Intergenic transcription causes repression by directing nucleosome assembly

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Transcription of non-protein-coding DNA (ncDNA) and its noncoding RNA (ncRNA) products are beginning to emerge as key regulators of gene expression. We previously identified a regulatory system in *Saccharomyces cerevisiae* whereby transcription of intergenic ncDNA (*SRG1*) represses transcription of an adjacent protein-coding gene (*SER3*) through transcription interference. We now provide evidence that *SRG1* transcription causes repression of *SER3* by directing a high level of nucleosomes over *SRG1*, which overlaps the *SER3* promoter. Repression by *SRG1* transcription is dependent on the Spt6 and Spt16 transcription elongation factors. Significantly, *spt6* and *spt16* mutations reduce nucleosome levels over the *SER3* promoter without reducing intergenic *SRG1* transcription, strongly suggesting that nucleosome levels, not transcription levels, cause *SER3* repression. Finally, we show that *spt6* and *spt16* mutations allow transcription factor access to the *SER3* promoter. Our results raise the possibility that transcription of ncDNA may contribute to nucleosome positioning on a genome-wide scale where, in some cases, it negatively impacts protein–DNA interactions.

[*Keywords*: ncDNA; intergenic transcription; chromatin; repression]

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Over the past decade, genome-wide expression studies in eukaryotes have revealed that transcription is not limited to protein-coding DNA, but rather occurs throughout entire genomes, often involving both DNA strands (Kapranov et al. 2007; Pheasant and Mattick 2007; Berretta and Morillon 2009; Jacquier 2009). Although the extent of transcription of non-protein-coding DNA (ncDNA) has been questioned recently (van Bakel et al. 2010), it is clear that eukaryotes produce many RNA molecules that do not encode proteins (noncoding RNAs [ncRNAs]) (Goodrich and Kugel 2009; Harrison et al. 2009; Mercer et al. 2009; Costa 2010). ncRNAs have diverse properties, ranging in size from short (microRNAs [miRNAs]) to long (long RNAs [lnRNAs]) and ranging in stability from stable to unstable. With the exception of several families of well-studied ncRNAs—including rRNAs, tRNAs, snRNAs, snoRNAs, and miRNAs—the biological functions of these ncRNAs are only beginning to be understood.

Although it is likely that some ncRNAs may represent transcriptional noise (Struhl 2007; Seila et al. 2009), it has become increasingly clear that transcription of noncoding regions of eukaryotic genomes plays important biological functions, primarily in regulating gene expression (Goodrich and Kugel 2009; Harrison et al. 2009; Mercer et al. 2009). Examples of this include the Xist/Tsix RNAs involved in mammalian X inactivation (Lee 2009), the roX1 and roX2 RNAs involved in dosage compensation in *Drosophila* (Gelbart and Kuroda 2009), the human HOTAIR involved in the regulation of developmental genes (Rinn et al. 2007), the mouse Air and Kcnq1ot1 RNAs involved in establishing genomic imprinting (Royo and Cavaille 2008), and the mouse VL30 RNA and human PSF-binding ncRNAs that regulate cell proliferation and tumorigenesis (Li et al. 2009; Wang et al. 2009).

Significant advances have been made in understanding widely diverse mechanisms by which transcription of ncDNAs regulate gene expression. In some cases, it is the ncRNA product that regulates gene expression. ncRNAs have been shown to recruit complexes that modify chromatin, interact with activator and coactivator proteins and modulate their function, and interact with RNA polymerase II (Pol II) and other basal transcription factors to control their activity (Goodrich and Kugel 2009; Harrison et al. 2009; Mercer et al. 2009). Alternatively, the act of transcribing ncDNA has also been shown to both positively and negatively regulate gene expression. In most of these cases, a transcription interference mechanism has been proposed. Examples include mouse and human globin genes (Ashe et al. 1997; Gribnau et al. 2000); the *Drosophila Hox* genes (Schmitt et al. 2005; Mazo et al. 2007); and *Saccharomyces cerevisiae SER3* (Martens et al. 2004), *ADH1/ADH3* (Bird et al. 2006), *IME4* (Hongay et al. 2006), and *FLO11* (Bumgarner et al. 2009) genes.

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Although several mechanisms of transcription interference have been described, most involving RNA Pol II directly, experiments that distinguish between these mechanisms at specific genes have not been performed.

Interestingly, several studies in yeast have implied that transcription of ncDNA may contribute to gene regulation by altering chromatin structure. Transcription of a series of ncRNAs 5′ of the *Schizosaccharomyces pombe* *fbp1*$^+$ gene was found to facilitate an open chromatin conformation, allowing transcription factors access to the *fbp1*$^+$ promoter during glucose induction (Hirota et al. 2008). Antisense transcription has been shown to silence the expression of *PHO84* by a mechanism that requires Hda1/2/3-dependent deacetylation of histones located at the *PHO84* promoter (Camblong et al. 2007, 2009). Finally, two recent studies provide evidence that transcription of DNA antisense to the *GAL10* gene alters post-translational modifications of histones that facilitate repression of the divergently transcribed *GAL10* and *GAL1* genes (Houseley et al. 2008; Pinskaya et al. 2009).

Previously, we showed that serine-dependent transcription of ncDNA (*SRG1*) in *S. cerevisiae* represses expression of the adjacent *SER3* gene (Martens et al. 2004, 2005). In the presence of serine, transcription of *SRG1* extends across the promoter of the adjacent *SER3* gene, yielding two short transcripts that terminate 75 base pairs (bp) 5′ and 25 bp 3′ of the *SER3* translational start (Thompson and Parker 2007), and a minor *SRG1–SER3* readthrough transcript that extends to the end of *SER3* (Martens et al. 2004; Thompson and Parker 2007). We provided evidence that it is the act of transcribing *SRG1* across the *SER3* promoter, rather than the *SRG1* RNA products, that represses *SER3* (Martens et al. 2004). In this study, we elucidate the mechanism whereby serine-dependent transcription of ncDNA (*SRG1*) in *S. cerevisiae* represses expression of the adjacent *SER3* gene. We show that *SER3* repression correlates with a broad region of strong micrococcal nuclease (MNase) protection spanning the entire *SRG1* transcription unit, suggesting that nucleosomes are loosely positioned across this region. Surprisingly, conditions that reduce *SRG1* transcription result in dramatically reduced MNase protection at the *SER3* promoter, indicating a loss of nucleosome occupancy. By analyzing mutations in *SPT6* and *SPT16*, two genes that encode subunits of the Spt6/Spn1(Iws1) and FACT elongation complexes, we provide evidence that it is the nucleosomes assembled at the *SER3* promoter by intergenic *SRG1* transcription, not RNA Pol II itself, that interfere with the binding of transcription factors to the *SER3* promoter. Our data are consistent with a general model in which transcription of ncDNA can assemble nucleosomes that occlude DNA from binding by sequence-specific DNA-binding proteins.

Results

Evidence that nucleosomes occupy the SER3 *promoter in repressing conditions*

Previously, we showed that transcription of intergenic *SRG1* DNA is required for *SER3* repression (Martens et al. 2004). Several pieces of data suggest that chromatin structure also plays an important role in *SER3* repression. First, we identified histones and two activators of histone gene expression, Spt10 and Spt21 (Dollard et al. 1994; Hess et al. 2004; Eriksson et al. 2005), in a genetic screen for repressors of *SER3* expression (J Pruneski, unpubl.). Second, DNA microarray experiments revealed that depletion of histone H4 resulted in strong *SER3* derepression (Wyrick et al. 1999). Third, a mutation in *SPT6*, a gene that encodes a protein required to maintain proper chromatin structure over genes during transcription (Kaplan et al. 2003; Cheung et al. 2008), also results in *SER3* derepression (Kaplan et al. 2003).

To investigate a possible role for chromatin structure in *SER3* repression, we first determined the positions of nucleosomes across the *SER3* locus in wild-type cells grown in *SER3*-repressing conditions (YPD) by a nucleosome scanning assay (Sekinger et al. 2005; Lee et al. 2007). Briefly, cells are treated with formaldehyde, spheroplasted, and then incubated with increasing amounts of MNase to digest nonnucleosomal DNA (see the Materials and Methods for details). As described previously (Brickner et al. 2007), we monitored MNase digestion of two sequences located in the *GAL1–10* promoter—one within a well-positioned nucleosome (*GAL1* NB), and one within an adjacent MNase-sensitive region (*GAL1* NUB)—by quantitative PCR (qPCR) (Supplemental Fig. S1). DNA isolated from the MNase concentration where we observed significant protection of *GAL1* NB relative to *GAL1* NUB was then used to assess MNase protection across *SRG1–SER3*. We performed qPCR with 38 unique primer pairs to amplify overlapping *SRG1–SER3* sequences (Fig. 1A) from both MNase-digested and undigested DNA. MNase protection for each of these sequences was quantified as the ratio of template present in MNase-digested DNA over undigested DNA that was then normalized to the amount of MNase-protected *GAL1* NB template. Using this method, we identified peaks of MNase protection, indicating the presence of a positioned nucleosome at the 3′ end of *AIM9* (the gene adjacent to *SRG1*) and two at the 5′ end of the *SER3* ORF (Fig. 1B). We also found a 200-bp MNase-sensitive region (from −750 to −550 with respect to the *SER3* ATG) corresponding to the *SRG1* promoter, indicating a nucleosome-depleted region that is a hallmark of many yeast promoters (Yuan et al. 2005; Albert et al. 2007; Lee et al. 2007). In addition, we identified a broad region of MNase protection that begins at the *SRG1* transcription start site (−475) and extends across the *SER3* promoter to the *SER3* translational start site, a region that defines the *SRG1* transcription unit. This pattern of strong MNase protection implies the presence of nucleosomes that are positioned randomly across the *SRG1* transcription unit. Therefore, the *SER3* promoter lacks the typical nucleosome-depleted region (Yuan et al. 2005; Albert et al. 2007; Lee et al. 2007). These results are consistent with our previously reported indirect-labeling experiments (Martens and Winston 2002) and with genome-wide nucleosome positioning experiments (Lee et al. 2007).

To determine if *SRG1* transcription affects the chromatin structure at *SER3*, we repeated the nucleosome

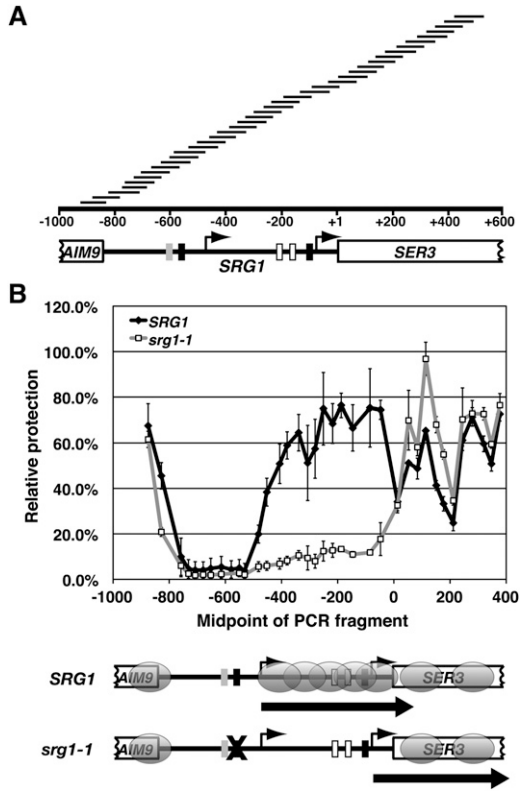

Figure 1. Nucleosome positions and relative occupancy at *SER3* in the presence and absence of *SRG1* transcription. (*A*) Schematic of *SER3* locus, including the 3′ 161 bp of *AIM9* (−1000 to −839 relative to *SER3* ATG) and the 5′ 600 bp of the *SER3* ORF. The arrows at −475 and −75 indicate the transcription start sites of *SRG1* and *SER3*, respectively. Blocks of intergenic sequence identity between *S. cerevisiae* and four related yeast strains are marked, including the *SRG1* and *SER3* TATAs (black boxes), sequences required for *SER3* activation (white boxes), and a Cha4-binding site (gray box). The scale represents the distance from the *SER3* translation start (+1). The tiled black bars *above* the scale indicate the DNA fragments amplified by qPCR to quantify nucleosome position and relative occupancy (see Supplemental Table S2 for details). (*B*) Nucleosome scanning assay was performed on wild-type (FY4, FY2097, and FY1350) and *srg1-1* (YJ582, FY2250, and YJ585) cells that were grown in YPD medium (*SER3* repressed) at 30°C. Using qPCR, the relative MNase protection of each *SER3* template was calculated as a ratio to the control *GAL1* NB template found within a well-positioned nucleosome in the *GAL1–10* promoter (see Supplemental Fig. S1). Each point on the graph shows the mean ± SEM from three independent experiments that are plotted at the midpoint of each PCR product. Results for amplicons SER3-5 to SER3-41 are shown. *Below* the graph, a diagram of the *SER3* locus indicates the positions of nucleosomes (gray ovals) extrapolated from the MNase protection data. The block arrows indicate the transcription activity of *SRG1* and *SER3*, respectively. *srg1-1* strains have a mutated TATA sequence (marked by an X) that inhibits *SRG1* transcription, causing *SER3* derepression.

scanning assay using *srg1-1* strains, which carry a mutation of the *SRG1* TATA sequence. This mutation severely reduces *SRG1* transcription, resulting in strong derepression of *SER3* (Martens et al. 2004). In the *srg1-1* cells, MNase protection was reduced specifically over the *SRG1* transcription unit as compared with wild-type cells, indicating a dramatic loss of nucleosome occupancy (Fig. 1B). Our results reveal a positive correlation between *SRG1* transcription and nucleosome occupancy across *SRG1*, an unexpected finding given the negative correlation between transcription and nucleosome occupancy generally observed for protein-coding genes (Lee et al. 2004; Schwabish and Struhl 2004).

Serine-dependent transcription of SRG1 *intergenic DNA controls nucleosome occupancy of the* SER3 *promoter*

We showed previously that *SER3* expression is tightly controlled by the serine-dependent regulation of *SRG1* transcription (Martens et al. 2005). Therefore, we also measured MNase accessibility at *SER3* in wild-type strains that were grown in synthetic complete (SC) + serine (*SRG1* induced; *SER3* repressed) and then shifted to SC − serine (*SRG1* repressed, *SER3* induced) for 25 min. Since the extent of the MNase digestion of the *GAL1* NB region was identical in these different growth conditions (Supplemental Fig. S2), we again normalized all *SER3* data to this region. As expected for cells grown in serine-rich media, the relative MNase protection across *SRG1–SER3* is nearly identical to that observed for cells grown in YPD (cf. wild-type strains in Figs. 1B, 2A). When cells were shifted to media lacking serine, we measured a significant decrease in MNase protection over the *SRG1* transcribed region. However, rather than extending across the entire *SRG1* transcription unit, as was observed for *srg1-1*, the reduced MNase protection was restricted to a 200-bp region that included sequences that had been determined previously to be required for *SER3* activation (Martens et al. 2004). An MNase-protected region of ~350 bp, consistent with two closely associated nucleosomes or possibly one nucleosome that adopts multiple positions, remains near the 5′ end of *SRG1*. This MNase-protected region begins at a more 5′ position, including the *SRG1* transcription start site and possibly the *SRG1* TATA, as compared with the beginning of the broad peak of MNase protection that was measured for cells grown in serine-rich media. Thus, in contrast to the complete loss of nucleosomes across *SRG1* that occurs in the *srg1-1* strains, serine starvation depletes nucleosomes specifically over sequences required for *SER3* activation. Therefore, in response to serine starvation, the *SER3* promoter adopts the typical promoter architecture, with +1 and −1 nucleosomes flanking a nucleosome-depleted UAS (Albert et al. 2007; Lee et al. 2007).

To determine if the loss of nucleosome occupancy at the *SER3* promoter is caused by a loss of *SRG1* transcription and is not simply an effect of the resulting increase in *SER3* transcription, we repeated the nucleosome scanning assay using strains that contain a mutation in the *SER3* TATA sequence (*ser3-100*). Although the *ser3-100* mutation strongly inhibits *SER3* activation when cells

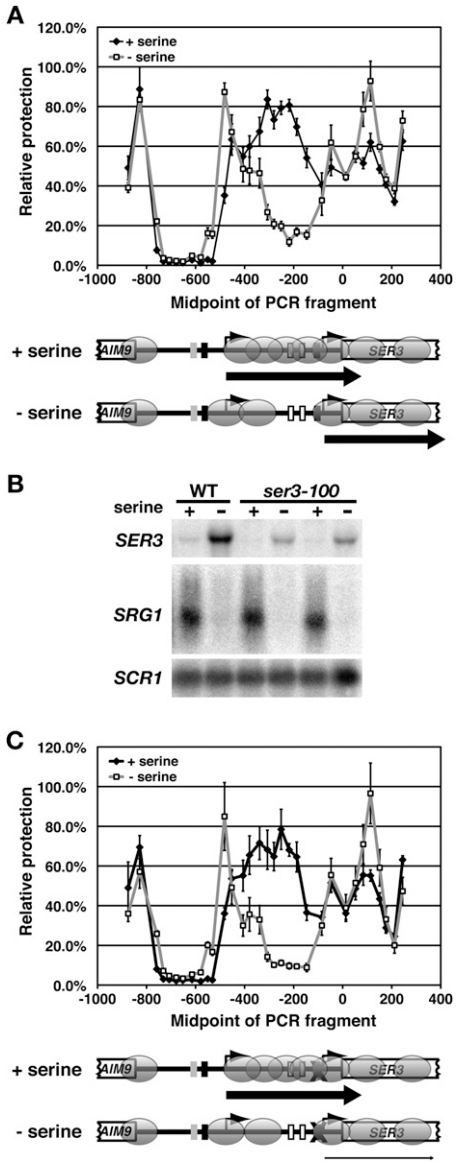

Figure 2. Effect of serine on nucleosome positions and relative occupancy at *SER3*. (*A*) Nucleosome scanning assay was performed on wild-type cells (FY2097 and FY4) that were grown at 30°C in SC + serine media (+ serine) and then shifted to SC − serine media (− serine) for 25 min as described in Figure 1. Each point on the graph shows the mean relative MNase protection ± SEM from four independent experiments (two for each strain) plotted at the midpoint of each PCR product. Results for amplicons SER3-7 to SER3-41 are shown. (*B*) Northern analysis of *SER3* and *SRG1* was performed on a wild-type (FY2097) and two *ser3-100* strains (YJ275 and FY2099) that have a mutated *SER3* TATA. Cells were grown at 30°C in SC + serine media (+ serine) and then shifted to SC − serine media (− serine) for 25 min. *SCR1* serves as a loading control. (*C*) Nucleosome scanning assay was performed on *ser3-100* strains (YJ275 and FY2099) as described in *A*.

are shifted from serine-rich to serine starvation media (10-fold decrease in *SER3* mRNA levels) (Fig. 2B), the changes in MNase protection between these growth conditions were identical to those observed for a wild type (Fig. 2, cf. A and C). Therefore, reduced nucleosome occupancy over the *SER3* promoter is not a consequence of increased *SER3* expression.

FACT and Spt6/Spn1(Iws1) are required to repress SER3

Our results thus far are consistent with two possible mechanisms for transcription interference at *SER3*. In the first possibility, similar to the conventional transcription interference mechanism (Greger et al. 2000), RNA Pol II elongating across *SRG1* competes with transcription factors for binding to the *SER3* promoter. In the second possibility, the nucleosomes maintained over the *SER3* promoter by *SRG1* transcription compete with transcription factor access to the *SER3* promoter. If the latter possibility is true, we reasoned that disrupting nucleosome reassembly during transcription might cause *SER3* derepression. Several studies have implicated the essential, highly conserved FACT and Spt6/Spn1(Iws1) transcription elongation complexes in transcription-dependent chromatin reassembly (Belotserkovskaya et al. 2003; Kaplan et al. 2003; Mason and Struhl 2003; Cheung et al. 2008; Jamai et al. 2009). Northern analyses were performed on several temperature-sensitive mutants of the Spt6/Spn1(Iws1) and FACT complexes that were grown in YPD at permissive (30°C) and nonpermissive (37°C) temperatures. Large increases in *SER3* mRNA levels were detected in multiple *spt6* and *spn1(iws1)* mutants at both 30°C and 37°C (Fig. 3A). While increases were more modest and variable in the FACT mutants (*spt16*, *pob3*, and *nhp6*), we did find that, in at least one mutant, *spt16-197*, a significant increase in *SER3* mRNA levels occurred at 30°C (Fig. 3B). Importantly, *SRG1* RNA levels were not significantly reduced in most of the mutant strains as compared with a wild type at 30°C.

We also performed chromatin immunoprecipitation (ChIP) experiments to measure RNA Pol II occupancy across the *SRG1/SER3* locus in a wild-type strain and two of these mutants (*spt6-1004* and *spt16-197*) that express either untagged Rpb1 (control) or a myc-tagged version of Rpb1 (Rpb1-13myc). The *spt6-1004* and *spt16-197* mutants have both been well characterized and share similar phenotypes characteristic of transcription defects, including sensitivity to the nucleotide analog 6-azauracil, suppression of Ty insertions, and cryptic intragenic transcription (Kaplan et al. 2003; Mason and Struhl 2003). Consistent with our Northern data, RNA Pol II strongly associates with the *SRG1* transcription unit (Fig. 3C) to similar levels in wild-type, *spt6-1004*, and *spt16-197* cells. Taken together, these results show that *SER3* repression is strongly dependent on both Spt6/Spn1(Iws1) and FACT. When these factors are mutated, *SER3* is derepressed without affecting RNA Pol II levels at *SRG1*. This result argues against a model in which it is the level of active transcription that confers transcription interference.

Beyond the primary sites of *SRG1* transcription termination, we found a twofold increase in RNA Pol II

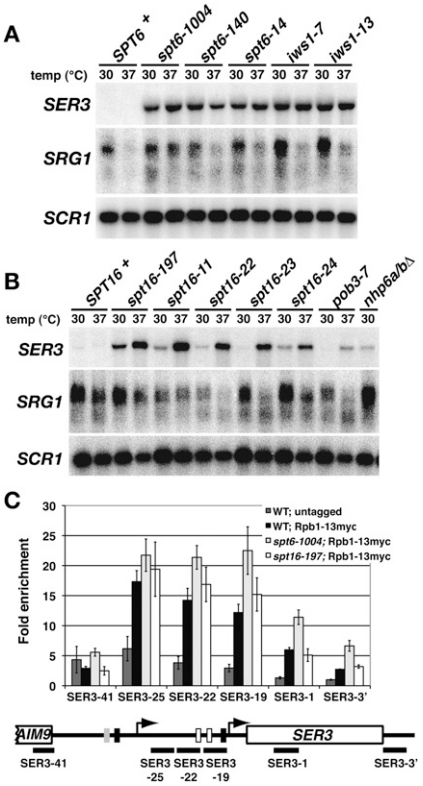

Figure 3. Repression of *SER3* is dependent on Spt6/Spn1(Iws1) and the FACT complex. (*A*) Northern analysis of *SER3*, *SRG1*, and *SCR1* (loading control) was performed on wild-type (FY4), *spt6-1004* (FY2425), *spt6-140* (FY111), *spt6-14* (FY1221), *iws1-7* (GHY1199), and *iws1-13* (GHY1200) strains. Cells were grown in YPD at 30°C to mid-log and then shifted for 60 min to 37°C. (*B*) Northern analysis of *SER3*, *SRG1*, and *SCR1* (loading control) was performed on wild-type (FY4), *spt16-197* (FY346), *spt16-11* (TF8030-1), *spt16-22* (YJ832), *spt16-23* (YJ833), *spt16-24* (TF7783-24), *pob3-7* (TF8031-1), and *nhp6aΔ::URA3 nhp6bΔ::URA3* (FY1411) strains that were grown in YPD. (*C*) ChIP analysis was performed on chromatin isolated from wild-type (YJ877, YJ878, YJ879, and YJ884), *spt6-1004* (YJ886, YJ887, YJ888, and YJ892), and *spt16-197* (YJ841, YJ842, and YJ843) strains expressing Rpb1-C13myc and untagged control strains (FY4, FY5, and YJ586). Rpb1-C13myc was immunoprecipitated with α-myc A14 antibody from chromatin prepared from cells that were grown in YPD at 30°C. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material and is expressed as the fold enrichment over a control region of chromosome V that lacks ORFs (Supplemental Table S2, No ORF). Each bar represents the mean ± SEM from at least three independent experiments. *Below* the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR (see Supplemental Table S2 for details).

occupancy in the *spt6-1004* cells as compared with wild-type cells, which is consistent with our Northern data (Fig. 3C). However, we did not detect an increase in RNA Pol II in the *spt16-197* cells. Although surprising given the increase in *SER3* mRNA levels in this mutant, this result may be reconciled if we consider that *SRG1* transcription does not always terminate properly, resulting in the production of a minor readthrough that extends to the end of *SER3* (Martens et al. 2004; Thompson and Parker 2007). Importantly, we found that the level of *SRG1–SER3* readthrough product is reduced in both *spt6-1004* and *spt16-197* mutants (S Hainer, unpubl.), which is likely due to increased initiation at the *SER3* promoter. Therefore, increased RNA Pol II occupancy in these mutant strains that would better reflect the observed increases in *SER3* transcription are likely masked by the RNA Pol II that occupies *SER3* as a result of the synthesis of an *SRG1–SER3* readthrough product.

Nucleosome occupancy of the SER3 *promoter is reduced in* spt6-1004 *and* spt16-197 *mutants at the permissive temperature*

To test whether the level of nucleosomes over *SRG1* affects *SER3* repression, we next performed nucleosome scanning assays to compare MNase accessibility across *SRG1* in wild-type, *spt6-1004*, and *spt16-197* cells that were grown in YPD at 30°C. We again normalized MNase protection of each *SRG1–SER3* region to the *GAL1* NB region, as the MNase accessibility of the *GAL1* control regions was indistinguishable between these strains (Supplemental Fig. S3A). Compared with wild-type cells, we measured a significant reduction of MNase protection specifically across the *SRG1* transcribed unit in *spt6-1004* cells (fourfold decrease) and to a slightly lesser extent in *spt16-197* cells (threefold decrease) (Fig. 4A), indicating nucleosome depletion across *SRG1*. These results are strikingly similar to the nucleosome scanning results we obtained for the *srg1-1* mutant (Fig. 1B). However, while *SRG1* transcription was greatly reduced in *srg1-1* strains, it remained at wild-type levels in the *spt6-1004* and *spt16-197* mutants.

To complement our MNase experiments, we performed histone H3 ChIP assays in these same strains grown under the same conditions (Fig. 4B). In wild-type cells, we detected significant histone H3 occupancy over the *SER3* promoter as compared with the *SRG1* promoter, which is consistent with nucleosomes occupying the *SER3* promoter. Moreover, at least for *spt6-1004* cells, there is a twofold to threefold decrease in histone H3 occupancy specifically over the *SER3* promoter that parallels the increase in MNase sensitivity over this region. Curiously, we did not observe a similar decrease in histone H3 occupancy over the *SER3* promoter in *spt16-197* cells. Since the loss of MNase protection is less pronounced in the *spt16-197* mutants as compared with the *spt6-1004* mutants, it is possible that histone H3 ChIP is not sensitive enough to detect a change in histone occupancy between wild-type and *spt16-197* strains. Alternatively, nucleosomes may only partially reassemble in the *spt16-197* mutant in a manner that makes them more accessible to MNase without altering histone H3 occupancy. Based on previous studies (Belotserkovskaya et al. 2003; Xin et al. 2009), an intriguing possibility is that reassembly of the H2A/H2B dimers at the *SER3* promoter may be specifically reduced by the *spt16-197* mutation. Taken together, these data support a model

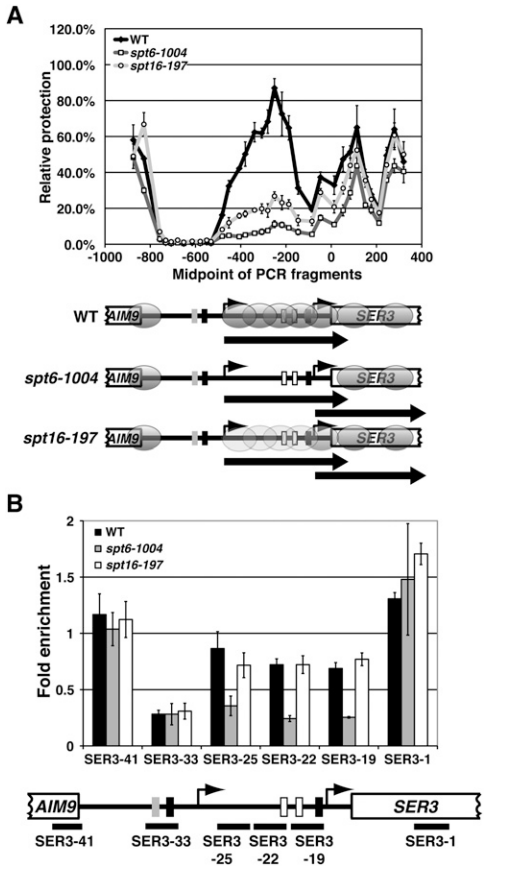

Figure 4. Nucleosome positions and relative occupancy at *SER3* in *spt6-1004* and *spt16-197* mutants. (*A*) Nucleosome scanning assay was performed on wild-type (FY2134, YJ864, and YJ847), *spt6-1004* (FY2180, YJ855, YJ862), and *spt16-197* (FY346, YJ859, and YJ916) strains that were grown in YPD at 30°C as described in Figure 1. The light-gray ovals over the *SRG1* transcription unit in the *spt16-197* strain reflect that this region is slightly more protected from MNase digestion as compared with the *spt6-1004* strain. (*B*) Histone H3 ChIP was performed on chromatin isolated from wild-type (FY4, FY5, and YJ586), *spt6-1004* (YJ886, YJ887, and YJ888), and *spt16-197* (YJ844, YJ845, and YJ846) cells that were grown in YPD. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material and is expressed as the fold enrichment over *GAL1* NB (see Supplemental Fig. S1). Each bar represents the mean ± SEM of at least three independent experiments. *Below* the graph is a schematic of *SER3* with black bars corresponding to the regions amplified by qPCR (see Supplemental Table S2 for details).

whereby FACT and Spt6/Spn1(Iws1) are required for *SRG1* transcription-dependent assembly of nucleosomes that repress *SER3*.

spt6-1004 *and* spt16-197 *mutants are defective for transcription interference at* SER3

To test whether *SRG1* transcription-dependent nucleosomes interfere with transcription factor binding to the *SER3* promoter, we performed ChIP experiments in *spt6-1004* and *spt16-197* mutants. Because sequence-specific activators of *SER3* remain unknown, we first used a previously described *ser3::GAL7UAS* allele in which the putative *SER3* UAS is replaced with two binding sites for the Gal4 transcription activator (Martens et al. 2004). We then measured Gal4 occupancy by ChIP in wild-type, *srg1-1*, *spt6-1004*, and *spt16-197* strains that all contain the *ser3::GAL7UAS* allele and were grown in YPgal (Fig. 5A). Consistent with our previous data (Martens et al. 2004), Gal4 occupancy at the *SER3* promoter increases eightfold in the *srg1-1* control strain where *SRG1* is no longer transcribed and the *SER3* promoter is depleted of

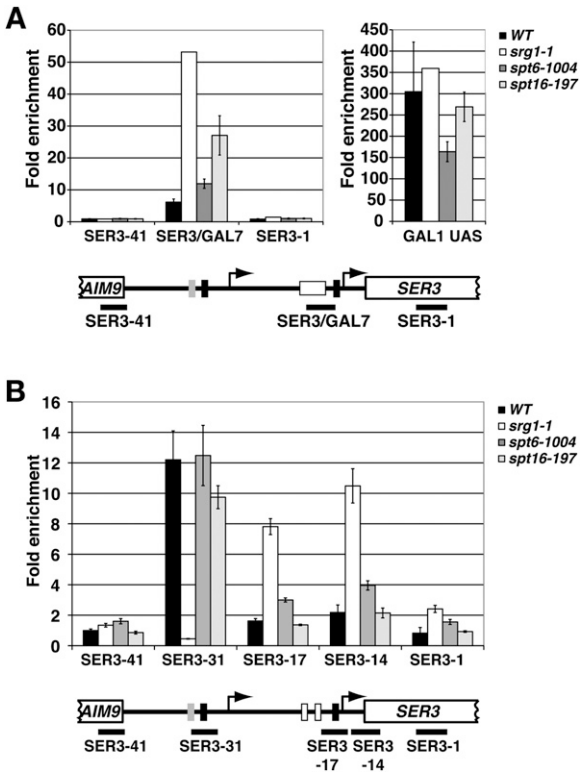

Figure 5. *spt6-1004* and *spt16-197* mutants are defective for transcription interference at *SER3*. (*A*) Gal4 ChIP was performed on wild-type (YJ871, YJ872, and YJ873), *spt6-1004* (YJ875, YJ876, and YJ850), *spt16-197* (YJ867, YJ868, and YJ869), and positive control *srg1-1* (FY2260) cells that all contain the *ser3::GAL7UAS* allele. Chromatin was prepared from cells grown at 30°C in YPraf to 0.8×10^7 cells per milliliter, and then for an additional 4 h at 30°C after the addition of 2% galactose. Gal4 ChIP signals were determined by qPCR at the three *SER3* locations (*left* histogram), and at *GAL1* as a positive control (*right* histogram). All values were normalized to a control region located near the telomere of chromosome VI (TELVI) (Supplemental Table S2) and represent the mean ± SEM. *Below* the graph is a diagram of the *ser3::GAL7UAS* allele in which the putative *SER3* UAS region was replaced with the *GAL7* UAS region containing two Gal4-binding sites (white box). The black bars indicate the regions of *SER3* amplified by qPCR. (*B*) TBP ChIP was performed on chromatin isolated from wild-type (FY4, FY5, YJ586, and KY719), *spt6-1004* (YJ886, YJ887, YJ888, and YJ892), *spt16-197* (YJ841, YJ842, YJ843, and YJ844), and positive control *srg1-1* (FY2471, YJ582, YJ583, and YJ585) strains that were grown in YPD at 30°C as described in Figure 3C.

nucleosomes. In the *spt6-1004* and *spt16-197* strains where *SRG1* is transcribed at wild-type levels but nucleosome occupancy at the *SER3* promoter is reduced, Gal4 occupancy at the *SER3* promoter was also increased twofold and fourfold, respectively (Fig. 5A, left panel). Based on our *SER3* expression and nucleosome occupancy data (Figs. 3A, 4A), the twofold increase in Gal4 occupancy at the *SER3* promoter in the *spt6-1004* strains was lower than expected. However, this result is likely related to the fact that we also found reduced Gal4 occupancy at the control *GAL1* promoter in *spt6-1004* cells as compared with wild-type, *srg1-1*, and *spt16-197* cells (Fig. 5A, right panel).

We also compared TBP occupancy by ChIP at the *SRG1* and *SER3* TATA sequences in wild-type, *srg1-1*, *spt6-1004*, and *spt16-197* strains that contain the endogenous *SRG1–SER3* locus (Fig. 5B). The *SRG1* and *SER3* TATA sequences are both conserved among related yeast strains, bind TBP, and are required for *SRG1* and *SER3* transcription, respectively (Martens and Winston 2002; Martens et al. 2004). At the *SRG1* TATA, there is little difference in TBP occupancy in the *spt6-1004* and *spt16-197* mutants as compared with the wild-type strains, which agrees with our Northern and RNA Pol II ChIP data (see Fig. 3). At the *SER3* TATA, TBP occupancy increased twofold in *spt6-1004* cells as compared with a fourfold increase in *srg1-1* control cells, suggesting that the loss of nucleosomes over the *SER3* promoter in the *spt6-1004* mutants either increases TBP binding directly or possibly indirectly by allowing an unknown *SER3* activator protein better access to the *SER3* promoter. Interestingly, we did not observe a significant difference in TBP occupancy in the *spt16-197* mutant. This result may not be surprising, considering the increase in *SER3* expression is significantly lower in this mutant as compared with the *spt6-1004* mutant (Fig. 3; see Supplemental Fig. S4B for a direct comparison). Therefore, this assay may lack the sensitivity to detect a significant difference in TBP occupancy between wild-type and *spt16-197* cells.

From these data, we conclude that transcription interference at *SER3* is dependent, at least in part, on Spt6 and Spt16. Taken together with results described earlier, our findings suggest that transcription interference of *SER3* is partially mediated by nucleosomes that occupy the *SER3* promoter as a consequence of *SRG1* transcription from intergenic DNA.

Histone modifications that suppress cryptic intragenic transcription are not required for SER3 *repression*

Spt6 and Spt16 have been shown previously to suppress transcription initiation from cryptic promoters that are located within protein-coding regions (Mason and Struhl 2003; Kaplan et al. 2009). Cryptic intragenic transcription is also suppressed by a cascade of transcription-dependent post-translational histone modifications (Lee and Shilatifard 2007; Li et al. 2007a). During transcription, Set2 methylates Lys 36 of histone H3, thereby marking nucleosomes associated with recently transcribed DNA (Pokholok et al. 2005; Rao et al. 2005). Dimethylated H3K36 acts as a binding site for the Rpd3S histone deacetylase complex (Youdell et al. 2008). Upon recruitment, Rpd3S deacetylates the reassembled nucleosomes on the N-terminal tails of histones H3 and H4, which suppresses cryptic intragenic transcription, presumably by occluding transcription factor access (Carrozza et al. 2005; Joshi and Struhl 2005; Keogh et al. 2005). Recently, Set1-dependent methylation of H3K4 has also been implicated as a signal for transcription-dependent histone deacetylation by Rpd3S (Pinskaya et al. 2009) and the Set3 complex (Kim and Buratowski 2009). Because of these observations, a likely hypothesis is that Set1 and Set2 may contribute to *SER3* repression by regulating similar histone modifications over the *SER3* promoter in response to *SRG1* transcription. To test this possibility, we performed a Northern analysis to measure the effect of deleting the genes encoding the Set1, Set2, and Dot1 histone methyltransferases; the Rco1 subunit of Rpd3S; and the Set3 subunit of the Set3 complex on *SER3* and *SRG1* expression. Deletions of any one of these genes or a *set1Δset2Δ* double deletion has no effect on *SER3* or *SRG1* mRNA levels (Fig. 6). Moreover, mutations of histone H3 Lys 4 (methylated by Set1), Lys 36 (methylated by Set2), or Lys 79 (methylated by Dot1) also has little to no effect on *SER3* repression (S Hainer, unpubl.). Therefore, our results suggest that the relative contribution of these histone reassembly mechanisms may vary at different loci throughout the genome.

Discussion

In this study, we provide evidence that intergenic transcription represses adjacent gene transcription by assembling a repressive chromatin structure, rather than by the act of transcription. First, we showed that *SRG1* intergenic transcription is required not only for repression of the adjacent *SER3* gene, but also to maintain MNase protection of the *SER3* promoter. Second, we determined that changes in the MNase protection of the *SER3* promoter are caused by changes in *SRG1* transcription and are not an effect of the changes to *SER3* transcription. Third, we found that cells expressing mutant versions of the Spt6 and Spt16 elongation factors derepress *SER3* and reduce MNase protection across the *SER3* promoter

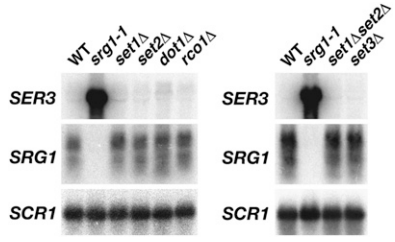

Figure 6. Repression of *SER3* does not require histone methyltransferases or the Rpd3S and Set3C histone deacetylase complexes. Northern analysis of *SER3*, *SRG1*, and *SCR1* (loading control) was performed on wild-type (YJ586), *srg1-1* (FY2471), *set1Δ* (KY938), *set2Δ* (KY912), *dot1Δ* (KY934), *rco1Δ* (KY1235), *set1Δset2Δ* (KY1822), and *set3Δ* (KY1806) strains that were grown in YPD at 30°C.

without altering *SRG1* RNA levels or RNA Pol II occupancy across *SRG1*. These results clearly implicate the nucleosomes assembled on the *SER3* promoter as the key factor in *SER3* repression. Finally, we found that Spt6 and Spt16 are required to inhibit transcription factor binding to the *SER3* promoter, which suggests that the nucleosomes assembled at the *SER3* promoter by these factors interfere with the binding of transcription factors to their sites on DNA.

Taken together with our previous studies (Martens and Winston 2002; Martens et al. 2004, 2005), we propose the following model for *SER3* regulation (Fig. 7). When cells are grown in serine-rich medium, the Cha4 DNA-binding protein recruits the Swi/Snf and SAGA complexes, resulting in the induction of *SRG1* transcription. RNA Pol II transcribes *SRG1* across the *SER3* promoter, disassembling nucleosomes in its path and then reassembling them in its wake by a mechanism that involves both Spt6 and Spt16. *SRG1* transcription is thus required to maintain nucleosomes across the *SER3* promoter, interfering with transcription factor binding. When cells are then transferred to serine starvation conditions, Cha4 no longer recruits Swi/Snf and SAGA, resulting in decreased *SRG1* transcription. Without intergenic transcription to maintain them, nucleosomes are depleted over the *SER3* UAS, allowing transcription factors—either an as yet unknown site-specific DNA-binding activator or possibly TBP and RNA Pol II—to bind and activate *SER3*. Two positioned nucleosomes remain at the 5′ end of *SRG1*, where they are likely to inhibit *SRG1* transcription.

In addition to its role in nucleosome assembly during transcription, Spt6 has also been reported to reassemble

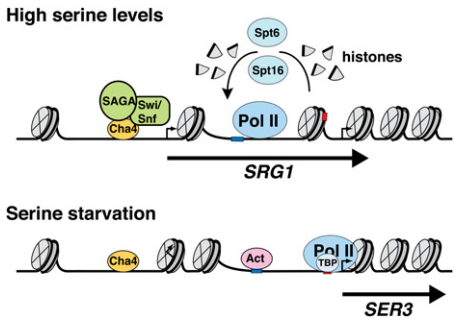

Figure 7. A model for *SER3* regulation by *SRG1* intergenic transcription. When serine is available to the cells, DNA-bound Cha4 recruits SAGA and Swi/Snf to initiate *SRG1* transcription, possibly by remodeling the two nucleosomes located at the 5′ end of *SRG1* to expose the *SRG1* transcription start site. RNA Pol II transcribes *SRG1* and, through Spt6 and Spt16, disassembles nucleosomes in its path and then reassembles them in its wake. As a result, nucleosomes continuously occupy the *SER3* UAS where they repress *SER3* by occluding the *SER3* promoter from transcription factor binding. In the absence of serine, *SRG1* transcription is repressed, possibly due to the presence of two nucleosomes at its 5′ end that encompass its transcription start site. In the absence of *SRG1* transcription, the *SER3* UAS is depleted of nucleosomes, allowing an as yet unknown activator (Act) and/or TBP and RNA Pol II to bind and activate *SER3* transcription.

nucleosomes at the promoters of *PHO5* and several other yeast genes during repression (Adkins and Tyler 2006). Therefore, an alternative model for *SER3* repression is that Spt6 and, possibly, Spt16 reassemble nucleosomes over the *SER3* promoter independently of *SRG1* transcription. Thus, mutations in these factors may bypass the normal role for *SRG1* transcription, which is to interfere with the recruitment of chromatin remodeling factors needed to displace the repressive nucleosomes at the *SER3* promoter. A prediction of this model is that the increased levels of *SER3* expression observed in the *spt6-1004* and *spt16-197* mutants would no longer be dependent on sequence-specific activators to recruit chromatin remodeling factors, analogous to what has been observed for *PHO5* (Adkins and Tyler 2006). To test this alternative model, we first identified a 37-bp sequence within the *SER3* promoter (from −192 to −228; *SER3* ATG = +1) that is required for *SER3* activation in response to serine starvation (Supplemental Fig. S4A). When this sequence was deleted in the *spt6-1004* and *spt16-197* strains, *SER3* mRNA levels were reduced as compared with similar strains expressing wild-type *SER3* (Supplemental Fig. S4B). Therefore, *spt6-1004* and *spt16-197* mutations do not bypass the requirement of the *SER3 UAS* for *SER3* activation, which argues against this alternative model.

Although MNase accessibility has been used extensively to predict nucleosome occupancy in eukaryotic organisms (for examples, see Yuan et al. 2005; Lee et al. 2007), we cannot rule out the possibility that DNA-binding proteins may contribute to the protection of the *SER3* promoter from MNase digestion in serine-rich conditions. However, our observation that MNase protection over the *SER3* promoter was reduced in *spt6-1004* and *spt16-197* mutants without affecting RNA Pol II occupancy suggests that at least RNA Pol II and its associated factors do not affect MNase digestion.

If *SRG1* transcription from intergenic DNA is required to maintain nucleosomes over the *SER3* UAS, then from where might these nucleosomes originate? An intriguing source of these nucleosomes would be those positioned over the *SRG1* transcription start site and TATA (Fig. 7) that likely inhibit *SRG1* transcription in the absence of serine. Based on this study and our previous work (Martens and Winston 2002; Martens et al. 2004, 2005), Swi/Snf, when recruited to the *SRG1* promoter in response to serine, may slide these nucleosomes toward *SER3* to facilitate preinitiation complex assembly and *SRG1* transcription. Once RNA Pol II begins to transcribe *SRG1*, the nucleosomes originally moved by Swi/Snf are disassembled to allow passage of RNA Pol II, and then are reassembled behind RNA Pol II by Spt6 and Spt16. Therefore, the activities of Swi/Snf, Spt6/Spn1, and FACT may combine to establish and maintain nucleosomes over the *SER3* promoter that interfere with transcription factor binding to this region. This scenario would also explain the difference in nucleosome occupancy at the 5′ end of *SRG1* observed for wild-type cells grown in the serine starvation media as compared with *srg1-1* cells grown in serine-rich media, two conditions in which

SER3 is strongly derepressed (Figs. 1, 2A). In contrast to wild-type cells grown in serine starvation medium, where it is no longer recruited, Swi/Snf is presumably still recruited by Cha4 in the *srg1-1* (*SRG1* TATA mutant) cells that are grown in serine-rich media. Thus, Swi/Snf can remodel the nucleosomes at the 5′ end of *SRG1*; however, these nucleosomes cannot be maintained in the absence of *SRG1* transcription.

In addition to the nucleosome reassembly activity of Spt6/Spn1 and FACT, it has been well documented that a cascade of transcription-dependent post-translational modifications of histones found within nucleosomes over protein-coding genes contributes to the repression of intragenic transcription initiation (Lee and Shilatifard 2007; Li et al. 2007a). However, our studies show that *SER3* repression appears to be independent of at least some of these marks, including Set1-mediated methylation of histone H3 K4, Set2-mediated methylation of K36, and the removal of histone H3 and H4 acetylation by the Rpd3S and Set3C histone deacetylase complexes. Although we cannot rule out the possibility that other post-translational histone modifications may be involved, our results indicate a difference in the requirement of transcription-dependent post-translational histone modifications between *SER3* repression by *SRG1* transcription and repression of cryptic intragenic transcription. This difference may be related to the fact that *SRG1* is a relatively short transcription unit (~400 bp) that is highly transcribed. It has been reported recently that cryptic intragenic transcription preferentially occurs at lowly transcribed genes (Li et al. 2007b; Cheung et al. 2008; Lickwar et al. 2009). Therefore, it is possible that highly transcribed *SRG1* may not be dependent on H3K36 methylation and subsequent histone deacetylation for protection from intragenic transcription, because of the frequent passage of RNA Pol II. Alternatively, short, highly transcribed genes may never establish this histone mark, since histone H3K36 methylation predominates toward the 3′ ends of transcribed genes (Pokholok et al. 2005). In support of this possibility, genome-wide analyses of K36 methylation indicate little K36 trimethylation at *SRG1* (Pokholok et al. 2005).

In contrast to the characteristic transcription-dependent depletion of nucleosomes seen at protein-coding genes (Yuan et al. 2005; Lee et al. 2007), we show transcription-dependent assembly of nucleosomes across intergenic *SRG1*. How does one account for this apparent contradiction between nucleosome occupancy and transcription? Several recent studies have indicated that DNA sequences can either favor or refract nucleosome formation, thereby influencing genome-wide nucleosome positioning (Yuan et al. 2005; Ioshikhes et al. 2006; Segal et al. 2006; Peckham et al. 2007; Field et al. 2008; Kaplan et al. 2009). As has been proposed for yeast genes containing nucleosome-depleted promoter regions (Segal and Widom 2009), one possibility is that the underlying DNA sequence of the *SER3* promoter may normally disfavor nucleosome formation to facilitate transcription factor binding. Therefore, by reassembling nucleosomes after each passage of RNA Pol II, *SRG1* transcription effectively maintains nucleosomes over DNA that is normally refractory to nucleosomes. Several observations support this possibility. First, the *SER3* UAS region that is nucleosome-depleted in the absence of *SRG1* transcription contains poly(dA:dT) tracts, a sequence motif that resists bending and thus disfavors nucleosome formation (Segal and Widom 2009). Second, the *SER3* UAS sequence is predicted to have a low nucleosome-forming potential by an algorithm developed using comparative genomics (Ioshikhes et al. 2006). Finally, the *SER3* UAS sequence failed to form a stable nucleosome in a genome-wide in vitro nucleosome reconstitution assay (Kaplan et al. 2009).

In *S. cerevisiae*, cells respond to changes in serine availability by rapidly inducing or repressing transcription of *SER3*. This response involves a dynamic competition between nucleosomes and transcription factors that is controlled by the transcription of *SRG1* from intergenic ncDNA. Our findings raise the intriguing possibility that widespread transcription of ncDNA may impact genome-wide chromatin architecture. In doing so, transcription of ncDNA may influence not only gene expression, but also other cellular processes that are dependent on protein–DNA interactions.

Materials and methods

S. cerevisiae *strains and media*

All *S. cerevisiae* strains used in this study (Supplemental Table S1) are isogenic with a *GAL2*$^+$ derivative of S288C (Winston et al. 1995). Strains were constructed using standard genetic crosses or by transformation (Ausubel et al. 1991). The C termini of *RPB1* and *SPT16* were tagged with 13 copies of the c-Myc epitope by PCR-mediated transformation of diploid strains using pFA6a-13myc-KanMX and pFA6a-13myc-HIS3MX, respectively (Longtine et al. 1998). The *spt16-22* and *spt16-23* alleles (Formosa et al. 2001) were integrated into a diploid strain by two-step gene replacement using SnaBI-digested pTF142-23 and pTF142-22 plasmids (kindly provided by T. Formosa, University of Utah, Salt Lake City, UT). The *ser3ΔUAS* mutation was constructed by replacing 37 bp of *SER3* promoter sequence (from −228 to −198; *SER3* ATG = +1) with an AvrII restriction site by QuikChange mutagenesis (Agilent Technologies) to yield pRM08 plasmid. The *ser3ΔUAS* allele was then integrated into a diploid strain by two-step gene replacement using AfeI-digested pRM08. Several strains contain a *KanMX*-marked deletion of the *SER33* gene, which is a paralog of *SER3*. Based on previous studies (Martens and Winston 2002; Martens et al. 2004) and the results presented in this study, the deletion of *SER33* does not affect *SER3* regulation. Strains were grown in the following media as indicated in the figure legends: YPD (1% yeast extract, 2% peptone, 2% glucose), YPgal (1% yeast extract, 2% peptone, 2% galactose), YPraf (1% yeast extract, 2% peptone, 2% raffinose), and SC with 1 mM serine (SC + serine) or without serine (SC − serine) (Rose et al. 1990).

Nucleosome scanning assay

Nucleosome scanning experiments were performed using a method adapted from those described previously (Whitehouse and Tsukiyama 2006; Brickner et al. 2007; Lee et al. 2007). Cells were grown to 2×10^7 to 3×10^7 cells per milliliter and were

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 at pH 7.5, 1 M sorbitol, 10 mM β-mercaptoethanol) containing 1.5 mg of zymolase 20T for 30 min at 30°C 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 at pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.075% NP-40, 1 mM β-mercaptoethanol, 500 μM spermidine), and resuspended in 1.8 mL of NP buffer. Samples were divided into six 300-μL aliquots that were then digested with 0, 1, 2.5, 5, 10, and 20 U of MNase (Nuclease S7 from Roche) for 45 min at 37°C. Digestions were stopped with 75 μL of Stop buffer (5% SDS, 50 mM EDTA) and were treated with 100 μg of proteinase K for 12–16 h at 65°C. DNA was extracted by phenol/chloroform using PLG-H tubes (5 Prime), and was incubated with 50 μg of RNase A for 1 h at 37°C. DNA was re-extracted with phenol/chloroform, precipitated with an equal volume of isopropanol, washed with 80% ethanol, and resuspended in 100 μL of 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 (Lohr 1984; Brickner et al. 2007; Floer et al. 2010)—one within a positioned nucleosome (*GAL1* NB), and a second 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 (see Supplemental Fig. S1) with a *GAL1* NUB/NB ratio of <15% was subjected to further qPCR using tiled *SER3* primer pairs (SER3-1 to SER3-41) (Supplemental Table S2). For each *SER3* primer set, the amount of protected template was calculated as a ratio between 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.

Northern analysis

Northern analysis was performed as described previously (Ausubel et al. 1991) on 20 μg of total RNA isolated from cells grown to 1×10^7 to 2×10^7 cells per milliliter. DNA probes were generated by random prime-labeling PCR fragments for *SER3* (ChrV: 324059–324307), *SRG1* (ChrV: 322258–322559), and *SCR1* (ChrV: 441741–442266). *SCR1* serves as a loading control, since its RNA levels are unaffected by the mutations and growth conditions used in this study.

ChIP analysis

For histone H3, TBP, and Rpb1-C13myc ChIPs, cells were grown in YPD at 30°C to 1×10^7 to 2×10^7 cells per milliliter. For Gal4 ChIPs, cells were grown in YPraf at 30°C to 0.8×10^7 cells per milliliter, and then an additional 4 h at 30°C after addition of 2% galactose. Chromatin preparation and treatment were preformed as described previously (Shirra et al. 2005). Gal4, histone H3, TBP, and Rpb1-13myc were immunoprecipitated by incubating sonicated chromatin overnight at 4°C with 1 μL of anti-GAL4 DBD antibody (sc-577, Santa Cruz Biotechnology), 5 μL of anti-histone H3 antibody (ab1791, Abcam), 2 μL of anti-TBP antibody (kind gift from G. Prelich, Albert Einstein College of Medicine), and 4 μL of anti-c-myc A-14 antibody (sc-789, Santa Cruz Biotechnology), respectively. Dilutions of input and immunoprecipitated DNA were subjected to qPCR. All ChIP signals were normalized to a control: either *GAL1* NB template (histone H3 ChIP), TELVI template located within a telomeric region on chromosome VI (Gal4 ChIP), or "No ORF" template located within a region of chromosome V that lacks ORFs (Rpb1-C13myc and TBP ChIPs). Details regarding the primers used for qPCR in each ChIP experiment are listed in Supplemental Table S2.

qPCR

All qPCR data were obtained using an ABI 7300 or StepOnePlus Real-Time PCR system, SYBR green reagents (Fermentas), and the primer sets listed in Supplemental Table S2. All calculations were performed using Pfaffl methodology for relative quantitation of real-time PCR (Pfaffl 2001).

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