Transcription Regulation by the Noncoding RNA *SRG1* Requires Spt2-Dependent Chromatin Deposition in the Wake of RNA Polymerase II[▽]

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Spt2 is a chromatin component with roles in transcription and posttranscriptional regulation. Recently, we found that Spt2 travels with RNA polymerase II (RNAP II), is involved in elongation, and plays important roles in chromatin modulations associated with this process. In this work, we dissect the function of Spt2 in the repression of *SER3*. This gene is repressed by a transcription interference mechanism involving the transcription of an adjacent intergenic region, *SRG1*, that leads to the production of a noncoding RNA (ncRNA). We find that Spt2 and Spt6 are required for the repression of *SER3* by *SRG1* transcription. Intriguingly, we demonstrate that these effects are not mediated through modulations of the *SRG1* transcription rate. Instead, we show that the *SRG1* region overlapping the *SER3* promoter is occluded by randomly positioned nucleosomes that are deposited behind RNAP II transcribing *SRG1* and that their deposition is dependent on the presence of Spt2. Our data indicate that Spt2 is required for the major chromatin deposition pathway that uses old histones to refold nucleosomes in the wake of RNAP II at the *SRG1-SER3* locus. Altogether, these observations suggest a new mechanism of repression by ncRNA transcription involving a repressive nucleosomal structure produced by an Spt2-dependent pathway following RNAP II passage.

In eukaryotes, modulation of the chromatin structure is a key aspect of important processes involving DNA. The basic unit of chromatin structure is the nucleosome, which consists of 146 bp of DNA wrapped around an octamer of histones (20). In addition to histones, many nonhistone proteins play important roles in regulating chromatin structure and chromatin-related processes (41). In *Saccharomyces cerevisiae*, one such nonhistone chromatin component is the HMG-like protein Spt2/Sin1, which was first identified genetically, by analysis of secondary mutations that suppress Ty and δ insertion mutations (*spt2* for "suppressor of Ty 2"), in the *HIS4* promoter (43).The sequence identity of Spt2 to HMG proteins is weak. However, like these proteins, it binds DNA nonspecifically and has significant affinity to four-way junction DNA, a structure similar to that found at the entrance/exit point of DNA from a nucleosome (25, 46). Several studies indicated that this factor could play a negative role in transcription initiation. Indeed, Spt2 was identified in a second screen for mutations that suppress the loss of the Swi/Snf chromatin remodeling complex and was called *SIN1* (39). In addition, mutations in *SPT2* suppress defects caused by mutations of SAGA histone acetyltransferase complex components (27, 31) and by deletion mutations in the RNA polymerase II (RNAP II) largest subunit gene (*RPB1*) (28). Finally, *spt2* mutations have been shown to derepress the heat shock *SSA3* gene (2).

While these mutant phenotypes suggested a negative role for Spt2 in transcription initiation, our recent work showed that it has an important function in transcription elongation (24). We demonstrated that Spt2 plays a significant role in the maintenance of proper chromatin structure over transcribed regions of the two active genes *PMA1* and *GAL1*. Similarly to other elongation factors, it is required for the repression of transcription from the *FLO8* cryptic promoter within its coding region (24). This factor is also involved in 3′-end processing of RNA and has been shown to specifically affect the polyadenylation of mRNA (11). Finally, in addition to its role in transcription, the *spt2Δ* mutation enhances recombination where transcription is active and causes defects in chromosome segregation and stability (16, 24, 38). Collectively, these observations suggest that Spt2p protects genome integrity at transcriptionally active regions, presumably by maintaining chromatin structure.

Global analyses of Spt2 localization showed that it is generally associated with coding regions of actively transcribed genes (24). However, a few exceptions have been observed, and among these infrequent Spt2 localizations is the *SRG1-SER3* intergenic region. Interestingly, the *SER3* gene is regulated by a transcription interference mechanism involving the transcription of a noncoding RNA (ncRNA) (21), produced from the intergenic region (*SRG1*) where Spt2 is localized. Noncoding RNAs were shown to be major players in gene expression regulation. They are produced by transcription across entire genomes, including intergenic regions, and regulate gene expression by different mechanisms, including RNA interference (RNAi)-mediated pathways of gene repression (10). However, production of some ncRNAs, as is the case for the ncRNA *SRG1*, regulates gene expression in *cis*. Indeed, recent observations indicated that, rather than the ncRNA

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product itself, it was the act of transcription and its associated processes that were important for the regulation of adjacent genes. Moreover, a few studies showed that the chromatin modulations associated with transcription of ncDNA play a major regulatory role. The activation of the *Schizosaccharomyces pombe fbp1*[+] gene requires displacement by ncRNA transcription of key nucleosomes at the *fbp1*[+] promoter (12). Similarly, antisense transcription enhances *PHO5* activation by facilitating nucleosome displacement from the *PHO5* promoter (40). Other studies have highlighted the roles of specific chromatin marks induced by the transcription of ncRNAs in the regulation of adjacent genes (reviewed in reference 10). Specifically, the histone posttranslational modifications directed to *GAL1* and *GAL10* regulatory regions by transcription of the ucut Gal ncRNA were shown to directly affect the expression of these genes (13, 30).

In the *SRG1-SER3* system, the act of transcribing *SRG1*, rather than the ncRNA product, mediates regulation of the *SER3* gene (21). The current model posits that in serine-rich medium *SRG1* transcription interferes with the downstream *SER3* promoter, thereby blocking *SER3* expression and unnecessary serine biosynthesis (see Fig. 1A) (23, 37). Although the specific molecular mechanism of transcription interference is unknown, several models were proposed, including promoter occlusion by the transcription machinery, collisions between RNAP II complexes, and promoter competition (37). In the case of the *SRG1-SER3* regulatory system, the current preferred model postulates that the passage of RNA polymerase II transcribing *SRG1* ncDNA through the *SER3* promoter inhibits the assembly of the preinitiation complex and represses *SER3* transcription (21). Importantly, in the absence of *SRG1* ncRNA, Spt2 is completely delocalized from the intergenic region, suggesting a tight association between the regulation by *SRG1* ncRNA and Spt2 targeting to the intergenic DNA (24). Moreover, deletion of the *SPT2* gene affects dramatically the transcription of *SER3*, apparently by disturbing the regulation by transcription interference associated with *SRG1* ncRNA production (24).

In this work, we analyzed the mechanism by which the elongation factor Spt2 modulates expression of the *SER3* gene by ncRNA *SRG1* transcription. We found that the change in ncRNA *SRG1* transcription in the *spt2Δ* background cannot explain the loss of *SER3* repression. Importantly, we show that Spt2 is involved in the shaping of a particular nucleosomal structure at the *SRG1-SER3* locus. Our data indicate that nucleosomes are deposited specifically within the ncDNA *SRG1* region, corresponding to the *SER3* promoter regulatory elements, following the passage of RNAP II. Interestingly, this deposition is severely impaired by the *spt2Δ* mutation, suggesting that the loss of nucleosomes at this region could be the consequence of disrupted nucleosome deposition in the wake of transcription. Therefore, in contrast to previous observations and models involving nucleosome displacement, histone posttranslational modifications, and promoter occlusion by RNAP II, our data suggest a model in which specific nucleosomes, deposited by a transcription-dependent mechanism involving Spt2, play a central role in the repression by ncRNA transcription.
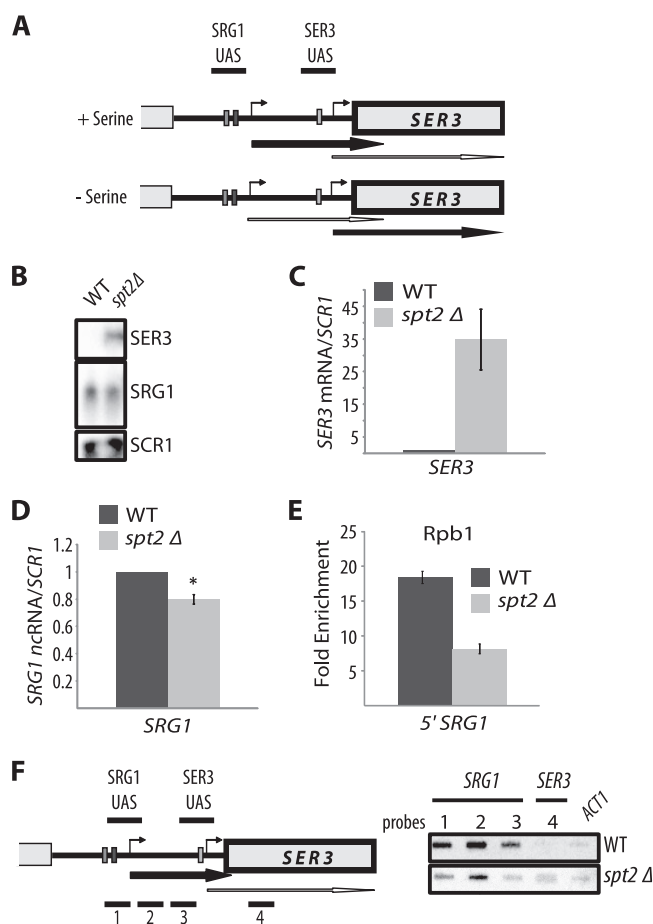

FIG. 1. Deletion of the *SPT2* gene affects *SER3* regulation and reduces ncRNA *SRG1* levels. (A) Diagram explaining the complex regulation of *SER3* by the intergenic transcription of the ncDNA *SRG1*. In the presence of high levels of serine in the medium, ncDNA *SRG1* is actively transcribed and SER3 is inhibited by transcription interference. In the absence of serine, *SRG1* ncDNA transcription activity is reduced and SER3 is then induced. (B) *spt2Δ* mutation affects the transcript levels of *SER3* and ncDNA *SRG1*. Wild-type (WT) (YAN1034) and *spt2Δ* (YAN1035) strains were grown in YPD at 30°C. Total RNA was extracted and analyzed by Northern blotting with a probe against *SER3* and *SRG1*. *SCR1* served as a loading control. (C and D) Quantification of the *SRG1* ncRNA and *SER3* mRNA by RT-qPCR. Total RNA analyzed by Northern blotting as shown in panel B was used to produce cDNA and quantified by qPCR. The relative level is the ratio of the indicated RNA to the *SCR1* transcript level. All values shown are the average results with standard errors from three independent experiments. ∗, $P < 0.05$. (E) Association of RNAP II with ncDNA *SRG1* is partially dependent on Spt2. Yeast cells from the wild-type (YAN1034) or *spt2Δ* (YAN1035) strains were grown in YPD medium to mid-log phase and then cross-linked with 1% formaldehyde. Chromatin immunoprecipitations were performed using the 8WG16 antibody against Rpb1. The fold enrichment is the ratio of the percent immunoprecipitation (%IP) of the ncDNA *SRG1* region to the %IP of the nontranscribed control region (NoORF). (F) Run-on assay showing that deletion of *SPT2* results in a decrease of transcription activity at intergenic ncDNA *SRG1*. Diagram showing the different probes used in the run-on assay conducted on the *SRG1-SER3* locus. Radiolabeled RNA extracted from wild-type (YAN1034) or *spt2Δ* (YAN1035) strains was hybridized on membranes containing immobilized probes spanning the *SRG1-SER3* locus. The *ACT1* probe was used as a control.

***S. cerevisiae* strains, media, and genetic methods.** All *S. cerevisiae* strains (see Table ST1 at http://www.crc.ulaval.ca/nourani/supplemental_data) are isogenic to a *GAL2* derivative of S288C. Strains (44) were constructed by standard methods, either by crosses or by transformation. The *HIS3-pGAL1::SRG1* and *HIS3-pGAL1::FMP27* alleles were constructed by replacing the promoter of the corresponding gene with the *HIS3-pGAL1* cassette (1, 19). *SPT15-3HA*, marked with *HIS3*, was generated by integrating DNA encoding three copies of the HA epitope at the 3′ end of the gene (19). For experiments involving galactose induction, cells were grown to an optical density at 600 nm (OD_{600}) of 0.5 in YP (1% yeast extract, 2% peptone) supplemented with 2% raffinose (YPraf). The cells were then centrifuged, resuspended in YP medium containing 2% galactose (YPgal), and grown for 2 h before being harvested. In the experiments involving glucose repression, cells were grown to an OD_{600} of 0.5 in YPgal; glucose was then added to reach a concentration of 4% in the medium. Efficient G_1 arrest (at least 95%) of cells was achieved by adding 500 ng/ml of α-factor for 2 to 3 h. Sequences of all oligonucleotides used in strain constructions, Northern blotting, reverse transcription (RT)-PCR, run-on transcription, nucleosome scanning assays, and chromatin immunoprecipitation (ChIP) assays are available upon request.

Chromatin immunoprecipitation experiments. Chromatin immunoprecipitation experiments were performed as previously described (22). For the immunoprecipitation of Spt2-13Myc, TBP-3HA, and Gal4, we used, respectively, the antibodies 9E10 anti-Myc (1 μl per immunoprecipitation; Covance), HA.11 anti-HA (1.5 μl per immunoprecipitation; Covance), and Gal4 (DBD):sc-577 (1 μl per immunoprecipitation; Santa Cruz Biotechnology). Immunoprecipitation of Rpb1 was performed using the 8WG16 anti-CTD antibody (2 μl per immunoprecipitation; Covance). The histone H3 immunoprecipitation was done using rabbit anti-H3 antibody (0.2 μl per immunoprecipitation; Abcam). The PCR amplification was performed with 1% of the precipitated material and 0.05% of the input DNA using the LightCycler 480 Sybr green I master kit from Roche.

RNA analyses. Total RNA was isolated using the hot-phenol method (35). In Northern blot analyses, 20 to 40 μg of RNA were separated on a 1% agarose formaldehyde-MOPS gel and transferred to a nylon membrane. The *SRG1*, *SER3*, and *SCR1* probes were amplified by PCR and radiolabeled by random priming. The *SRG1*, *SER3*, and *SCR1* probes were also quantified by RT-quantitative PCR (qPCR). For that process, cDNAs were generated using the Invitrogene M-MLV reverse transcriptase kit and their levels were measured by real-time PCR using LightCycler 480 Sybr green I master kit purchased from Roche. The run-on experiments were conducted as described in reference 21.

Nucleosome scanning assay. Nucleosome scanning experiments were performed using a method adapted from those previously described (3, 17, 42). Cells were grown to 2×10^7 to 3×10^7 cells/ml and treated with formaldehyde (2% final concentration) for 30 min at 30°C and then glycine (125 mM final concentration) for 10 min at room temperature. Formaldehyde-treated cells (1.2×10^9) were harvested by centrifugation, washed with Tris-buffered saline, and then incubated in ZDB buffer (50 mM Tris Cl, pH 7.5, 1 M sorbitol, 10 mM β-mercaptoethanol) containing 1.5 mg Zymolase 20T at 30°C for 30 min on a rocker platform. Spheroplasts were pelleted by low-speed centrifugation, gently washed with NP buffer (1 M sorbitol, 50 mM NaCl, 10 mM Tris Cl, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.075% NP-40, 1 mM β-mercaptoethanol, and 500 μM spermidine), and resuspended in 1.8 ml NP buffer. Samples were divided into six 300-μl aliquots that were then digested with 0, 1, 2.5, 5, 10, and 20 units of micrococcal nuclease (MNase) (Nuclease S7; Roche) for 45 min at 37°C. Digestions were stopped with 75 μl Stop buffer (5% SDS, 50 mM EDTA) and treated with 100 μg proteinase K for 12 to 16 h at 65°C. DNA was extracted by phenol-chloroform using PLG-H tubes (5 Prime) and incubated with 50 μg RNase A for 1 h at 37°C. DNA was reextracted with phenol-chloroform, precipitated with an equal volume of isopropanol, washed with 100% ethanol, and resuspended in 100 μl TE. MNase digestions were evaluated by two methods. First, one-fifth of digested DNA was separated by gel electrophoresis. Second, previously characterized *GAL1* promoter sequences (3, 8, 18), one within a positioned nucleosome (*GAL1* NB) and a second in an adjacent region (*GAL1* NUB) that is rapidly digested by MNase, were amplified by qPCR from MNase-treated and untreated samples. The MNase concentration that resulted in mostly mononucleosome-sized DNA with a *GAL1* NUB/NB ratio of <15% was subjected to further qPCR using tiled *SER3* primer pairs that amplify 38 unique *SER3* sequences that range from 90 to 114 bp in size, with an average overlap of 69 bp between sequences. For each *SER3* primer set, the amount of protected template was calculated as a ratio between amounts of MNase-digested and undigested samples and then normalized to the amount of protected *GAL1* NB template. All nucleosome scanning assays were done in triplicate using at least two independent strains as

indicated in the figure legends. The qPCR data were obtained using an ABI 7300 real-time PCR system with Sybr green reagents (Fermentas). All calculations were performed using Pfaffl methodology for relative quantitation of real-time PCR (29).

RESULTS

Spt2 is required for normal *SER3* repression and affects ncRNA *SRG1* production. The *SER3* gene is regulated by a complex mechanism involving noncoding RNA *SRG1* (ncRNA *SRG1*) transcription (21). As indicated in Fig. 1A, in the presence of serine in the medium, the *SER3* gene is inhibited by the active transcription of the *SRG1* gene. This gene produces a noncoding RNA and its transcription interferes with that of *SER3*. In the absence of serine from the medium, the *SRG1* gene is repressed and *SER3* is in turn rapidly activated (23). Spt2 is recruited to the ncDNA *SRG1*, and its association to this location is tightly linked to the presence of the ncRNA *SRG1* (24). We decided to further study the role of the elongation factor Spt2 in the complex regulation of *SER3*. Since *SER3* regulation is tightly associated with the transcription of the ncRNA *SRG1*, we wanted to know the effect of *SPT2* deletion on the levels of *SER3* mRNA and *SRG1* ncRNA. For that, we extracted total RNA from a wild-type or *spt2Δ* strain and performed Northern blot analyses. We observed a high level of *SER3* transcript in the *spt2Δ* strain, while no detectable amount of such transcript was observed in the wild-type cells (Fig. 1B). This is consistent with our previous observations (24). Interestingly, although the *SER3* mRNA level is dramatically increased in *spt2Δ* cells, the amount of ncRNA *SRG1* appears only slightly reduced in this mutant (Fig. 1B). We decided to quantify precisely the level of both *SER3* and *SRG1* transcripts by RT-qPCR. As indicated in Fig. 1C and D, the deletion of *SPT2* resulted in a dramatic increase of *SER3* mRNA (30- to 40-fold induction) but only a 20% reduction of the noncoding *SRG1* RNA steady-state level.

Recent observations showed that Spt2 plays a significant role in the posttranscriptional events and that it is necessary for the polyadenylation of RNAP II transcripts (11). Therefore, it is possible that the slight reduction of ncRNA *SRG1* is the consequence of a posttranscriptional defect linked to the *SPT2* deletion. To explore the possible role of Spt2 in the stability of the noncoding *SRG1* RNA transcripts, we conducted an experiment to measure the half-life of the ncRNA *SRG1* in wild-type or *spt2Δ* strains (see Fig. S1 at http://www.crc.ulaval.ca/nourani/supplemental_data). Our data indicate that *SRG1* ncRNA stability is not affected by the loss of Spt2.

Our results show that *SRG1* ncRNA levels are slightly affected by the loss of Spt2. Importantly, this effect is not associated with the posttranscriptional function of Spt2, since no defect in the stability of this transcript was observed (see Fig. S1 at the URL above). This finding suggests that a *SRG1* ncRNA drop in the *spt2Δ* strain could be the consequence of a decrease in transcription activity. We wanted to analyze, using different methods, the transcription activity at the *SRG1* region in wild-type and *spt2Δ* strains. For that, we first measured the transcription activity in different regions of the *SRG1-SER3* locus using run-on assays. Radiolabeled RNA was extracted from the wild-type strain or the *spt2Δ* mutant and blotted on membranes containing four probes covering the *SRG1-SER3*

locus and a control probe located in the *ACT1* gene (Fig. 1F). Not surprisingly, as a consequence of the *SER3* derepression in the *spt2*Δ strain, we detected a higher signal at the *SER3* open reading frame (probe 4, Fig. 1F) in this mutant. Importantly, deletion of the *SPT2* gene is associated with a reduction of probe 1, 2, and 3 run-on signals, indicating a lower transcription activity at the intergenic region (*SRG1*) in the absence of Spt2 (Fig. 1F).

In order to confirm our data, we extended our analysis and asked whether the reduction of transcription activity in the absence of Spt2 is associated with the reduction of the RNAP II level at *SRG1*. For that we measured the RNAP II occupancy in the wild-type or *spt2*Δ strain by Rpb1 chromatin immunoprecipitation assays (Fig. 1E). The deletion of *SPT2* led to a decrease of RNAP II association at the 5′ region of *SRG1*, indicating a correlation between the transcriptional activity and the level of RNAP II at this location. Therefore, we concluded that Spt2 is necessary for normal association of the transcription machinery at this location.

A different hypothesis could explain the reduction in transcription activity at this location. Spt2 could be required for the first steps of initiation or during early elongation of transcription at the intergenic region *SRG1*. One of the most important events during initiation is the recruitment of TBP to the TATA box (32). We analyzed the recruitment of TBP-HA to both *SRG1* TATA and *SER3* TATA in the wild-type and *spt2*Δ strains (see Fig. S2 at http://www.crc.ulaval.ca/nourani /supplemental_data). Our experiment shows clearly that TBP-HA association at *SRG1* TATA is similar in both strains, indicating that Spt2 loss affects transcription of ncDNA *SRG1* at a post-TBP recruitment step.

Spt2 role on *SRG1* transcription cannot explain the loss of *SER3* repression. The *SER3* gene is repressed presumably by transcription interference mechanism, whereby passages of RNAP II transcribing the *SRG1* intergenic region obstruct the *SER3* promoter, inhibiting the interaction of activators and initiation factors with their respective binding sites (21). If the transcription interference, in this case, was achieved merely by maintaining a certain transcription rate, then it is possible that the small reduction in transcriptional activity observed at the intergenic region in the *spt2*Δ mutant can disturb the transcription interference and consequently compromise *SER3* repression. We reasoned that, if this hypothesis was true, increasing the *SRG1* ncRNA transcription in the *spt2*Δ mutant to wild-type level would suppress the phenotype associated with this mutation and restore *SER3* repression. We therefore asked whether a higher level of transcriptional activity at the *SRG1* intergenic region is capable of restoring *SER3* repression in *spt2*Δ. To address this, we used an experimental system (Fig. 2A) in which we replaced the *SRG1* promoter by the strong inducible *GAL1* promoter in the wild type and the *spt2*Δ mutant (*pGAL1-SRG1*) and induced transcription of *SRG1*. Our goal was to increase the transcription activity at the intergenic region in the *spt2*Δ mutant in order to reach levels equal to or higher than those found in wild-type cells. We first compared the level of transcription activity under inducing conditions in wild-type, *spt2*Δ, and *spt2*Δ *pGAL1-SRG1* strains by analyzing RNAP II occupancy at the *SRG1* intergenic region via Rpb1 ChIP assays (Fig. 2B). RNAP II occupancy at the *SRG1* intergenic region in the *spt2*Δ *pGAL1-SRG1* strain was significantly

higher than that in wild-type or *spt2*Δ cells, where *SRG1* is transcribed from its own promoter. This indicates that replacing the *SRG1* promoter by that of *GAL1*, in *spt2*Δ cells, results in higher transcription activity at the *SRG1* intergenic region. We next wanted to know whether this higher transcription activity correlated with higher ncRNA levels. Total RNA was extracted from wild-type, *spt2*Δ, and *spt2*Δ *pGAL1-SRG1* cells grown under galactose-inducing conditions, and the ncRNA *SRG1* levels were analyzed by Northern blotting (Fig. 2C). As expected, in the *spt2*Δ mutant, the *GAL1* promoter produced a higher level of the ncRNA *SRG1* transcript than the *SRG1* promoter in both wild-type and *spt2*Δ mutant strains (compare lanes 1 and 2 to 4 in Fig. 2C). This observation was confirmed by RT-qPCR (see Fig. S4 at http://www.crc.ulaval.ca/nourani /supplemental_data). We conclude that the *GAL1* promoter induces a higher transcription level at *SRG1* in *spt2*Δ cells. Surprisingly, analyses of the *SER3* transcript level by Northern blotting or RT-qPCR (Fig. 2C and D; also see Fig. S3 at the URL above) indicated that this higher level of transcription activity observed in *spt2*Δ *pGAL1-SRG1* was not associated with repression of the *SER3* gene. We observed instead that higher transcription activity at ncDNA *SRG1* resulted in further derepression of *SER3*. Therefore, suppression of the *SRG1* transcription defect associated with the *spt2*Δ mutation does not restore *SER3* repression. We conclude that the reduction of transcription activity observed in *spt2*Δ cells cannot explain the failure of *SER3* repression by the transcription interference mechanism. Interestingly, higher transcription activity in wild-type cells was also associated with *SER3* activation (Fig. 2C, compare lanes 1 to 3). This indicates that high *SRG1* transcription induced by the *GAL1* promoter is not sufficient by itself for the repression of *SER3*.

Remarkable changes in intergenic *SRG1* transcription are observed when different serine levels are present in the growth media (23). Therefore, we analyzed the *SRG1* and *SER3* transcript levels during a time course with different serine concentrations (see Fig. S5 at http://www.crc.ulaval.ca/nourani /supplemental_data). Our data clearly show that the absence of serine results in a decreased level of *SRG1* and induction of *SER3* transcription in the wild-type strain. When serine was added back, we observed a dramatic increase of *SRG1* level that correlated with *SER3* repression (see Fig. S5). Interestingly, in the *spt2*Δ mutant, the serine regulation of ncRNA *SRG1* is similar to that in the wild-type strain. However, in this mutant *SER3* is never completely repressed, even after a dramatic increase of the ncRNA level in the presence of serine (see Fig. S5). This observation is highlighted in Fig. 2E, where we compare conditions in which the level of ncRNA *SRG1* is significantly higher in the *spt2*Δ strain than in the wild-type strain. Our Northern blot (Fig. 2E) and RT-qPCR (data not shown) analyses demonstrate that the levels of the *SRG1* ncRNA are lower in wild-type cells in the presence of low levels of serine (YPD medium) than those in the *spt2*Δ mutant in the presence of high levels of serine (SD medium with added serine). However, in contrast to the level in the wild type, the higher level of the *SRG1* ncRNA in *spt2*Δ cells is not associated with better repression of *SER3*. This indicates a disconnection between intergenic *SRG1* transcription levels and *SER3* regulation in the *spt2*Δ mutant and confirms that the small reduction of ncRNA transcription observed in these cells cannot
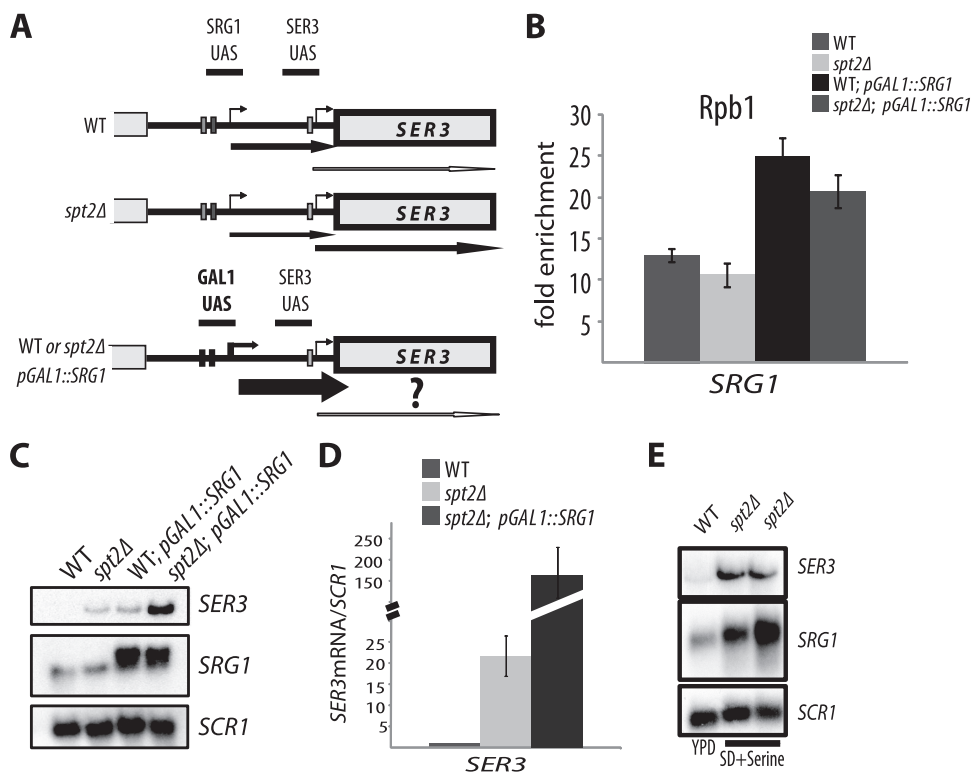
FIG. 2. Increasing the intergenic transcription of ncDNA *SRG1* does not restore *SER3* repression in the *spt2Δ* mutant. (A) Diagram showing the p*GAL1-SRG1* construct. WT, wild type. (B) *GAL1* promoter drives a higher level of transcription at ncDNA *SRG1*. Yeast cells from the wild-type strain (YAN1034), the *spt2Δ* mutant (YAN1035), wild-type *pGAL1-SRG1* (YAN 1040), or the *spt2Δ* strain containing the *pGAL1-SRG1* construct (YAN1039) were grown in YP galactose medium to mid-log phase and then cross-linked with 1% formaldehyde. Chromatin immunoprecipitations were performed using the 8WG16 antibody against Rpb1. The fold enrichment is the ratio of the percent immunoprecipitation (%IP) of ncDNA *SRG1* region to the %IP of the nontranscribed control region (NoORF). The values shown represent the average results with standard errors from three independent experiments. (C) Overproduction of the ncRNA *SRG1* in the *spt2Δ* strain containing the *pGAL1-SRG1* construct is not associated with repression of *SER3*. Total RNA was extracted from cells of wild-type (YAN1034), *spt2Δ* (YAN1035), wild-type *pGAL1-SRG1* (YAN 1040), and *spt2Δ pGAL1-SRG1* (YAN1039) strains grown as indicated above. The RNA was analyzed by Northern blotting with probes against *SER3* and *SRG1. SCR1* served as a loading control. (D) Quantification of the SER3 mRNA by RT-qPCR. Total RNA analyzed by Northern blotting as shown in panel C was used to produce cDNA that was quantified by qPCR. The relative level is the ratio of the indicated *SER3* mRNA to the *SCR1* transcript level. The values shown represent the average results with standard errors from three independent experiments. (E) High ncRNA *SRG1* levels in serine-rich media are not associated with *SER3* repression in the *spt2Δ* mutant. Yeast cells from the wild-type (YAN1034) or *spt2Δ* (YAN1035) strains were grown, respectively, in YPD or SD medium supplemented with serine. Total RNA was extracted and analyzed by Northern blotting with probes against *SER3* and *SRG1. SCR1* served as a loading control. Lanes 2 and 3 are duplicates that represent results of two independent experiments.

explain by itself the loss of *SER3* repression. The transcription of the *SRG1* ncDNA alone is not able to prevent expression of *SER3* when *SPT2* is mutated. Conversely, the presence of Spt2 in wild-type cells is not by itself sufficient for repression of *SER3* when the intergenic *SRG1* region is not transcribed (21). Together, these observations indicate that both intergenic transcription and Spt2 are necessary for repression of *SER3*. Importantly, it also follows that the mere passage of RNAP II and production of ncRNA are not sufficient for silencing of *SER3*. Other transcription-associated events involving Spt2 must be required to achieve full repression of the downstream gene.

Mutation of the histone chaperone *SPT6* has a similar defect in the repression of *SER3*. Our present data show that *SER3* regulation in *spt2Δ* cells is not linked to the modulation of ncRNA *SRG1* transcription. Therefore, the mechanism used by the elongation factor Spt2 to repress *SER3* remains unclear. Interestingly, we previously showed a functional link between the histone chaperone Spt6 and Spt2 (24). Moreover,

we found that Spt6 is required for the recruitment of Spt2 to the transcribed regions of some active genes (24). To further understand how Spt2 regulates the *SER3* gene, we asked if the recruitment of this factor to the *SRG1* intergenic region is under the control of Spt6. To address this question, we analyzed the association of Spt2-13Myc with SRG1 by ChIP assays in the wild-type strain, the *spt6-1004* strain, and a control untagged strain (Fig. 3A). In the wild-type strain, the Spt2-13Myc signal at the *SRG1* intergenic region was 10-fold higher than that of the untagged control, confirming a strong specific recruitment to *SRG1*. In contrast, the association of Spt2-13Myc with *SRG1* in *spt6-1004* was very weak, indicating that Spt6 plays an important role in the recruitment of Spt2 to the *SRG1* intergenic region. We next asked if Spt6 recruitment to *SRG1* is dependent on the Spt2 protein. As shown in Fig. 3B, deletion of *SPT2* is associated with partial loss of Spt6-Flag occupancy at *SRG1*. We therefore conclude that Spt2 and Spt6 are dependent on each other for their recruitment to *SRG1*.
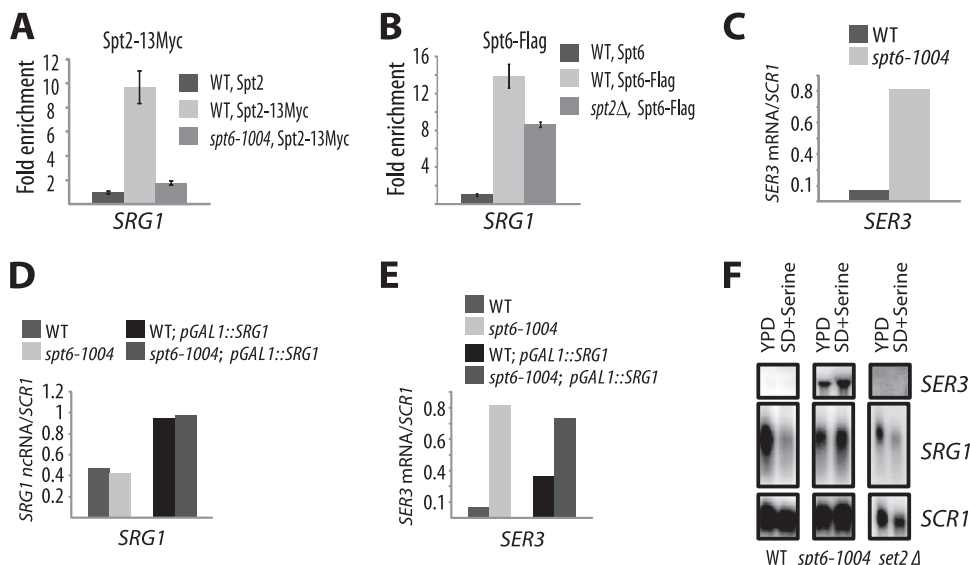
FIG. 3. Spt6 regulates *SER3* independently of *SRG1* transcription level. (A) Recruitment of Spt2 to the ncDNA *SRG1* is dependent on Spt6. ChIP assays of Spt2-13Myc were performed with chromatin extracted from wild-type (WT) (YAN1040), *spt6-1004* (YAN1041), and untagged (YAN1042) strains. The fold enrichment is the ratio of the percent immunoprecipitation (%IP) of the ncDNA *SRG1* region to the %IP of the nontranscribed control region (NoORF). The values shown represent the average results with standard errors from three independent experiments. (B) Recruitment of Spt6 to the ncDNA *SRG1* is partially dependent on Spt2. ChIP assays of Spt6-Flag were performed with chromatin extracted from wild-type (YAN1057), *spt2Δ* (YAN1058), and untagged (YAN1042) strains. The fold enrichment is the ratio of %IP of the ncDNA *SRG1* region to the %IP of the nontranscribed control region (NoORF). The values shown represent the average results with standard errors from three independent experiments. (C) *SER3* is derepressed in the *spt6-1004* mutant. Total RNA extracted from the wild-type (YAN1034) or the *spt6-1004* (YAN1043) strain was used to produce cDNA that was subsequently quantified by qPCR. The *SER3* mRNA relative level is the ratio of the *SER3* mRNA to the *SCR1* transcript level. The values shown represent the average results of two independent experiments. (D) *GAL1* promoter increases the level of the ncDNA *SRG1* transcripts in the *spt6-1004* mutant. Total RNAs extracted from the wild-type (YAN1034), *spt6-1004* (YAN1043), wild-type *pGAL1-SRG1* (YAN 1040), and *spt6-1004 pGAL1-SRG1* (YAN1044) strains were used in reverse transcription reactions to produce cDNA that was quantified by qPCR. The relative level is the ratio of *SRG1* ncRNA to the *SCR1* transcript level. The values represent the average results of two independent experiments. (E) Overproduction of the ncRNA *SRG1* in the *spt6-1004* strain does not lead to repression of *SER3*. The *SER3* mRNA was quantified using the cDNA obtained in the experiment shown in panel D. The *SER3* relative level is the ratio of *SER3* mRNA to the *SCR1* transcript level. (F) Deletion of the *SET2* gene has no effect on *SER3* repression. Yeast cells from the wild-type (YAN1034), *spt2Δ* (YAN1035), and *set2Δ* (YAN1051) strains were grown, respectively, in YPD or SD medium supplemented with serine. Total RNA was extracted and analyzed by Northern blotting with probes against *SER3* and *SRG1*. *SCR1* served as a loading control. All experiments using *spt6-1004* strains and described in this figure were performed with cells grown at 30°C.

Given the role of Spt2 in the regulation of *SER3* and the fact that its function depends on Spt6 integrity, it became tempting to speculate that similarly to Spt2, Spt6 could be involved in the regulation of *SER3* by transcription of ncDNA *SRG1*. To test this, we first analyzed the *SER3* transcript levels in wild-type and *spt6-1004* strains by RT-qPCR and found a dramatic increase of the *SER3* transcript in *spt6-1004* cells (Fig. 3C). Under these conditions, the derepression of *SER3* was also associated with a small reduction in intergenic *SRG1* transcription, as evidenced by a decreased ncRNA level and a reduced RNAP II occupancy (data not shown). Interestingly, as observed for Spt2, artificially increasing *SRG1* intergenic transcription in *spt6-1004* cells by replacing the *SRG1* promoter with that of *GAL1* resulted in a higher transcription activity without restoring the expected repression of *SER3* (Fig. 3D and E). Thus, similarly to Spt2, Spt6 plays a major role in the regulation of *SER3* by ncRNA *SRG1*. The transcription of *SRG1* alone is not able to prevent expression of *SER3* when *SPT2* or *SPT6* is mutated. In addition, the presence of Spt2 or Spt6 in wild-type cells is not by itself sufficient for repression of *SER3* when intergenic *SRG1* region is not actively transcribed (21). Collectively, these observations indicate that in addition

to RNAP II passages and production of ncRNA, other transcription-associated events involving Spt2 and the histone chaperone Spt6 must be required to achieve full repression of the downstream gene.

Histone H3 methylation is not involved in the regulation of *SER3* by transcription of ncDNA *SRG1*. Spt6 is a transcription elongation factor, mainly known to be involved in chromatin modulation associated with elongation (15). In addition to functioning together with Spt2, Spt6 controls the histone H3-lysine 36 methylation by the Set2 enzyme (4, 5, 45). Interestingly, a number of recent studies have reported a link between regulation by transcription of ncRNA and chromatin modifications, specifically histone H3 methylation (reviewed in reference 10). Indeed, regulation of *GAL1* and *GAL10* by the ucut Gal1-10 ncRNA requires methylation of histone H3-K36 and H3-K4 (13). Therefore, the phenotype observed in *spt6-1004* could be associated with the known defects in histone H3-K36 methylation in this mutant (4, 5, 45). To address this possibility directly, we analyzed by Northern blotting the level of *SER3* and *SRG1* in wild-type, *spt6-1004*, and *set2Δ* strains in media containing low or high concentrations of serine (Fig. 3F). In both conditions, the *SER3* transcript was not detected
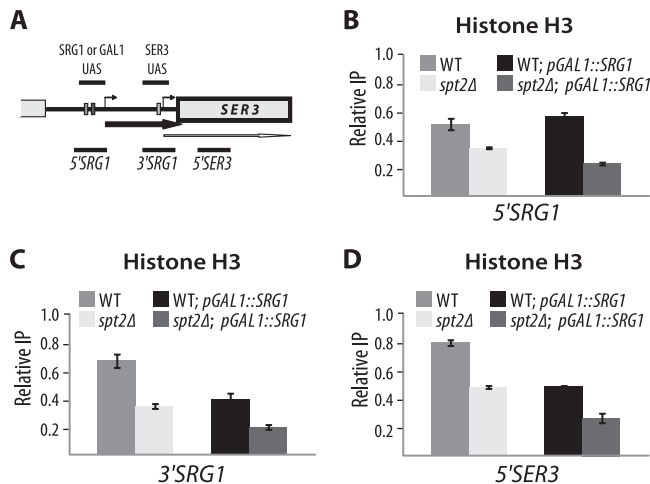
FIG. 4. Spt2 controls the histone H3 occupancy at the *SRG1-SER3* locus. (A) Diagram showing the *SRG1-SER3* regions analyzed by histone H3 ChIP. (B, C, and D) Spt2 is required to maintain normal histone H3 level at the *SRG1* 3′-end region corresponding to the *SER3* promoter. Histone H3 ChIP assays were conducted using chromatin extracted from wild-type (WT) (YAN1034) and *spt2Δ* (YAN1035) strains with or without the *pGAL1-SRG1* construct. For each strain, the value shown represents the ratio of the percent immunoprecipitation (%IP) at the indicated region to the %IP at NoORF. The values shown represent the average results with standard errors from three to six independent experiments.
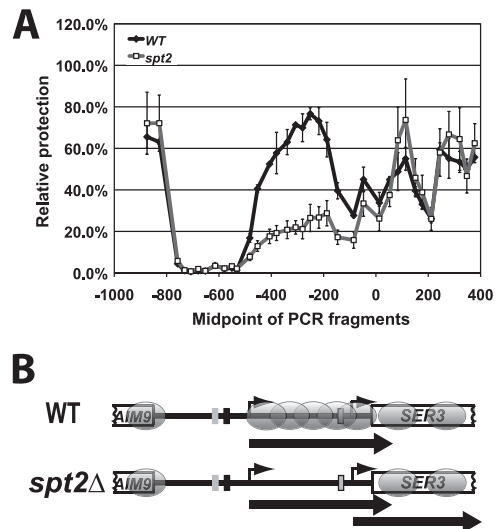

FIG. 5. Spt2 controls the nucleosomal structure at the ncDNA *SRG1*. (A) *spt2Δ* mutation is associated with loss of specific nucleosomes at the ncDNA *SRG1* region corresponding to the *SER3* promoter. A nucleosome scanning assay was performed on wild-type (WT) (OY8 or KY766) and *spt2Δ* (YAN16 or OY8*spt2*) cells that were grown in YPD (*SER3* repressed) at 30°C. Using qPCR, the relative MNase protection of each *SER3* template was calculated as a ratio to that of a *GAL1* promoter template (*GAL1* NB) found within a well-positioned nucleosome in the *GAL1-10* promoter. Each point on the graph shows the mean ± standard error of the mean result from three independent experiments that are plotted at the midpoint of each PCR product. (B) Diagram of the *SER3* locus showing nucleosome positions (ovals) extrapolated from the nucleosome scanning experiments.

in wild-type strains, whereas appreciable amounts were visible in *spt6-1004* cells. Importantly, no *SER3* transcripts were observed in cells bearing the deletion of the *SET2* gene. Moreover, we observed a similar regulation of *SER3* and *SRG1* in wild-type and *set2Δ* strains, while the *SPT6* mutation resulted in constitutive transcription of *SER3* (see Fig. S6 at http://www.crc.ulaval.ca/nourani/supplemental_data). Thus, the Spt6 function in regulating *SER3* cannot be attributed to its role in methylation of H3-K36. In addition, we also analyzed the role of histone H3-lysine 4 methylation in *SER3* regulation by measuring the *SER3* mRNA levels by RT-qPCR in wild-type cells and *spt2Δ* and *set1Δ* mutants (see Fig. S7 at the URL above). This experiment showed that loss of H3-K4 methylation had no significant impact on *SER3* repression. Our observations clearly indicate that in contrast to regulation of *GAL1* and *GAL10* by ncRNA, histone H3-K4 and H3-K36 methylation by Set1 and Set2, respectively, are not required for normal *SER3* repression by ncRNA *SRG1*.

Spt2 is required for maintaining proper nucleosomal structure at the *SRG1* intergenic region. Mutations in *SPT2* or *SPT6* result in spurious transcription from cryptic promoters located within coding regions (15, 24). Presumably, in the absence of normal Spt2 activity, the chromatin structure modified by the passage of transcription machinery is not properly restored, leading to the derepression of cryptic promoters. Importantly, Spt2 was shown to control the level of histone H3 at transcribed regions of *GAL1* and *PMA1* genes (24). This led us to imagine that the role of Spt2 in regulation of *SER3* could have a link with its function in modulating chromatin structure at transcribed regions. To address this question, we first determined whether the deletion of *SPT2* gene affects the histone H3 levels at the *SRG1-SER3* locus (Fig. 4A). To this end, we

conducted histone H3 ChIP assays at three different locations around *SRG1-SER3* in wild-type and *spt2Δ* strains. As shown in Fig. 4B, C, and D, H3 occupancy is significantly reduced at this locus in Spt2-deficient cells. This result indicates that chromatin structure within the *SRG1* transcribed region corresponding to the *SER3* promoter could be significantly affected by the loss of Spt2. Interestingly, increasing transcription activity at *SRG1* by inserting the strong *GAL1* promoter does not suppress the loss of histone H3 associated with mutation of *SPT2* at the *SRG1* transcribed region (see Fig. 4B, C, and D). Therefore, the significant histone H3 occupancy change observed in *spt2Δ* cells is independent of the transcription rate. This important observation indicates clearly that *spt2Δ* mutation effect on *SRG1-SER3* chromatin structure is not mediated through the modulation of the *SRG1* transcription activity. It should be noted that inserting the *GAL1* promoter at *SRG1* in wild-type cells resulted in the reduction of histone H3 level in the 3′*SRG1* and 5′*SER3* regions (see Fig. 4C and D). This effect on nucleosome occupancy at *SRG1* could explain the *SER3* derepression observed in wild-type cells and reported in Fig. 2C.

To further study the role of Spt2 in maintaining the nucleosomal structure of *SRG1-SER3*, we determined precise nucleosome positions at this locus by a nucleosome scanning assay similar to those previously described (3, 17, 42). Using this approach first in wild-type cells, we identified two sharp peaks of MNase protection within the 5′ end of the *SER3* open reading frame, indicating the presence of two well-positioned nucleosomes (Fig. 5A, black line, and Fig. 5B). In addition, we
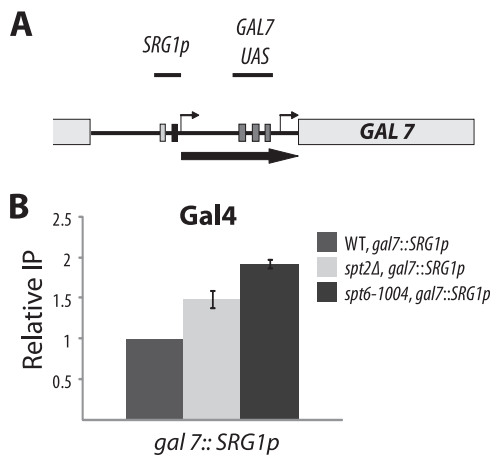
A

B

FIG. 6. Mutations of *SPT2* or *SPT6* enhance the accessibility of p*SRG1-GAL7* UAS to the Gal4 activator. (A) Diagram showing the p*SRG1-GAL7* construct used to analyze Gal4 binding in the presence of *SRG1* transcription. (B) Gal4 binding to the p*SRG1-GAL7* UAS is increased in the *spt2Δ* and *spt6-1004* mutants. ChIP assays using anti-Gal4 antibody were conducted on chromatin extracted from wild-type (WT) (FY2257), *spt2Δ* (YAN1045), and *spt6-1004* (YAN1046) strains. For each strain, the fold enrichment shown is relative to the fold enrichment calculated for the wild-type strain and arbitrarily set at 1.0.

observed a broad region of MNase protection that overlaps the *SRG1* transcribed region, which is consistent with the presence of nucleosomes that are randomly positioned. Importantly, this broad region of MNase protection is greatly reduced in *spt2Δ* cells (Fig. 5A, gray line), indicating a loss of nucleosomes over the *SRG1* transcribed region. Taken together with our histone H3 ChIP results, our data suggest that there is a significant disruption of nucleosomal structure at *SRG1* in *spt2Δ* cells. Moreover, our data also show that this effect is independent of the level of *SRG1* transcription.

Mutations in *SPT2* and *SPT6* compromise *SRG1*-mediated inhibition of activator binding. Spt2 is required for normal positioning of nucleosomes in the *SRG1* transcribed region that corresponds to the *SER3* promoter (here called SRG1 3′). In the absence of these nucleosomes, it is possible that transcription activators governing the induction of *SER3* can freely bind their target sites. Because the *SER3* regulators are not known, we decided to use a strain (see Fig. 6A) in which the *SRG1* promoter was inserted in front of the *GAL7* promoter region (21). It was previously shown using this construction that *SRG1* transcription reduces Gal4 binding to the *GAL7* upstream activation sequence (UAS) (21). We asked if *SPT2* or *SPT6* mutations impaired the inhibition of Gal4 binding by *SRG1* transcription. Therefore, we performed Gal4 ChIP assays in wild-type, *spt6-1004*, and *spt2Δ* strains containing the *SRG1* promoter in front of the *GAL7* UAS region. As shown in Fig. 6B, Gal4 binding is significantly increased in the *spt2Δ* and *spt6-1004* mutants. Thus, *SPT2* or *SPT6* mutations compromise the Gal4 inhibition achieved by *SRG1* transcription at the *GAL7* UAS and enhance significantly the accessibility of this region to activators. Importantly, in both *spt2Δ* and *spt6-1004* mutants, Gal4 binding does not increase at the normal *GAL7* promoter (see Fig. S8 at http://www.crc.ulaval.ca/nourani/supplemental_data). We next asked whether

these mutations alter the chromatin structure at the *SRG1::GAL7* promoter. As shown in Fig. S9 at the URL above, both mutations are associated with a significant loss of histone H3 occupancy at *SRG1::GAL7*. Collectively, these observations suggest that a loss of nucleosomes at the *SRG1* region observed in Spt2-deficient cells compromise the *SER3* promoter occlusion to activators.

Nucleosome deposition in the wake of RNAP II passage at the *SRG1* intergenic region is significantly affected in *spt2Δ* cells. We established that Spt2 has an important impact on the chromatin structure of the *SRG1* intergenic region. However, how this elongation factor participates in the maintenance of this chromatin structure remained an open question. Interestingly, this genomic region is robustly transcribed and thus RNAP II activity could lead to displacement of key nucleosomes in this region that must be subsequently reassembled. One could imagine that Spt2 plays a role in nucleosome redeposition in the wake of transcription machinery and that the absence of this factor could lead to a deficient restoration of nucleosomes. To directly test this hypothesis we first used an experimental system described in a number of studies (7, 36). Briefly, through histone H3 ChIP assays, we measured the kinetics of histone H3 redeposition in the transcribed region of the large (8-kb) model gene *FMP27* that is driven by the *GAL1* promoter (36). The rapid repression of *GAL1-FMP27* obtained by adding glucose to the growth medium allowed us to study the rate with which histone H3 is deposited following the last wave of transcription (Fig. 7A). In wild-type cells, upon the addition of glucose, histone H3 levels increased rapidly and reached their maximum at the middle region of *GAL1-FMP27* within 4 min (Fig. 7B). This is consistent with previous studies (36). Deletion of *SPT2* was associated with a significant defect in the H3 redeposition at the *GAL1-FMP27* region after glucose repression. As shown in Fig. 7B, although glucose repression was associated with a small increase of histone H3 occupancy in *spt2Δ* cells, the recovery of nucleosomes in this strain is slower and did not reach the wild-type level even after 20 min of repression. We therefore conclude that the loss of Spt2 has a substantial effect on the nucleosome deposition associated with transcription elongation at *GAL1-FMP27*.

Altogether, our findings suggest the possibility that Spt2 is required for nucleosome redeposition in the wake of transcription at the *SRG1* ncDNA. To address this possibility, we used the same approach described in the legend to Fig. 7A by replacing the *SRG1* promoter with that of *GAL1* and following the histone H3 deposition after the last wave of transcription upon glucose addition (Fig. 7C). We analyzed these kinetics at the *SRG1* transcribed region corresponding to the *SER3* promoter, and the results are reported in Fig. 7D. In wild-type cells, we observed a very rapid increase in histone H3 occupancy that reached its maximum level after 4 to 6 min of repression. Surprisingly, in contrast to the results for *GAL1-FMP27*, the level of histone H3 started to drop and returned to a low level quickly, indicating that nucleosomes that reassembled following RNAP II passage at the ncDNA *SRG1* are very unstable compared to those at *FMP27*. Interestingly, the histone H3 deposition associated with the last wave of transcription was severely impaired in *spt2Δ* cells, indicating an important role of Spt2 in transcription-dependent nucleosome deposition at the region corresponding to the *SER3* promoter.
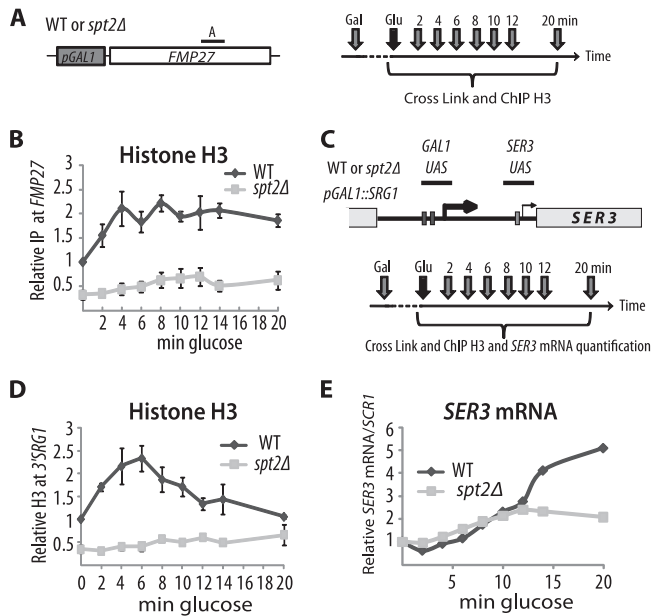
FIG. 7. Spt2 is required for nucleosome reassembly in the wake of RNAP II at the *SRG1* intergenic region. (A) Diagram explaining the experimental procedure designed to analyze histone H3 recovery upon repression of *GAL1-FMP27*. WT, wild type. (B) *spt2Δ* mutation affects significantly histone H3 redeposition associated with the last wave of transcription at the *GAL1-FMP27* transcribed region. Yeast cells from wild-type (YAN1047) or *spt2Δ* (YAN1048) strains were grown in galactose medium to mid-log phase. Glucose was then added to the medium, and the cells were cross-linked and harvested at the indicated time points. Histone H3 level was analyzed by ChIP assays using chromatin extracted from wild-type or *spt2Δ* strains. For each strain, the value of the ratio IP/input calculated for the time zero min was arbitrarily set at 1. (C) Diagram explaining the experimental procedure designed to analyze histone H3 recovery at the *SRG1* 3′ end upon repression of *pGAL1-SRG1*. (D) *spt2Δ* mutation affects significantly histone H3 redeposition associated with the last wave of transcription at the *SRG1* region corresponding to the *SER3* promoter. The experiment was conducted as described for panel B with chromatin extracted from wild-type (YAN1040) or *spt2Δ* cells (YAN1039). The ratio IP/input calculated for time zero in the wild type was arbitrarily set at 1. (E) Spt2 delays maximal *SER3* induction. The *SER3* mRNA and *SCR1* levels were analyzed by RT-qPCR using total RNA extracted from cells treated as described for panel B. For each strain, the value of the ratio *SER3* mRNA/*SCR1* calculated for time zero was arbitrarily set at 1. The values represent the average of two to three independent experiments. Note that at time zero, as expected, the absolute levels in the *spt2Δ* strain are higher than those observed in wild-type cells.

Importantly, the histone H3 steady-state level in the *pGAL1-SRG1 spt2Δ* strain grown in glucose almost reaches the wild-type level (see Fig. S10 at http://www.crc.ulaval.ca/nourani /supplemental_data).

In addition to monitoring histone H3, we analyzed *SER3* transcript levels upon glucose repression of *GAL1-SRG1* (Fig. 7E). In this experiment, we focused on the degree of induction in each strain and not on the absolute levels, which are higher in *spt2Δ* cells. Upon glucose addition, we observed a rapid induction of *SER3* transcripts in wild-type and *spt2Δ* cells. This induction quickly reached a plateau in the *spt2Δ* mutant, while it continued to increase in wild-type cells. The graph in Fig. 7E shows that maximum induction of *SER3* was reached in cells

lacking Spt2 after only 10 min. In contrast to this, *SER3* induction continued for at least 10 more minutes in wild-type cells and did not reach a plateau even after 20 min. This delay could be due to the deposition of nucleosomes in the wild-type cells during the first 4 to 6 minutes after *GAL1* promoter shutdown. Later, these nucleosomes are displaced, allowing a further induction of *SER3*. Therefore, our observation suggests that nucleosome deposition associated with the last wave of transcription delays *SER3* induction in wild-type cells. Since the *spt2Δ* mutant is deficient in nucleosome deposition, we did not observe any delay in maximal induction of *SER3* and a plateau was reached after 10 min of glucose repression. Altogether, our data strongly suggest that Spt2 is required for the assembly of nucleosomes associated with transcription of ncDNA *SRG1* and that these nucleosomes play a crucial role in the normal repression of *SER3*.

Our data strongly suggest that Spt2, together with *SRG1* transcription, maintains higher nucleosome occupancy over the *SER3* promoter and therefore represses its transcription. To confirm this, we analyzed histone H3 occupancy in the *srg1-1* mutant. In this strain (Fig. 8A), mutation of the *SRG1* TATA box abolishes its transcription (21). As shown in Fig. 8B, in the absence of *SRG1* transcription, the levels of histone H3 at the *SRG1* region corresponding to the *SER3* promoter dropped significantly, indicating that chromatin structure in this region is dependent on transcription. Interestingly, deletion of the *SPT2* gene did not result in a further decrease in histone H3 occupancy (Fig. 8B). This observation shows that the role of Spt2 in maintaining chromatin structure at the *SER3* promoter is dependent on *SRG1* transcription. Moreover, we found that deletion of *SPT2* did not affect significantly the *SER3* transcript level in the *srg1-1* mutant (Fig. 8C), suggesting therefore that the role of Spt2 in the regulation of *SER3* is associated with *SRG1* transcription.

Spt2 contributes to redeposition of nucleosomes displaced by *SRG1* ncRNA transcription. As shown earlier, there are nucleosomes randomly positioned at the *SRG1* ncDNA region corresponding to the *SER3* promoter. These nucleosomes are displaced and reassembled by continuous passages of RNAP II. We first wanted to know whether this chromatin modulation involves a mechanism that recycles the histones of these nucleosomes or uses new histones. As our data show that Spt2 participates in the deposition of these nucleosomes, we asked specifically whether Spt2 favors the recycling or the use of new histones. To answer these questions, we used an experimental system previously described by us and others (6, 33, 34). In this system, there are two different sources of histone H3 in the cell: the endogenous untagged histone H3 and a galactose-inducible form fused to the Flag tag that is coexpressed with histone H4 (Fig. 9A). In order to eliminate the contribution of DNA replication-dependent histone deposition, exponentially growing cells containing the described construction (Fig. 9B) are blocked in G_1 with α-factor. After incubation with α-factor, cells are either left untreated or induced to express Flag-H3 prior to formaldehyde treatment to cross-link chromatin. Next, the levels of Flag-H3 are assayed by standard ChIP-qPCR at the *SRG1* ncDNA region corresponding to the *SER3* promoter and at a control nontranscribed intergenic region of chromosome V (NoORF) (Fig. 9C and D). As shown in Fig. 9C and D, after induction of the new histone H3 in wild-type cells, we
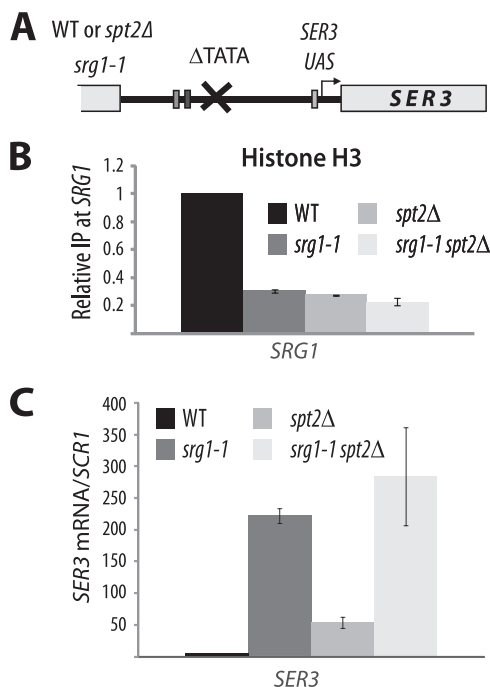
FIG. 8. Chromatin structure at SRG1 depends on active transcription. (A) Diagram showing the *srg1-1* construct. WT, wild type. (B) Mutation of *SRG1* TATA is associated with a significant loss of histone H3 at the *SRG1* 3′-end region corresponding to the *SER3* promoter. Histone H3 ChIP assays were conducted using chromatin extracted from wild-type (YAN1053), *srg1-1* (YAN1054), *spt2Δ* (YAN1055), and *srg1-1 spt2Δ* (YAN1056) strains. For each strain, the value shown represents the ratio IP/input relative to the same ratio calculated for the corresponding wild-type strain and arbitrarily set at 1.0. The values shown are the averages and standard errors of results from three independent experiments. (C) The *spt2Δ* mutation in the *srg1-1* strain does not increase significantly *SER3* derepression. Total RNA extracted from wild-type (YAN1053), *srg1-1* (YAN1054), *spt2Δ* (YAN1055), and *srg1-1 spt2Δ* (YAN1056) strains was used to produce cDNA that was subsequently quantified by qPCR. The *SER3* mRNA relative level is the ratio of the *SER3* mRNA to the *SCR1* transcript level. The values shown are the averages and standard errors of results from three independent experiments.

detected higher levels of its incorporation at *SRG1* than in the control region (NoORF). Thus, *SRG1* ncDNA is associated with a significant replication-independent histone H3 turnover, indicating the existence of a transcription-dependent chromatin assembly mechanism that deposits new nucleosomes. Surprisingly, deletion of *SPT2* resulted in higher deposition of new histone H3 at ncDNA *SRG1*. Moreover, the low level of total H3 in Spt2-deficient cells suggests that, in the *spt2Δ* mutant, the vast majority of nucleosomes at *SRG1* are refolded using newly synthesized histones. This indicates that Spt2 inhibits nucleosome assembly that uses newly synthesized histones. Because the overall nucleosome deposition is reduced in *spt2Δ* cells (Fig. 7), our data suggest that, similarly to yFACT at the transcribed regions of some genes (14), Spt2 favors a dominant nucleosome assembly pathway that uses old histones displaced by elongating RNAP II. In Spt2-deficient cells, the induction of another chromatin assembly pathway that uses new histones is not able to overcome this loss. Thus, Spt2 participates in the major mechanism that refolds chromatin structure in the wake
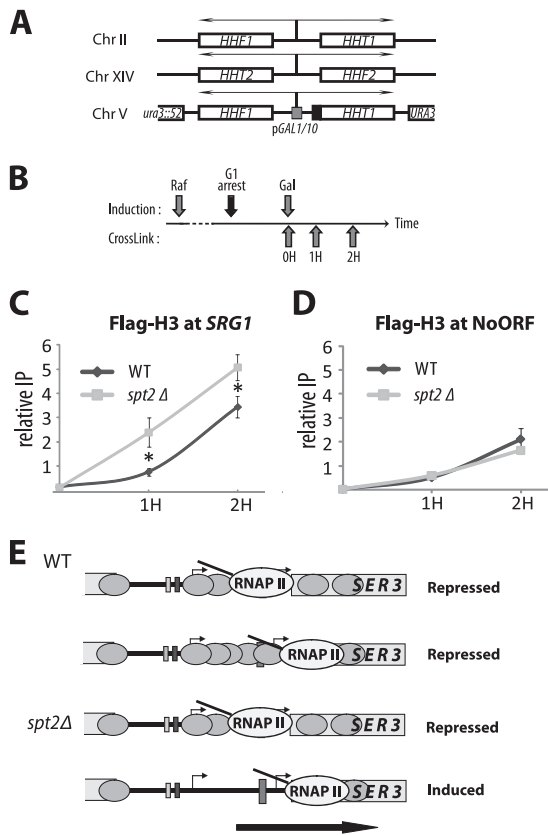

FIG. 9. Spt2 is involved in a nucleosome reassembly pathway that uses old histones at *SRG1*. (A) Diagram representing the different sources of histone H3 and H4 in the yeast strains used in the experiment. (B) Diagram representing the experimental procedure. (C and D) Spt2 inhibits the incorporation of new histone H3 at the *SRG1* region corresponding to the *SER3* promoter. Yeast cells from the wild-type (WT) (YAN1049) or *spt2Δ* strains (YAN1050) containing the construction encoding histone Flag-H3 under the control of the *GAL1* promoter were grown in raffinose-containing medium to mid-log phase. After G$_1$ arrest by α-factor, the cells were formaldehyde fixed or shifted to galactose medium for 60 or 120 min prior to formaldehyde treatment. ChIP assays were then performed using anti-Flag antibody. The values shown (relative IP/input) represent the averages and standard errors of results from three independent experiments. ∗, $P < 0.05$. (E) Model of *SER3* regulation by ncRNA *SRG1* transcription.

of RNAP II at *SRG1*. This mechanism recycles histones of nucleosomes previously displaced by the transcription machinery and shapes specific chromatin structure that is crucial for *SER3* repression.

DISCUSSION

The modulation of gene expression by ncRNA is an emerging field that has focused mostly on transregulatory mechanisms, including repression by RNAi pathways. However, a growing amount of evidence points toward the existence of various *cis*-directed mechanisms where the process of ncRNA production itself, and not the product, contributes to the regulation of a gene. Interestingly, several observations introduced the notion that the chromatin environment created by the transcription of ncRNA may have a regulatory role (10).

These studies involved different mechanisms where ncRNA transcription displaces nucleosomes or modifies histones to regulate a nearby gene (12, 13, 30). Here we show that *SRG1* ncRNA transcription deposits nucleosomes that participate in the repression of the adjacent *SER3* gene. Our study also brings new insights into the molecular mechanism of transcription interference. Different models of transcription interference have been proposed, including promoter competition, occlusion by elongating RNAP II, or collision between transcription machineries (reviewed in reference 37). We provide evidence that transcription interference in the *SRG1-SER3* system involves deposition of specific nucleosomes in the wake of an elongating RNAP II. Importantly, and in contrast to previous observations, our work shows that histone H3-K4 methylation and H3-K36 methylation are not required for the normal *SER3* regulation by ncRNA transcription. This suggests that the most important factor in this repression is the deposition of nucleosomes and not their posttranslational modifications that could alter their dynamic properties. Finally, we show that the elongation factor Spt2 and the histone chaperone Spt6 play central roles in this regulatory system. Our data indicate that Spt2 participates in the shaping of the transcription-dependent chromatin structure over the *SRG1* transcribed region that controls the activity of *SER3* promoter.

Before this work was completed, the working model of *SER3* regulation had been based on the canonical mechanism of transcription interference, whereby successive passages of RNAP II transcribing the *SRG1* intergenic region obstruct the *SER3* promoter, inhibiting the interaction of activators and initiation factors with their respective binding sites. In the *spt2Δ* or *spt6-1004* mutant strains grown in standard rich medium, we observed a small but consistent reduction of transcription activity at the *SRG1* intergenic region that produces the ncRNA. It was easy to imagine that if regulation by transcription interference was achieved merely by maintaining a certain transcription rate involving the passage of a specific number of RNAP II complexes, a small reduction in the number of these complexes traversing the *SER3* promoter could compromise the interference. However, direct and indirect experimental evidence quickly challenged this simple view. First, our work showed that under conditions where *SRG1* transcription in the *spt2Δ* mutant is higher than that in wild-type cells, the *SER3* gene remained active (Fig. 2E; also see Fig. S5 at http://www.crc.ulaval.ca/nourani/supplemental_data). Second, increasing artificially the transcription rate of the *SRG1* ncRNA in the *spt2Δ* mutant did not restore normal repression (Fig. 2C and D). Third, in *spt6-1004* cells, we observed similar defects in *SER3* repression. This phenotype cannot be explained by variations in *SRG1* transcription (Fig. 3). Fourth, changing the transcription in the wild type from a high level (*SRG1* promoter) to a very high level (*GAL1* promoter) did not further repress *SER3* transcription. In fact, we observed the opposite result, since insertion of the *GAL1* promoter was associated with *SER3* derepression (Fig. 2C, 2D, and 3E; also see Fig. S3 at the URL above). These data suggest a more complicated mechanism of transcriptional interference in which the passages of RNA polymerase II alone are not sufficient to repress *SER3* transcription. Thus, the presence or absence of normal Spt2 and Spt6 function could slightly affect the production of

the ncRNA under some conditions, but this marginal role could not explain the loss of *SER3* repression. Finally, taken together, these observations indicate that *SER3* repression is not only mediated by the production of the *SRG1* ncRNA or the frequency of transcription machinery passages over the *SER3* promoter. Instead, it is the entire process of transcription and its associated events such as chromatin modulation that are involved in the interference with the *SER3* promoter. Our observations support an important role of Spt2 in the chromatin modulations associated with *SRG1* transcription that contributes directly to the repression of *SER3*.

Perhaps the most significant observation in this work is the one related to the chromatin structure in the *SRG1* intergenic region (Fig. 4 and 5). Nucleosome scanning assays conducted in wild-type cells indicate that this region contains a large peak representing the presence of nucleosomes randomly positioned within this short DNA segment (Fig. 5). Importantly, our data indicate clearly that nucleosomes are assembled in the wake of transcription at this region (Fig. 7). However, shortly after their deposition, these nucleosomes appear to be displaced. Therefore, our findings suggest that continuous transcription of intergenic *SRG1* is required for the maintenance of nucleosomes at this region. Interestingly, mutation of the *SRG1* TATA box abolishes its transcription and results in a clear depletion of histone H3 at this region (Fig. 8). This is consistent with a recent study showing that nucleosomes over the *SER3* UAS (upstream activation sequence) are present when *SRG1* is transcribed, while inhibition of the intergenic transcription in the absence of serine is associated with a loss of nucleosomes at this position (9a).

The precise mechanism by which Spt2 contributes to the shaping of the nucleosomal structure at the *SRG1* ncDNA remains an interesting question. Several hypotheses could be proposed. It is possible that Spt2 acts after chromatin synthesis and stabilizes the nucleosomes deposited in the wake of RNAP II. Alternatively, Spt2 could be directly involved in transcription-dependent chromatin assembly associated with *SRG1* transcription. The latter possibility is likely to be the case for several reasons. First, Spt2 has two HMG-like domains and could, similarly to other HMG-box proteins, assist histone chaperones in nucleosomal assembly (9, 26). Second, it has functional genetic and physical interactions with the histone chaperone Spt6 which are consistent with such a role (reference 24 and unpublished data). Third, it binds four-way junction DNA, a structure similar to that found at the entrance/exit point of DNA from a nucleosome (25, 46). Consistently, we found that it interacts with mononucleosomes *in vitro* (unpublished data). Future studies focused on Spt2 should help to determine its precise role in chromatin remodeling in the wake of RNAP II.

Our analyses of the replication-independent histone H3 incorporation indicate the existence of at least two different mechanisms involved in the control of transcription-dependent nucleosome deposition at the ncDNA *SRG1*. First, we showed that *SRG1* ncDNA incorporates a high level of new histones in G$_1$-arrested cells, indicating that transcription at this region is coupled to a replication-independent chromatin assembly pathway that uses new histones. Second, our analyses of histone H3 deposition outside S-phase show that the absence of Spt2 is associated with both a preferential deposition of newly

synthesized H3 and an overall loss of histone H3 (Fig. 4 and 9) at the *SRG1* ncDNA. This indicates that the loss of Spt2 affects an additional mechanism(s) that recycles old histone H3 to reassemble nucleosomes displaced by RNAP II at *SRG1*. This observation is similar to the data recently reported on the yFACT histone chaperone (14). Indeed, the Strubin group clearly showed that newly synthesized histone H3 deposition is induced on a number of genes in the *spt16-197* mutant, while nucleosomal occupancy decreases overall (14). Without excluding other possibilities, one simple explanation of such observations is that two different chromatin assembly pathways coexist at *SRG1*. One uses old histones and recycles them to redeposit nucleosomes displaced by RNAP II and requires Spt2. The other pathway uses newly synthesized histones and is not dependent on Spt2. At *SRG1*, one possibility is that the recycling pathway is dominant, and the induction of another pathway using newly synthesized histone H3 cannot overcome the loss of Spt2. Whether this effect is more general and could be applied to other genomic loci would be interesting to assess. It is likely that this mechanism also functions outside the *SRG1-SER3* locus. Indeed, analyses at other transcribed regions showed that *spt2Δ* mutation is also associated with the induction of newly synthesized H3 deposition (P. Thebault, unpublished data). Finally, given the fact that Spt2 and Spt16 defects have similar phenotypes on histone H3 dynamics at different transcribed regions, it will be interesting to test whether yFACT depletion has any role on *SRG1* histone H3 turnover or chromatin structure and therefore on *SER3* regulation.

Finally, our data suggest a new model of regulation by ncRNA transcription that involves nucleosomes deposited in the wake of the transcription machinery (Fig. 9E). We propose that ncRNA *SRG1* transcription achieves *SER3* repression mainly by creating a repressive chromatin structure. Therefore, in contrast to what has been imagined, transcription interference within the *SRG1-SER3* system is not only achieved by RNAP II passing through the *SER3* promoter but is also mediated by the nucleosomes inserted behind RNAP II complexes as they leave this regulatory region. These nucleosomes are important for the repression of *SER3* transcription that could possibly be initiated in the time frame between two successive rounds of ncRNA *SRG1* transcription. In the *spt2Δ* mutant, these nucleosomes are not assembled correctly, creating an open chromatin context for the *SER3* activator(s). Importantly, and in contrast to previous observations, our work shows that histone H3-K4 and H3-K36 methylation is not required for the normal *SER3* regulation by ncRNA transcription, suggesting that the most important factor is the deposition of nucleosomes and not modifications that play a role in the dynamic properties of these nucleosomes. Overall, we provide strong evidence that Spt2 participates in a regulation by transcription interference via a novel mechanism involving a complex interplay between RNAP II and chromatin dynamics associated with its passage.

ACKNOWLEDGMENTS

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