, Leo1-HA, and HA-Cdc73 (A) and of Ctr9-MYC (B) within the coding sequence of two highly transcribed genes, PMAI and ADH1, and two lowly transcribed genes, GAL1 and CYC1, were determined by qPCR using the ChIP assay shown in Fig. 3. (C to E) Relative occupancies of Spt6-FLAG (C), Spt16 (D), and Rpb3-HA (E) at these four genes were determined by qPCR by using the immunoprecipitated chromatin shown in Fig. 6. (F) Relative occupancy of histone H3 was determined by qPCR by using the immunoprecipitated chromatin assayed in Fig. 5B. The occupancies of each of these factors at these genes were normalized to their occupancies at a control region on chromosome V that contained no open reading frames (no ORF). Each value represents the mean ± standard error of the mean for at least three biological replicates, and asterisks indicate statistical significance (*, P < 0.05; **, P < 0.01). (G and H) Spt6-FLAG (G) and Spt16 (H) ChIP data from wild-type, paf1Δ, and rtf1Δ strains (C and D) were normalized to Rpb3-HA ChIP data (E).
found that histone H3 occupancy was significantly reduced at 
*MA1* in *paf1Δ* mutants; however, we did not detect any change in histone H3 occupancy at *ADH1* (Fig. 7F). Taken together, these data indicate that the requirement of Paf1 and Ctr9 for transcription-dependent nucleosome occupancy and recruitment of Spt6 and Spt16 at *SRG1* is not universal for all highly transcribed genes.

**DISCUSSION**

In this study, we sought to further our understanding of the repression of *SER3* by *SRG1* intergenic transcription. We uncovered a role for the multifunctional Paf1 transcription elongation complex in *SER3* repression. We showed that the Paf1 complex colocalizes with Pol II during *SRG1* transcription, and we provided genetic data indicating that the Paf1 complex functions in a previously characterized pathway with Bur1/2 and Spt4/5. Our data indicate that *SER3* repression requires the Paf1 and Ctr9 subunits to promote *SRG1* transcription-dependent nucleosome occupancy across the *SER3* promoter, possibly by facilitating the association of the histone chaperones Spt6 and Spt16.

Our nucleosome scanning and histone ChIP experiments provided evidence that Paf1 and Ctr9 repress *SER3* by facilitating *SRG1* transcription-dependent nucleosome occupancy of the *SER3* promoter (Fig. 5). Previous studies have indicated that a primary function of the Paf1 complex is to establish several histone modifications that are important for chromatin dynamics at actively transcribed genes. However, these marks do not appear to play a major role in *SER3* repression by *SRG1*. First, we have shown that both an *rfl1Δ* mutant and mutations that prevent Rtf1-dependent monoubiquitylation of histone H2B at K123—either an arginine substitution of lysine 123 or deletion of the *RAD6* or *BRE1* genes responsible for this mark—only weakly derepress *SER3* compared to *paf1Δ* and *ctr9Δ* mutants (Fig. 2 and 4). Second, we determined that a parallel set of mutations preventing the subsequent methylation of histone H3 K4 and K79 have no effect on *SER3* repression (26, 27). Finally, we had previously shown that *SER3* repression is also unaffected by mutations that prevent methylation of histone H3 K36 (26, 27), a modification that is dependent on Paf1, Ctr9, and to a lesser extent Cdc73 (14).

The Paf1 complex has also been shown to play a role in regulating phosphorylation events occurring in the heptapeptide repeats in the CTD of RNA Pol II and transcription termination (34, 56, 61, 64, 75, 87). Although we cannot absolutely rule out a role for these Paf1 complex-dependent activities in regulating *SER3* repression, our data suggest that these activities are unlikely to account for the role of the Paf1 complex in maintaining *SRG1* transcription-dependent nucleosome occupancy of the *SER3* promoter. First, there are distinct differences in the subunit requirements for these activities. Whereas nucleosome occupancy of the *SER3* promoter is primarily dependent on Paf1 and Ctr9, additional subunits of this complex are required for the phosphorylation of the serines at position 2 within the heptapeptide repeats (Ser2-P) and proper transcription termination (34, 56, 61, 64, 75, 87). Second, the termination of *SRG1* has been mapped to two distinct sites, 75 bp 5' and 25 bp 5' of the *SER3* translation start site, along with a minor read-through product to the end of *SER3* (86). Among Paf1 complex subunit deletions, we did not observe any increase in this read-through product, as might be expected if the Paf1 complex affected *SRG1* termination (data not shown). Taken together, our studies of *SER3* repression suggest a role for the Paf1 complex in controlling transcription-coupled nucleosome occupancy that is primarily dependent on the Paf1 and Ctr9 subunits and independent of previously characterized activities of this complex.

Interestingly, a possible role for the Paf1 complex in regulating chromatin dynamics during transcription, independent of its roles in promoting histone modifications, was recently proposed by Kim et al. based on an *in vitro* transcription system where the human Paf1 complex was shown to stimulate elongation through nucleosomes (37). Our studies of *SER3* regulation suggest this role may be specific to the Paf1 and Ctr9 subunits, involving a more direct role for these two factors in promoting nucleosome occupancy during transcription. One study previously implicated the Paf1 complex in altering nucleosome occupancy during gene induction. However, rather than promoting nucleosome occupancy, the earlier study showed that Paf1 and Ctr9 were required for efficient histone eviction at the *GAL1-10* promoter during the induction of these genes in response to galactose (51). Several studies have also linked efficient induction of *GAL1-10* and H2B K123ub by Rad6/Bre1 (29, 33, 97), suggesting that the effect of the Paf1 complex on *GAL1-10* induction is likely through its role in promoting histone modifications.

How might Paf1 and Ctr9 promote *SRG1* transcription-dependent nucleosome occupancy at the *SER3* promoter? One possibility is that Paf1 regulates the histone chaperones Spt6 and/or Spt16, which are required for the maintenance of nucleosomes over the *SER3* promoter (27). In yeast, the Paf1 complex has genetic interactions with Spt6 (34) and both physical and genetic interactions with Spt16 (63, 79). The Paf1 complex has also been shown in *Drosophila* to be required for full recruitment of Spt6 and the FACT subunit SSRP1 during transcription (1). These connections led us to examine whether the recruitment of these factors is affected in Paf1 complex mutants in yeast (Fig. 6). ChIP experiments revealed that Spt6 occupancy over *SRG1* is strongly dependent on Paf1 but not Rtf1, which correlates with the effect that each of these factors has on *SER3* repression. Western analyses and RNA Pol II ChIP data indicated that this reduction in Spt6 occupancy in *paf1Δ* cells is not caused by a reduction in Spt6 protein levels or by a reduction in RNA Pol II levels across *SRG1* (Fig. 6). Taken together, our results support a role for Paf1 and Ctr9 in promoting FACT occupancy across actively transcribed *SRG1*. In contrast to Spt6, we found Spt6 occupancy at *SRG1* to be only modestly dependent on Paf1. Therefore, while Paf1 and Ctr9 may play a prominent role in Spt6 occupancy at *SRG1*, other factors are likely to contribute significantly to Spt6 occupancy of this region. Spt6 is known to associate with elongating Pol II through a direct interaction with Pol II CTD containing Ser2-P (21, 81). Although it is unlikely to be part of the Paf1-dependent pathway that represses *SER3* as we discussed earlier, this mark may contribute to Spt6 occupancy at *SRG1* in a Paf1-independent pathway. Interestingly, Thebault et al. recently reported that Spt6 occupancy at *SRG1* is also partially dependent on Spt2, an HMG-like transcription elongation factor (85). Although we cannot
rule out the possibility that Spt2 contributes to Spt6 occupancy in a pathway with Paf1, Spt2 may also facilitate Spt6 recruitment independently of Paf1. Overall, our results are consistent with those observed in Drosophila, in which depletion of Paf1, and to a lesser extent Rtf1, led to reduced association of Spt6 and FACT over an actively transcribed gene without affecting Pol II association or global protein levels (1). Interestingly, localization of the Paf1 complex to actively transcribed genes has also been shown to be partially dependent on Spt6 and Spt16 (34, 63). Therefore, it is possible that recruitment of Spt6, Spt16, and the Paf1 complex is interdependent, where the disruption of one of these factors results in reduced associations of the others.

Cells lacking Paf1 not only reduce the association of Spt6 and Spt16 across the SRG1 transcription unit but also reduce nucleosome occupancy over this region that overlaps the SER3 promoter. Because both Spt6 and Spt16 interact with histones (5, 6, 25) and have been implicated in restoring nucleosome occupancy after the passage of RNA Pol II at transcribed genes (5, 13, 31, 35, 52), it is also possible that the loss of these factors over SRG1 is a consequence, rather than a cause, of reduced nucleosomes over this region in the paf1Δ strains. In this case, the Paf1 complex may be required for the transcription-dependent nucleosome reassembly activity of Spt6 and Spt16 rather than recruitment of these factors. Interestingly, Spt2 is also required to promote nucleosome occupancy across the SRG1 transcription unit to repress SER3 transcription (62, 85). In addition, Spt2 has been genetically linked to Paf1 and Ctr9, and its colocalization with RNA Pol II across actively transcribed genes is dependent on Paf1, albeit weakly (62). It will be interesting to decipher the functional interplay between the Paf1 complex, Spt6, FACT, and Spt2 in promoting SRG1 transcription-dependent nucleosome occupancy and SER3 repression.

In addition to the well-characterized role of the Paf1 complex in promoting transcription, whole-genome expression analyses indicate that the Paf1 complex also functions as a negative regulator of transcription (64). Our finding that the Paf1 complex indirectly represses SER3 expression by positively regulating the chromatin dynamics associated with SRG1 intergenic transcription across the SER3 promoter has provided one of the first insights into understanding how this complex negatively regulates transcription. A negative regulatory role for the Paf1 complex has also been recently characterized for the yeast gene ARG1 (18). In this case, Paf1 complex members were found to associate with both the ARG1 promoter and ORF during repression conditions. Similar to SER3 repression, ARG1 repression is most strongly dependent on the Paf1 and Ctr9 subunits; however, Rtf1 and Rtf1-regulated histone modifications seem to have a greater effect at ARG1 than at SER3 (18). Although the mechanistic role of Paf1 and Ctr9 in ARG1 repression has yet to be defined, it is interesting that transcription antisense to ARG1 has been detected at this locus (19, 98). Additional experiments are required to determine if SER3 and ARG1 repression occur by a common mechanism involving Paf1-mediated chromatin dynamics during transcription of ncDNA.

In addition to SRG1, we assayed the effects of deleting PAF1 on histone H3, Spt6, and Spt16 occupancy over the transcribed regions of two other highly transcribed genes, PMA1 and ADH1 (Fig. 7). Similar to our results for SRG1, we detected reduced Spt6 and Spt16 occupancy levels in cells lacking Paf1. However, in contrast to what we observed at SRG1, RNA Pol II levels for these genes were also slightly reduced in paf1Δ cells. Gene-specific differences in RNA Pol II occupancy have been previously reported for paf1Δ mutants (56). When normalized to RNA Pol II levels, there is no longer a reduction in Spt6 occupancy, while Spt16 occupancy is reduced to similar levels in both paf1Δ and rtf1Δ mutants (Fig. 7G and H). Furthermore, histone H3 occupancy was unaffected at ADH1/ and only moderately reduced at PMA1 in paf1Δ cells. These studies suggest that there are likely to be additional factors that influence the role of the Paf1 complex in regulating transcription-coupled nucleosome assembly at specific genes. One factor that may contribute to these gene-specific differences is the properties of the transcribed DNA. SRG1 is transcribed across the promoter region of SER3. In general, promoter regions tend to be comprised of sequences that are refractory to nucleosome formation, whereas the sequences of ORFs generally do not contain these properties (73). This would explain the inherent instability of nucleosomes over the SER3 promoter in the absence of SRG1 transcription. This characteristic of the SRG1 transcription unit makes it unique compared to the transcription of most protein-coding genes and may have allowed us to uncover this new role for the Paf1 complex that may not be readily detectable for other transcribed regions of the genome.

In conclusion, our analysis of SER3 repression by intergenic SRG1 transcription supports a previously uncharacterized role for the Paf1 complex in promoting transcription-dependent nucleosome occupancy. This activity is primarily dependent on the Paf1 and Ctr9 subunits, possibly mediated by their requirement for the recruitment and/or activity of the Spt6 and Spt16 histone chaperones. Additional studies will be necessary to elucidate the precise mechanism by which Paf1 and Ctr9 regulate chromatin dynamics during SRG1 transcription, to determine how broadly these two factors function in a similar manner at other transcribed regions of the genome, and to determine the characteristic of a transcription unit that makes it susceptible to this new activity of the Paf1 complex.

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