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Identification of Histone Mutants That Are Defective for Transcription-Coupled Nucleosome Occupancy▽

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Our previous studies of *Saccharomyces cerevisiae* described a gene repression mechanism where the transcription of intergenic noncoding DNA (ncDNA) (*SRG1*) assembles nucleosomes across the promoter of the adjacent *SER3* gene that interfere with the binding of transcription factors. To investigate the role of histones in this mechanism, we screened a comprehensive library of histone H3 and H4 mutants for those that derepress *SER3*. We identified mutations altering eight histone residues (H3 residues V46, R49, V117, Q120, and K122 and H4 residues R36, I46, and S47) that strongly increase *SER3* expression without reducing the transcription of the intergenic *SRG1* ncDNA. We detected reduced nucleosome occupancy across *SRG1* in these mutants to degrees that correlate well with the level of *SER3* derepression. The histone chromatin immunoprecipitation experiments on several other genes suggest that the loss of nucleosomes in these mutants is specific to highly transcribed regions. Interestingly, two of these histone mutants, H3 R49A and H3 V46A, reduce Set2-dependent methylation of lysine 36 of histone H3 and allow transcription initiation from cryptic intragenic promoters. Taken together, our data identify a new class of histone mutants that is defective for transcription-dependent nucleosome occupancy.

Chromatin is a dynamic participant in regulating the function of both large genomic regions and individual genes (reviewed in references 6, 11, 58, and 59). Nucleosomes are the fundamental unit of chromatin, consisting of 147 bp of DNA wrapped around an octamer of histones, including two H2A/H2B heterodimers and one H3/H4 heterotetramer (52, 61). Not surprisingly, nucleosomes have a major impact on the regulation of transcription in several ways. At promoters, nucleosomes interfere with the binding of sequence-specific transcription factors. Across transcribed sequences, nucleosomes act both negatively as a barrier to elongating RNA polymerases and positively by inhibiting transcription factor access to cryptic intragenic promoters to prevent aberrant transcription. Therefore, a major strategy for gene regulation that is shared among eukaryotes is the control of nucleosome architecture (reviewed in references 4, 11, 71, and 87).

Eukaryotic cells have three major classes of proteins that contribute to transcription regulation by altering chromatin: chromatin remodelers, posttranslational histone modifiers, and histone chaperones. Chromatin remodelers, such as the yeast Swi/Snf complex, use the energy from ATP hydrolysis to reposition or remove nucleosomes primarily at promoter regions, thus allowing sequence-specific proteins to bind DNA (10, 16, 30). Posttranslational histone modifiers catalyze the covalent addition of methyl, acetyl, phosphoryl, and ubiquitin groups to the side chains of specific amino acids encoded by the histone genes (13, 84, 89). These modifications have been shown to impact gene regulation by facilitating the activity of chromatin remodelers and by providing a binding platform for additional regulatory proteins. Histone chaperones, including Asf1, Spt6, and Spt16, interact with histones and contribute to the disassembly and reassembly of nucleosomes at promoters and across coding sequences during transcription (27, 49, 95).

We recently described a new mechanism for controlling chromatin at promoters involving the transcription of noncoding DNA (ncDNA) (33). In the presence of serine, the transcription of *SRG1* ncDNA is initiated upstream of the adjacent *SER3* gene and extends across the *SER3* promoter (64, 65). We provided evidence that during *SRG1* transcription, Spt6 and Spt16 histone chaperones reassemble nucleosomes over the *SER3* promoter after the passage of RNA polymerase II (Pol II), which then interfere with transcription factor binding, resulting in *SER3* repression (33). In response to serine starvation, *SRG1* transcription is reduced, causing nucleosome depletion across the *SER3* promoter, which in turn allows transcription factors to bind the *SER3* promoter and activate *SER3* transcription.

Although histone chaperones, including Spt6/Spn1, FACT, and Asf1, have been implicated in mediating transcription-coupled nucleosome assembly, less is known about how histone proteins contribute to this mechanism (5, 7, 15, 27, 28, 41, 43, 47, 49, 66). Several studies have begun to identify specific histone residues that may be involved in this process (15, 23, 24, 100). Among these residues, lysine 36 on histone H3 and several other lysines within the amino-terminal tail of histone H4 are sites of posttranslational modifications that are required to protect recently transcribed DNA from aberrant transcription (22, 24, 60, 77, 78, 98). However, we have provided evidence that *SER3* repression by intragenic *SRG1* transcription is independent of these histone modifications (33). Here, we report the results of a modified synthetic genetic array (SGA) screen using a comprehensive library of histone H3 and H4 mutants (20) to identify histone residues required for *SER3* repression. Mutations altering five histone H3 (K122,

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Q120, V117, R49, and V46) and three histone H4 (S47, I46, and R36) residues that most strongly derepress *SER3* show reduced nucleosome occupancy across the *SER3* promoter. Chromatin immunoprecipitation (ChIP) assays of a subset of yeast genes suggest that these residues, in particular H3 K122, H3 Q120, H3 V117, H4 I46, and H4 R36, generally are required for transcription-dependent nucleosome occupancy at highly transcribed genes. In addition, we provide evidence that two of these residues, histone H3 R49 and V46, have a distinct role in repressing cryptic intragenic transcription by promoting the Set2-dependent methylation of lysine 36 of histone H3. Overall, our results have identified a subset of histone H3 and H4 residues that are required for normal transcription-dependent nucleosome occupancy.

MATERIALS AND METHODS

Strains and media. All *S. cerevisiae* strains used (see Table S1 at http://www.pitt.edu/~martens/supplemental_data/) are isogenic to a *GAL2*$^+$ derivative of S288C (96). All strains were constructed by transformation or by genetic crosses (3). The *lyp1*Δ::*SER3pr-lacZ* allele was generated by two successive PCR-mediated integrations. First, the *LYP1* open reading frame (ORF) was replaced with a 1,523-bp PCR product containing *SRG1* and *SER3* sequences [−713 to −1 relative to *SER3* ATG (+1)] and the *URA3* open reading frame. The *URA3* open reading frame at *lyp1* then was replaced with a 3,046-bp PCR product containing the *lacZ* open reading frame that was amplified from plasmid p180 (35, 69). Transformants were selected by growth on plates containing 5-fluoro-orotic acid, screened for β-galactosidase activity, and confirmed by PCR. The *snf2*Δ::*KANMX*, *snf2*Δ::*LEU2*, *spt6-1004*, and *spt16-197* alleles have been described previously (12, 47, 63, 64). All strains comprising the comprehensive histone mutant library are derivatives of JDY86 and were kindly provided by J. Boeke (20). YJ1082 is a derivative of JDY86 and was generated by gene replacement of the *URA3* gene 3′ of *HHTS-HHFS* with *KANMX*, which was PCR amplified from pRS400 (8). The *hhts-T118I* allele was generated by PCR-based site-directed mutagenesis followed by one-step gene replacement. First, a portion of the *HHTS-HHFS*::*KANMX* cassette beginning 50 bp 5′ of the T118 codon of *HHFS* and extending to the 3′ end of *KANMX* was PCR amplified from YJ1082 genomic DNA using a forward primer that contains a C-to-T base change converting the threonine codon at 118 to isoleucine. A second DNA fragment consisting of the 400 bp 5′ of the T118 codon was PCR amplified from YJ1082 genomic DNA. These two fragments were mixed together and subjected to PCR amplification to generate one long DNA fragment containing the mutation that converts the T118 codon to isoleucine, which then was used to transform YJ112. Transformants were selected by growth on plates containing G418 and confirmed by PCR and sequencing. Solid media used for the modified SGA screen were the following: synthetic complete lacking uracil and lysine (SC-Ura-Lys), synthetic complete lacking histidine, lysine, and uracil that was supplemented with 50 mg/liter thialysine (SC-His-Lys-Ura+thialysine), and sporulation medium supplemented with histidine, lysine, tryptophan, methionine, and cysteine (93). Thialysine is an analogue of lysine that is toxic to yeast cells expressing a functional Lyp1 lysine permease. Therefore, strains containing the *lyp1*Δ::*SER3pr-lacZ* reporter allele can be selected by their growth in the presence of thialysine (90). All other media were prepared as previously described (79). Yeast extract-peptone-dextrose (YPD), YPraff, and YPgal media contained 2% glucose, 2% raffinose, and 2% galactose, respectively, as the sole carbon sources. Solid YPraff and YPgal media also contained 1 mg/liter antimycin A.

SGA screen of histone H3/H4 mutant library. A previously described manual synthetic genetic array (SGA) screen (93) was modified to utilize a comprehensive library of histone H3 or H4 mutants (20). First, 422 histone mutant strains were systematically mated to YJ923 for 1 day at room temperature. Mated cells were pinned to SC-Ura-Lys plates and incubated at 30°C for 2 days to select for diploids. Diploid cells then were pinned to sporulation plates and incubated at 22°C for 5 days. After two successive rounds of selection on SC-His-Lys-Ura+thialysine plates, haploid cells containing both the histone substitution and the *lyp1*Δ::*SER3pr-lacZ* reporter were replica printed to YPD plates and subjected to an X-Gal overlay as described by Duttweiler et al. (26). Briefly, yeast cells that were grown as small patches on YPD plates at 30°C for 2 days were permeabilized by covering the patches with 5 to 10 ml of chloroform for 5 min. The chloroform was decanted, and excess chloroform was allowed to evaporate. A warm agarose solution containing 1% low-melting-point agarose, 0.1 M $NaPO_4$, and 25 mg/ml X-Gal was poured over the cells and allowed to set. After 35 min, each strain was scored for the appearance of blue color and compared to the appearance of the YJ980 control strain expressing one wild-type copy of histones H3 and H4.

Western analysis. Whole-cell extracts (WCE) were prepared from cells grown in YPD at 30°C to 3×10^7 to 4×10^7 cells/ml using trichloroacetic acid as previously described (19, 100). Equal amounts of WCE were separated by 15% acrylamide SDS-PAGE, transferred to Protean nitrocellulose (Whatman), and assayed by immunoblotting. The antibodies used to detect histone H3 and H4 levels and specific posttranslational modifications of these histones were the following: anti-H3 (1:20,000) (gift from LeAnn Howe), anti-H4 (1:2,500) (ab10158; Abcam), anti-H3K4me2 (1:3,000) (07-030; Millipore), anti-H3K4me3 (1:2,500) (ab8580; Abcam), anti-H3K36me2 (1:2,500) (39255; Active Motif), anti-H3K36me3 (1:500) (ab9050; Abcam), and anti-H3K79me2/3 (1:1,000) (ab2621; Abcam). After incubation with horseradish peroxidase (HRP)-conjugated IgG secondary antibody (1:5,000; GE Healthcare), the immunoreactive proteins were visualized by enhanced chemiluminescence detection (Perkin-Elmer).

Northern analysis. Cells were grown to 1×10^7 to 2×10^7 cells/ml in YPD at 30°C. Total RNA isolation and Northern analysis were performed as previously described (18). Radiolabeled DNA probes were generated by the random-primed labeling of PCR fragments for *SRG1* (−454 to −123 relative to *SER3* ATG), *SER3* (+111 to +1342), *FLO8* (+1515 to +2326), *STE11* (+1868 to +2110), *SYF1* (+2032 to +2525), and *SCR1* (−163 to +284) that were amplified from genomic DNA. RNA levels were quantified using a PhosphorImager (Instant Imager, Packard Instrument Co.) and normalized to the *SCR1* loading control.

Dilution growth assays. Cells were grown at 30°C to saturation and then washed twice with water. Starting at 1×10^8 cells/ml, cultures were serially diluted 10-fold. Three μl of each dilution was spotted onto YPD, YPraff, and YPgal media and incubated at 30°C for the indicated number of days.

Nucleosome scanning assay. Nucleosome scanning assays were performed as previously described (33) on cells grown at 30°C to 2×10^7 cells/ml in YPD. For each of the 38 *SER3* primer pairs, the amount of template protected from digestion by micrococcal nuclease was calculated as a ratio between micrococcal nuclease (MNase)-digested and undigested samples and then normalized to the amount of MNase-protected control template (*GAL1* NB) that is located within a well-positioned nucleosome in the *GAL1* promoter (9, 30).

ChIP. For H3 ChIP of galactose-induced *GAL1*, cells were grown in YPraff to approximately 1.5×10^7 cells/ml and then 2% galactose was added for 1 h. For all other ChIP experiments, cells were grown in YPD at 30°C to 1×10^7 to 2×10^7 cells/ml. Chromatin was prepared as previously described (85). Histone H3 was immunoprecipitated by incubating sonicated chromatin overnight at 4°C with 5 μl anti-histone H3 antibody (ab1791; Abcam) and followed by the addition of IgG-Sepharose beads (GE Healthcare) for 2 h at 4°C. Dilutions of input DNA and immunoprecipitated DNA were analyzed by quantitative PCR (qPCR). Primer sets that amplify the following regions were used to measure H3 occupancy by qPCR: *PYK1* (5′, +62 to +164; 3′, +1173 to +1279), *PMA1* (5′, +691 to +794; 3′, +1689 to +1791), *ADH1* (+845 to +943), *CYC1* (+122 to +217), *TUB2* (5′, +105 to +202; 3′, +1083 to +1189) and *GAL1* (5′, +79 to +175; 3′, +1366 to +1487). Histone H3 ChIP signals for each gene were normalized to a No ORF control template, which is located within a region of chromosome V that lacks open reading frames (51).

qPCR. All qPCR data for the nucleosome scanning assays were obtained by using an ABI 7300 real-time PCR system, SYBR green reagents (Fermentas), and primer sets tiling *SER3* (33). All qPCR data for the ChIP assays were obtained using a StepOnePlus real-time PCR system, SYBR green reagent (Fermentas), and the indicated primers. Calculations were performed using the methodology of Pfaffl (75).

RESULTS

Identification of histone mutations that derepress *SER3*. We have previously shown that the transcription of *SRG1* ncDNA represses *SER3* transcription by assembling nucleosomes across the overlapping *SER3* promoter (33). Although several studies have identified factors that contribute to transcription-coupled nucleosome reassembly, including the Spt6/Spn1(Iws1) and FACT transcription elongation complexes and the HMG-like Spt2 protein (33, 91), less is known about how

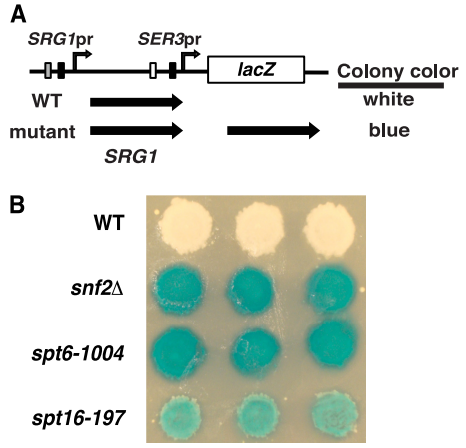

FIG. 1. Detection of *SER3* derepression from an ectopically expressed *SER3pr-lacZ* reporter. (A) Diagram of *SER3pr-lacZ* reporter. The *LYP1* ORF was replaced by *SER3* 5′ UTR sequence from −713 to −1, including *SRG1* and its promoter, fused to the *lacZ* ORF. Block arrows beneath the diagram indicate the expected *SRG1* and *SER3-lacZ* transcripts in wild-type and mutant strains grown in serine-rich medium (YPD). The table on the right indicates the expected results for an X-Gal overlay assay for wild-type and mutant strains. (B) X-Gal overlay detects *SER3pr-lacZ* derepression in *snf2*Δ (YJ924, YJ982, and YJ983), *spt6-1004* (YJ977, YJ978, and YJ979), and *spt16-197* (YJ974, YJ975, and YJ976) strains that is not seen in wild-type strains (YJ921, YJ980, and YJ981). Cells were grown on YPD medium and incubated with X-Gal for 32 min.

histone proteins themselves contribute to this mechanism. To investigate the role of histones in *SER3* repression, we performed a comprehensive genetic screen to identify mutations in the genes encoding histones H3 and H4 that derepress *SER3* in normal repressing conditions. We constructed an *SER3pr-lacZ* reporter gene to monitor *SER3* repression using a standard β-galactosidase assay by replacing the *SER3* coding sequence with the coding sequence for the *Escherichia coli lacZ* gene (Fig. 1A; also see Materials and Methods). Since the deletion of the *SER3* gene leads to increased expression from the *SER3* promoter (data not shown), the *SER3pr-lacZ* reporter is integrated at *LYP1*. *LYP1* encodes a lysine permease, and its depletion has no effect on *SER3* regulation (data not shown). A β-galactosidase overlay assay demonstrates that the *SER3pr-lacZ* reporter effectively detects mutations that are known to derepress endogenous *SER3* (Fig. 1B).

Using this *SER3pr-lacZ* reporter strain in a modified SGA strategy, we systematically screened a library of histone H3 and H4 mutants (kindly provided by J. Boeke) for those that display increased β-galactosidase activity compared to a control strain expressing wild-type histones. The histone H3 and H4 mutant library consists of 422 alleles, including alanine substitutions of all non-alanine residues, serine substitutions of all alanines, a number of additional substitutions that exploit the physical characteristics of several side chains (for example, lysine-to-arginine mutations maintain charge) or that mimic different posttranslational modifications (for example, lysine-to-glutamine mutations to mimic acetylation), and a series of histone tail deletions (20). In these strains, one copy of the histone genes, *HHT1-HHF1*, is deleted while the second copy, *HHT2-HHF2*, has been replaced with a synthetic version of these genes (*HHTS-HHFS*) that has been mutated (20). In the initial phase of the screen, we identified 139 histone H3 and H4 mutants that increase β-galactosidase activity. We then performed Northern analyses, in duplicate, on all 139 mutants to assay changes to the endogenous *SER3* and *SRG1* expression levels (see Table S2 at http://www.pitt.edu/~martens/supplemental_data/). Note that strains expressing a single copy of the synthetic histone (*HHTS-HHFS*) module increase *SER3* levels 3-fold compared to a strain with two wild-type copies of the histone H3 and H4 genes. This result is consistent with previous results indicating that *SER3* expression is sensitive to histone gene dosage (97). Therefore, the changes in RNA levels in the histone mutants were normalized to a control strain expressing a single copy of the synthetic histone genes (*HHTS-HHFS*). Of the initial 139 mutants, 12 mutants resulted in at least a 4-fold increase in *SER3* mRNA levels compared to the *HHTS-HHFS* control, while another 54 mutants resulted in more modest increases in *SER3* mRNA levels (1.5- to 4-fold). These data further emphasize the important role of chromatin in *SER3* regulation.

Of the 12 histone mutants that strongly derepress *SER3*, only alanine substitutions of histone H3 H39 and R72 significantly decrease *SRG1* RNA levels (see Table S2 at http://www.pitt.edu/~martens/supplemental_data/). Therefore, histone H3 H39 and R72 may contribute to *SER3* repression indirectly by impairing *SRG1* transcription. In contrast, *SRG1* RNA levels are either unaffected or slightly elevated by the other 10 mutants, which include histone H3 K122A, K122R, K122Q, Q120A, V117A, R49A, V46A and histone H4 R36A, S47D, and I46A (Fig. 2A and B). Since previous studies have shown that *SER3* is derepressed when histone H4 is depleted (97), we also tested the effect of these mutants on histone H3 and H4 protein levels by Western analysis (Fig. 2C). All 10 histone mutant strains express levels of histone H3 and H4 indistinguishable from a wild-type *HHTS-HHFS* strain. Taken together, these data identify eight amino acids, five in histone H3 and three in histone H4, that are strongly required to repress *SER3* by a mechanism that is independent of the regulation of *SRG1* transcription.

Recent large-scale phenotypic analyses have reported a range of phenotypes for mutations that alter these eight residues (Table 1) (28, 39, 67, 81, 83). Most notably, substitutions of some of these residues confer phenotypes linked to defects in chromatin structure, including telomeric silencing defects and the suppression of a *LYS2* transcriptional defect caused by a Ty retrotransposon insertion (*SPT*$^-$ phenotype of the *lys2-128*δ allele) (86). Of particular note is the *SPT*$^-$ phenotype; a phenotype that is caused by mutations that allow transcription to initiate from the Ty promoter inserted within the early coding region of *LYS2.* Mutations in several genes encoding transcription-related factors that cause *SPT*$^-$ phenotypes, including the *SPT6* and *SPT16* histone chaperones, also derepress *SER3* (17, 33, 63). The lack of correlation between *SER3* derepression and *SPT*$^-$ phenotype for these histone mutants suggests that the transcription interference mechanisms that regulate *SER3* and *lys2-128*δ are distinct.

Examination of the X-ray crystal structure indicates that all eight of these histone residues track DNA on the lateral surface of the nucleosome (Fig. 3) and therefore are unlikely to be involved in the general stability of the histone octamer. Five of

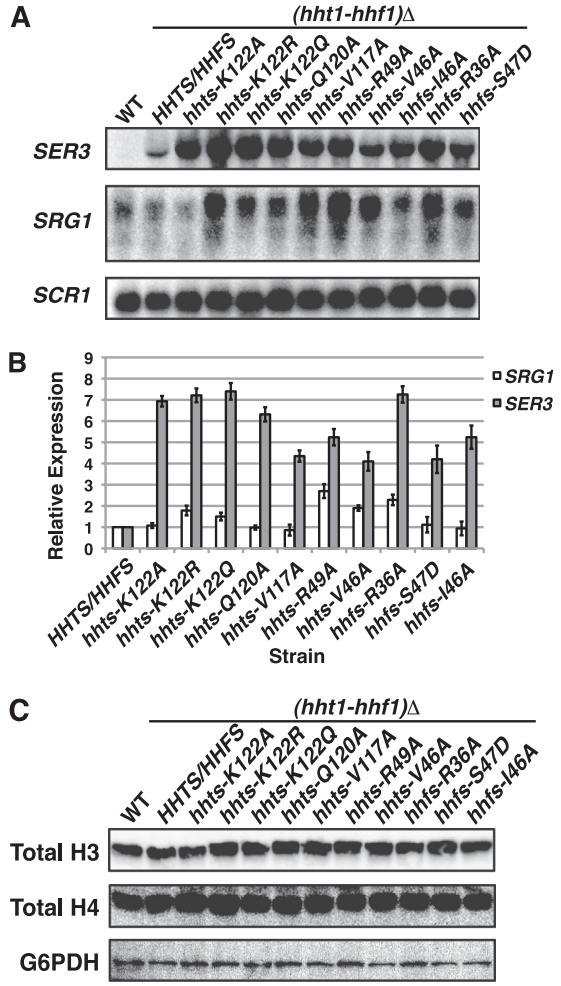

FIG. 2. Single-amino-acid substitutions in histones H3 and H4 strongly derepress *SER3*. (A) Northern blot analysis examining the effect of histone mutants on *SER3*, *SRG1*, and *SCR1* (loading control). Total RNA was isolated from a wild-type strain (FY4) and derivatives of JDY86 expressing either synthetic or wild-type copies of histone H3 and H4 (*HHTS*/*HHFS*) or mutants *hhts-K122A*, *hhts-K122R*, *hhts-K122Q*, *hhts-Q120A*, *hhts-V117A*, *hhts-R49A*, *hhts-V46A*, *hhfs-I46A*, *hhfs-R36A*, and *hhfs-S47D* that were grown to a density of 1×10^7 to 2×10^7 cells/ml in YPD at 30°C. (B) Quantitation of Northern analyses. *SRG1* and *SER3* RNA levels for the histone mutants are normalized to the *SCR1* loading control and are relative to the *SRG1* and *SER3* RNA levels measured in control *HHTS-HHFS* strains (arbitrarily set to 1). Each bar represents the means ± standard errors of the means (SEM) from three independent experiments involving JDY86 derivatives (which are shown in panel A) and related strains generated by genetic crosses (YJ925 to YJ946). (C) Western analysis examining the effect of histone mutants on total histone H3 and histone H4 protein levels. Strains expressing the indicated histone alleles were grown to $\sim 3 \times 10^7$ cells/ml in YPD at 30°C. Proteins were extracted with trichloroacetic acid and subjected to Western analysis using anti-H3, anti-H4, and anti-G6PDH (loading control). Similar results were obtained for three independent experiments using the strains listed in panel B.

the eight amino acids, histone H3 K122, Q120, and V117 and histone H4 S47 and I46, cluster at the nucleosome dyad. These residues are part of the L1L2 loop region of the $(H3\text{-}H4)_2$ tetramer that contacts DNA on either side of the nucleosome dyad (61). Interestingly, two of these residues, histone H3 K122 and H4 S47, are highly conserved among eukaryotes and are targets for posttranslational modifications, although the functional significance of these modifications is not known. H3 K122 has been shown to be methylated in mice and humans (74, 88) and acetylated in bovine (99); however, similar modifications in yeast have not yet been detected (32, 76). The phosphorylation of H4 S47 has been detected in yeast (32, 40, 99), and a phenotype associated with this residue is dependent on the substitution: S47A and S47E both confer sensitivity to HU, while S47D is not sensitive (40). Interestingly, we found that *SER3* expression also is affected differentially depending on the substitution at H4 S47. In this case, an H4 S47A mutant more weakly derepresses *SER3* than an S47D mutant (see Fig. S1 at http://www.pitt.edu/~martens/supplemental_data/). The other three residues, R49 and V46 on the N-terminal α-helix of histone H3 and R36 on α-helix 1 of histone H4, are located near the DNA entry/exit sites on the nucleosome (94). Although these eight histone residues share similar locations on the nucleosome and all are required to repress *SER3*, it is unclear whether they function together in a common mechanism.

Nine of 10 histone mutants that strongly derepress *SER3* do not confer a *sin* phenotype. Previous studies have genetically identified mutations in several genes, including the histone genes, that suppress transcriptional defects caused by the loss of a component of the Swi/Snf chromatin remodeling complex (36, 38, 54, 57). The *sin* mutations (for Swi/Snf independent) identified within the histone H3 and H4 genes had the following amino acid substitutions: H3 T118I, H3 R116H, H3 E105K, H4 R45H/C, and H4 V43I. Three of these residues, H3 T118, H3 R116, and H4 R45, lie within the L1L2 loop at the nucleosome dyad along with six of the eight H3 and H4 residues that derepress *SER3* (70). Moreover, mutations that change these amino acids to alanines confer lethality in *S. cerevisiae*, resulting in their absence from the library of histone mutations that we used for our screen (20). Therefore, we tested each of the histone mutants that confer strong *SER3* derepression for a *sin* phenotype using a previously described growth assay (36). Wild-type, *snf2*Δ, and *snf2*Δ strains expressing one wild-type copy of histone H3 and H4 genes (*HHT1-HHF1*) and having

TABLE 1. Known histone mutant phenotypes

Amino acid change	Known phenotype[a]
H3 K122A	Telomeric silencing, K56 hyperacetylation, zeocin^s
H3 K122R	Telomeric silencing
H3 K122Q	Telomeric silencing, K56 hyperacetylation, zeocin^s
H3 Q120A	Telomeric silencing, *SPT*$^-$
H3 V117A	Telomeric silencing, *SPT*$^-$
H3 R49A	Ribosomal and telomeric silencing, HU^s, ts, MMS, 6AU^s, *SPT*$^-$
H3 V46A	MMS
H4 R36A	Ribosomal and telomeric silencing, cs, ts, HU^s, MMS, *SPT*$^-$
H4 S47D	
H4 I46A	Ribosomal and telomeric silencing, ts

[a] Phenotypes were obtained from http://www.histonehits.org (39), English et al. (28), Matsubara et al. (67), and Sakamoto et al. (81). HU^s, sensitivity to hydroxyurea; cs, cold sensitivity; ts, temperature sensitivity; 6AU^s, sensitivity to 6-azauracil; MMS, sensitivity to methyl methanesulfonate; *SPT*$^-$, suppressor of Ty insertion phenotype.

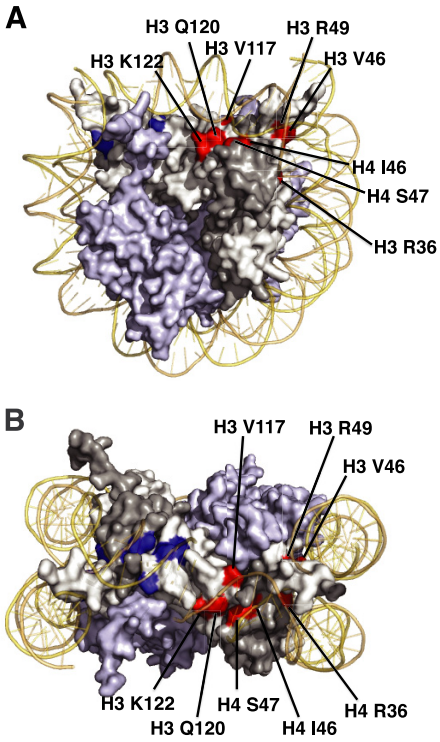

FIG. 3. Mapping of the eight H3/H4 histone residues that strongly derepress *SER3* onto the yeast nucleosome crystal structure. (A) A surface representation of the yeast nucleosome core particle viewed down the DNA superhelical axis. Histone proteins are color coded as follows: H3 in white, H4 in gray, and H2A/H2B in purple. The DNA helix is shown in yellow. The five histone H3 and three histone H4 residues required for *SER3* repression are highlighted in red (first H3-H4 dimer) and blue (second H3-H4 dimer). (B) Rotation of the view in panel A by 90° around the horizontal axis revealing the lateral surface surrounding the nucleosome dyad. These images were generated by Pymol (Protein Data Bank number 1ID3).

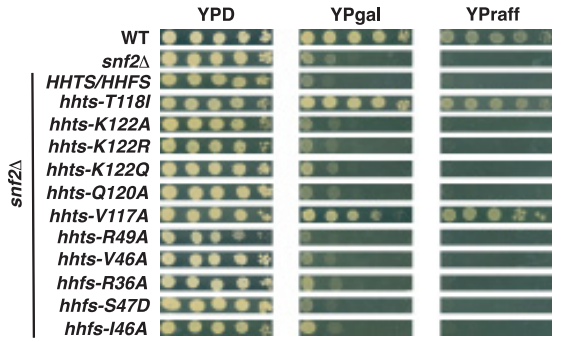

FIG. 4. Analysis of histone mutants for *sin* phenotype. Wild-type (FY4), *snf2*Δ (YJ112), *HHTS-HHFS snf2*Δ (YJ1049), *hhts-T118I snf2*Δ (YJ1081), *hhts-K122A snf2*Δ (YJ1051), *hhts-K122R snf2*Δ (YJ1054), *hhts-K122Q snf2*Δ (YJ1057), *hhts-Q120A snf2*Δ (YJ1060), *hhts-V117A snf2*Δ (YJ1063), *hhts-R49A snf2*Δ (YJ1066), *hhts-V46A snf2*Δ (YJ1069), *hhfs-R36A snf2*Δ (YJ1072), *hhfs-S47D snf2*Δ (YJ1075), and *hhfs-I46A snf2*Δ (YJ1078) were grown to saturation in YPD at 30°C. Three μl of 10-fold serial dilutions were spotted onto solid YPD, YPgal, and YPraff media and incubated for 3 days. A representative growth assay of three biological replicates that produced equivalent results is shown.

the second copy replaced by a synthetic copy of these genes that is either wild type (*HHTS-HHFS*) or contains 1 of the 10 mutations (for example, *hhts-K122A-HHFS*) were spotted on YPD, YPraff, and YPgal (Fig. 4). As expected, an *snf2*Δ strain failed to grow on YPraff and YPgal media, and this growth defect is suppressed by the *sin* mutant allele *hhts-T118I*. Of the 10 mutant histone alleles that strongly derepress *SER3*, only one, *hhts-V117A*, confers a *sin* phenotype similar to that of the T118I mutant. V117 lies between T118 and a second residue that confers a strong *sin* phenotype, suggesting that these three amino acids are, at least in part, functionally related. Importantly, these data indicate that, with the exception of V117A, the histone mutations that confer strong *SER3* repression appear to be distinct from those that confer a *sin* phenotype.

Effect of histone mutants on nucleosome occupancy across the *SER3* promoter. To examine the effect of these 10 histone mutants on nucleosome occupancy at *SER3*, we performed nucleosome scanning assays (Fig. 5) as previously described (33). MNase protection across *SER3* was normalized to the protection of a well-studied, nucleosome-bound region of the *GAL1* promoter whose digestion by MNase is unaffected by these histone mutants (see Materials and Methods for details; also see Fig. S2 at http://www.pitt.edu/~martens/supplemental_data/). Compared to control *HHTS-HHFS* strains, protection from MNase digestion was reduced across the *SRG1* transcribed region in all 10 histone mutants. MNase protection was more dramatically reduced in the H3 K122 (R/Q), H3 Q120A, H3 V117A, H4 R36A, and H4 I46A mutants than in the H3 K122A, H3 R49A, H3 V46A, and H4 S47D mutants. When plotted against our Northern analysis data, MNase protection across the *SRG1* transcription unit correlates well with *SER3* repression (Fig. 6). As a control, we show that an H3 K115A mutant, which has no effect on *SER3* expression (data not shown), does not alter the nucleosome architecture across the *SER3* locus (Fig. 5J). We performed histone H3 ChIP assays in these strains to complement our MNase experiments (Fig. 7). In strains showing a dramatic reduction in MNase protection, we detected a significant loss of histone H3 occupancy over the *SER3* promoter compared to that of the adjacent *AIM9* ORF. For the mutants having a more modest loss of MNase protection, we were unable to detect a significant decrease in histone H3 occupancy across the *SER3* promoter. Taken together, our data identify eight histone residues that contribute to *SER3* repression by facilitating *SRG1* transcription-dependent nucleosome occupancy across the *SER3* promoter.

Histone H3 V46 and R49 are required to repress cryptic intragenic transcription. A previous study has shown that transcription from cryptic promoters located within protein-coding genes is repressed by maintaining normal chromatin structure across these regions during transcription (47). Mutations that impair a number of factors that alter either nucleosome assembly or posttranslational modifications to histone proteins, such as the Spt6 and Spt16 transcription factors and the Set2 histone methyltransferase, have been shown to allow cryptic transcription (14, 15, 46, 47, 76, 78). In addition, several amino acid substitutions in histone H3 were identified in a genetic selection for mutations that promote cryptic transcription (15, 23–25, 77, 100). To test whether the 10 histone mutants that reduce nucleosome occupancy across the *SER3* promoter have

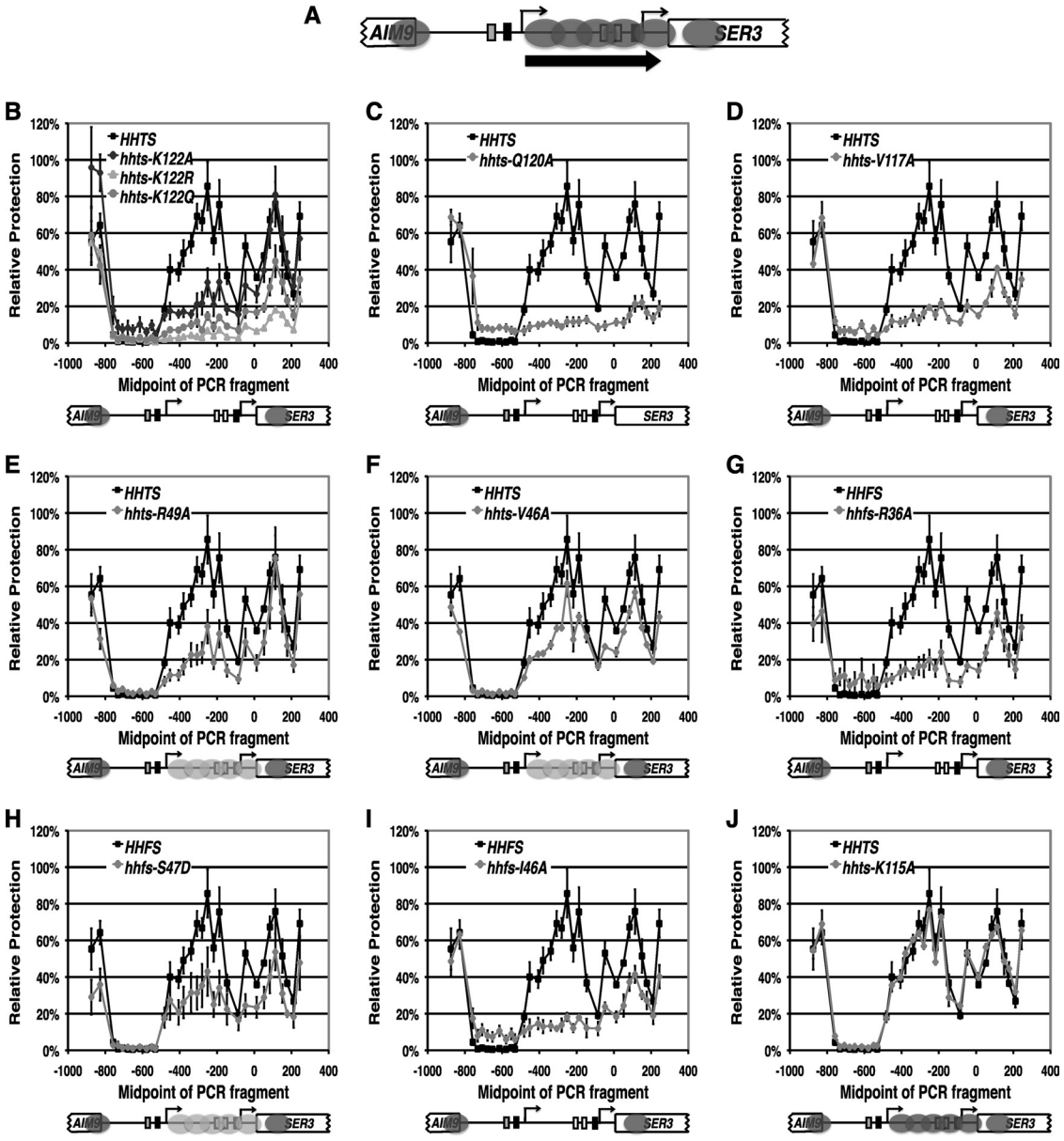

FIG. 5. Effect of histone mutants on nucleosome positions at *SER3*. (A) Diagram of the *SER3* locus. The gray ovals mark the position of nucleosomes when wild-type cells are grown in *SER3*-repressing conditions (YPD). The block arrow indicates *SRG1* transcription. (B to J) Nucleosome scanning assays were performed on (*hht1-hhf1*)Δ strains expressing either synthetic wild-type copies of histone H3 and H4 (*HHTS* and *HHFS*) or the indicated histone mutant alleles. Cells were grown in YPD medium at 30°C. Each experiment was done in triplicate using one set of strains from the original histone mutation library (JDY86 derivatives) and two additional sets of strains generated by genetic crosses (YJ925 to YJ946). MNase protection across the *SER3* locus relative to a positioned nucleosome within the *GAL1* promoter was determined by qPCR, and the means ± SEM for the three replicates is plotted at the midpoint for each PCR product. Shown below each graph is a diagram of the *SER3* locus indicating the positions of nucleosomes (gray ovals) extrapolated from the MNase protection data for each histone mutant. The light gray ovals are indicative of less dramatic reductions in MNase protections compared to that of the wild-type control shown in panel A.

a more general defect in transcription-dependent nucleosome assembly, we determined whether these mutants permit cryptic intragenic transcription by Northern analysis (Fig. 8A). We examined the production of previously observed aberrant transcripts that initiate within the protein-coding sequences of three genes, *FLO8*, *STE11*, and *SYF1* (15, 47). Two of the 10 histone mutants, H3 R49A and H3 V46A, produce aberrant transcripts similar to those previously described for an *spt6-1004* mutant (47). Therefore, histone H3 R49 and V46 may have a more general role in regulating chromatin structure during transcription.

The Set2-dependent methylation of histone H3 K36 has been shown to play a role in the repression of cryptic transcription by recruiting and/or directing activity of the Rpd3S histone deacetylase complex to remove acetylation marks from the amino-terminal tails of histone H4 after the passage of RNA Pol II (14, 15, 22, 46, 48, 76, 78, 98). The resulting hypoacetylated nucleosomes are thought to protect recently

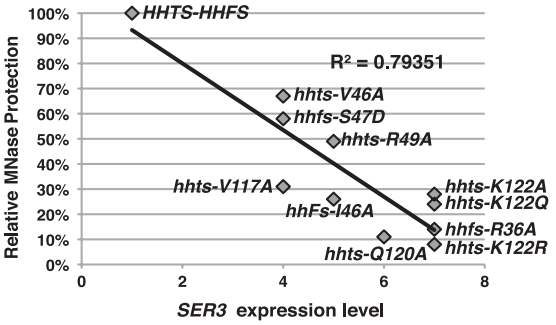

FIG. 6. Correlation between MNase protection of *SRG1* and *SER3* expression. The extent of MNase protection across the *SRG1*-transcribed unit for wild-type and histone mutant strains (Fig. 4) was plotted against the relative level of *SER3* expression in these strains as determined by Northern analysis (Fig. 1). Change in MNase protection was calculated by taking the area under the curve over the *SRG1* transcription unit in the histone mutant strain and subtracting this from the area under the curve over the *SRG1* transcription unit in the wild-type control. All values were normalized to those of strains expressing synthetic copies of wild-type histone H3 and H4 genes (*HHTS-HHFS*), where the MNase protection across *SRG1* was set to 100% and *SER3* expression was set to 1. The line of best fit and R^2 values were determined by linear regression.

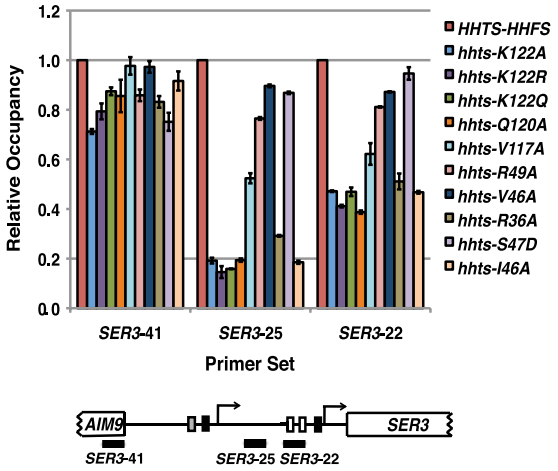

FIG. 7. Relative occupancy of histone H3 in histone mutants over *SER3*. Histone H3 ChIP was performed on chromatin isolated from (*hht1-hhf1*)Δ strains expressing *HHTS-HHFS* alleles (JDY86, YJ927, and YJ928) or the indicated histone mutant alleles (JDY86 derivative, YJ925, YJ926, and YJ930 to YJ946) that were grown in YPD at 30°C. The amount of immunoprecipitated DNA was determined by qPCR as a percentage of the input material normalized to a control region in chromosome V and represent the means ± SEM of three experiments. Histone H3 occupancy at each genomic location, determined for the strains expressing wild-type histone H3 and H4, was arbitrarily set to 1. Below the graph is a schematic of *SER3*, with black bars corresponding to the regions amplified by qPCR.

transcribed DNA from the binding of transcription factors and intragenic transcription. Therefore, we tested whether the histone H3 R49A and V46A mutants were defective in H3 K36 methylation by Western analysis (Fig. 8B). In agreement with our cryptic intragenic transcription data, the H3 R49A and H3 V46A substitutions dramatically reduced global levels of histone H3 K36 di- and trimethylation. These defects were specific to histone H3 K36, as the methylation at two other sites on histone H3, K4 and K79, were unaffected (Fig. 8C and D). In contrast, the remaining eight histone mutants had little to no effect on the methylation state of all three of these histone H3 lysines. These data suggest that histone H3 R49 and V46 repress cryptic intragenic transcription by maintaining normal Set2-dependent methylation of histone H3 K36. However, since we have shown previously that *SER3* repression is independent of the methylation states of histone H3 K4, K36, and K79 (33), histone H3 R49 and V46 likely repress *SER3* by a mechanism independent from their involvement in histone H3 K36 methylation.

Effect of histone mutants on histone H3 occupancy at other genes. Several studies have indicated that cryptic intragenic transcription is more common within genes that are lowly transcribed (15, 60). In contrast, transcription run-on and RNA Pol II ChIP experiments have indicated that *SRG1* is a highly transcribed region of the yeast genome (64, 65). Therefore, we performed histone H3 ChIP assays to test whether these 10 histone mutants cause a more general defect in nucleosome occupancy at other highly transcribed yeast genes (Fig. 9). At three highly transcribed genes, *PMA1* (100 mRNA/h), *PYK1* (95 mRNA/h), and *ADH1* (125 mRNA/h) (37 and data obtained from http://web.wi.mit.edu/young/pub/orf_transcriptome.txt), histone H3 levels were reduced in 7 of the 10 mutants corresponding to those that show the strongest effects on nucleosome occupancy at the *SER3* promoter [H3 K122(A/R/Q), H3 Q120A, H3 V117A, H4 R36A, and H4 I46A]. The only exception is the H3 V117A mutant, which results in reduced histone occupancy at *PMA1* and *PYK1* but not *ADH1*. Conversely, histone H3 occupancy at three lowly transcribed genes, *GAL1* (repressed), *TUB2* (12 mRNA/h), and *CYC1* (10 mRNA/h) (37), was either unaffected or slightly increased in 9 of the 10 histone mutants. The only exception was the H4 S47D mutant, where we found a surprising 2-fold increase in histone H3 levels toward the 3′ end of all three lowly transcribed genes. Furthermore, inducing high levels of *GAL1* expression by growing cells in the presence of galactose uncovered histone H3 occupancy defects in the histone mutants similar to those detected at the other highly transcribed genes (see Fig. S3 at http://www.pitt.edu/~martens/supplemental_data/). Taken together with our analysis at *SRG1*, we have identified a new set of histone mutants that are defective for transcription-coupled nucleosome occupancy specifically at highly transcribed genes. These data support recent studies which suggest that high levels of transcription result in nucleosome displacement, whereas regions with low levels of transcription maintain nucleosome occupancy (21, 41, 42, 45, 50, 53, 55, 56, 80, 82, 92).

DISCUSSION

Previously, we reported that the transcription of *SRG1* ncDNA represses *SER3* transcription by assembling nucleosomes across the promoter of *SER3*, which interfere with transcription factor binding (33). In this work, we systematically tested the contribution of all nonessential amino acids in histones H3 and H4 to *SER3* repression. We identified changes of 52 histone H3 and H4 residues that derepress *SER3* where the increase in *SER3* RNA levels ranged from modest (1.5-fold) to strong (7-fold), further emphasizing the prominent role of nucleosomes in *SER3* repression by the transcription of *SRG1*

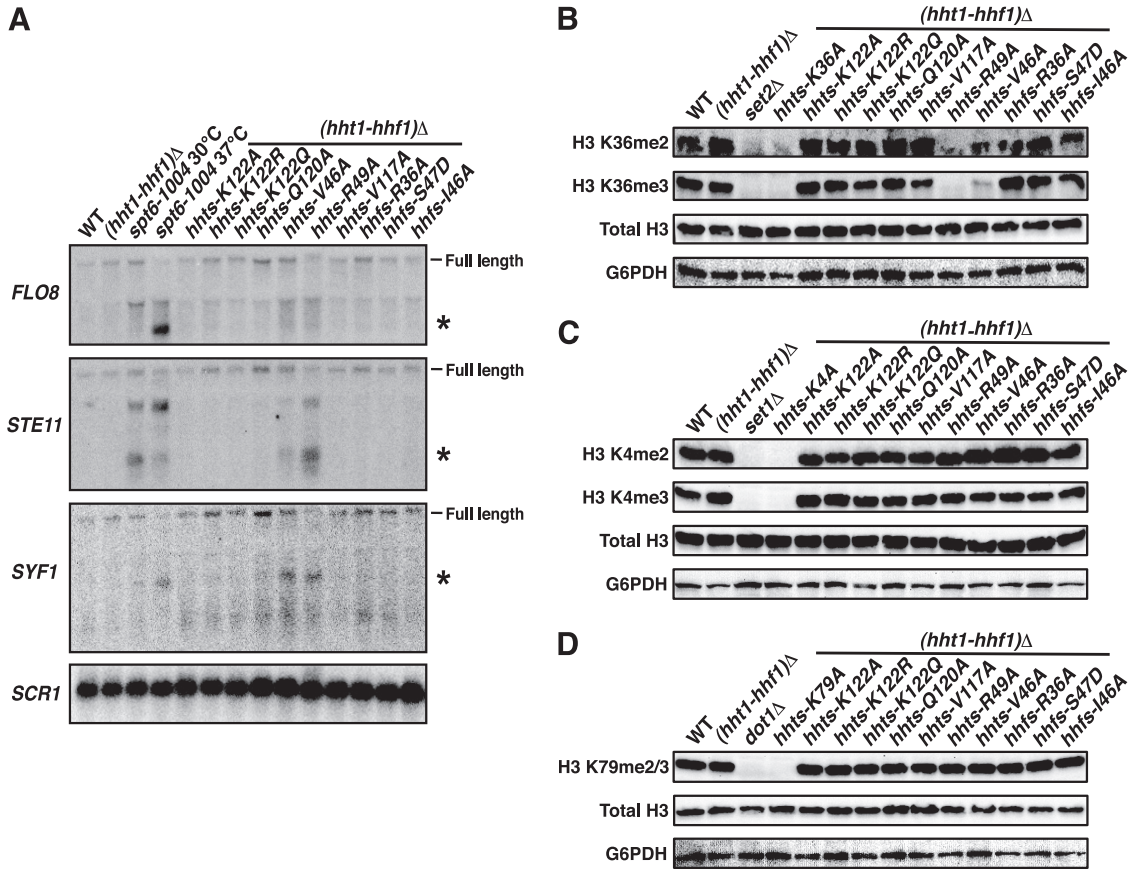

FIG. 8. Effect of histone mutants on cryptic intragenic transcription and posttranslational histone modifications. (A) Northern analysis of *FLO8*, *STE11*, and *SYF1* for cryptic intragenic transcription. Total RNA was isolated from (*hht1-hhf1*)Δ strains that express either synthetic wild-type copies of histone H3 and H4 or the indicated histone mutant alleles (JDY86 derivatives). Strains wild type for both copies of histone H3 and H4 (WT) and expressing either a normal copy of *SPT6* (WT) or the *spt6-1004* mutant allele were included as negative and positive controls for cryptic transcription. All strains were grown in YPD at 30°C except for the *spt6-1004* mutant, which also was shifted to 37°C for 60 min as indicated. Cryptic transcripts for each gene are marked with an asterisk. *SCR1* serves as a loading control. (B to D) Western analyses of posttranslational histone modifications. Whole-cell extracts were prepared from wild-type (FY4), *set2*Δ (KY1716), *set1*Δ (KY1755), and *dot1*Δ (KY1907) strains and (*hht1-hhf1*)Δ strains expressing either synthetic wild-type copies of histone H3 and H4 or the indicated histone mutant alleles (JDY86 derivatives) that were grown in YPD at 30°C. Immunoblots of WCEs were probed with H3 K36 (B), H3 K4 (C), or H3 K79 (D) methyl-specific antibodies. Immunoblots of total H3 and G6PDH are provided as loading controls. Similar results were observed for two distinct sets of strains (YJ925 to YJ946).

ncDNA. Of particular interest were substitutions of eight histone residues that most strongly derepress *SER3* (>4-fold) without reducing *SRG1* transcription. All eight residues are located on the lateral surface of the nucleosome; five residues, H3 K122, H3 Q120, H3 V117, H4 I46, and H4 S47, track the DNA binding surface near the nucleosome dyad, and three residues, H3 R49, H3 V46, and H4 R36, are near the DNA entry/exit point (20, 94). Our nucleosome scanning experiments show that substitutions of these nonessential residues impair nucleosome occupancy at the *SER3* promoter, thus resulting in *SER3* derepression. Moreover, substitutions of all but three of these residues reduce histone H3 occupancy over the open reading frames of highly transcribed, but not lowly transcribed, genes. Of note, the three exceptions, H3 R49A, H3 V46A, and H4 S47D mutations, more modestly reduce MNase protection at *SER3* compared to the other mutants, which was not evident by histone H3 ChIP. Therefore, these three mutants also are likely to cause modest effects on nucleosome occupancy at other highly transcribed genes that may only be detected by the more sensitive nucleosome-scanning assay. Taken together, our data reveal a class of histone residues that are required for nucleosome occupancy specifically at locations of high transcription activity with a greater dependence on H3 K122, Q120, V117, and H4 R36 and I46 than on H3 R49, V46, and H4 S47.

Results from this study and others strongly suggest that the histone mutants that strongly derepress *SER3* are defective for the *SRG1* transcription-dependent deposition of nucleosomes over the *SER3* promoter. First, we had previously established that the nucleosome occupancy of the *SER3* promoter is dependent on *SRG1* transcription and the Spt6 and Spt16 histone chaperones, which are required to restore nucleosome occupancy after the passage of RNA Pol II (33). Second, nucleosome occupancy across other highly transcribed genes, but not lowly transcribed genes, is reduced by mutations that alter these eight histone residues. Finally, at *GAL1* we showed that these histone residues are required to maintain nucleosome occupancy across its open reading frame in cells that were

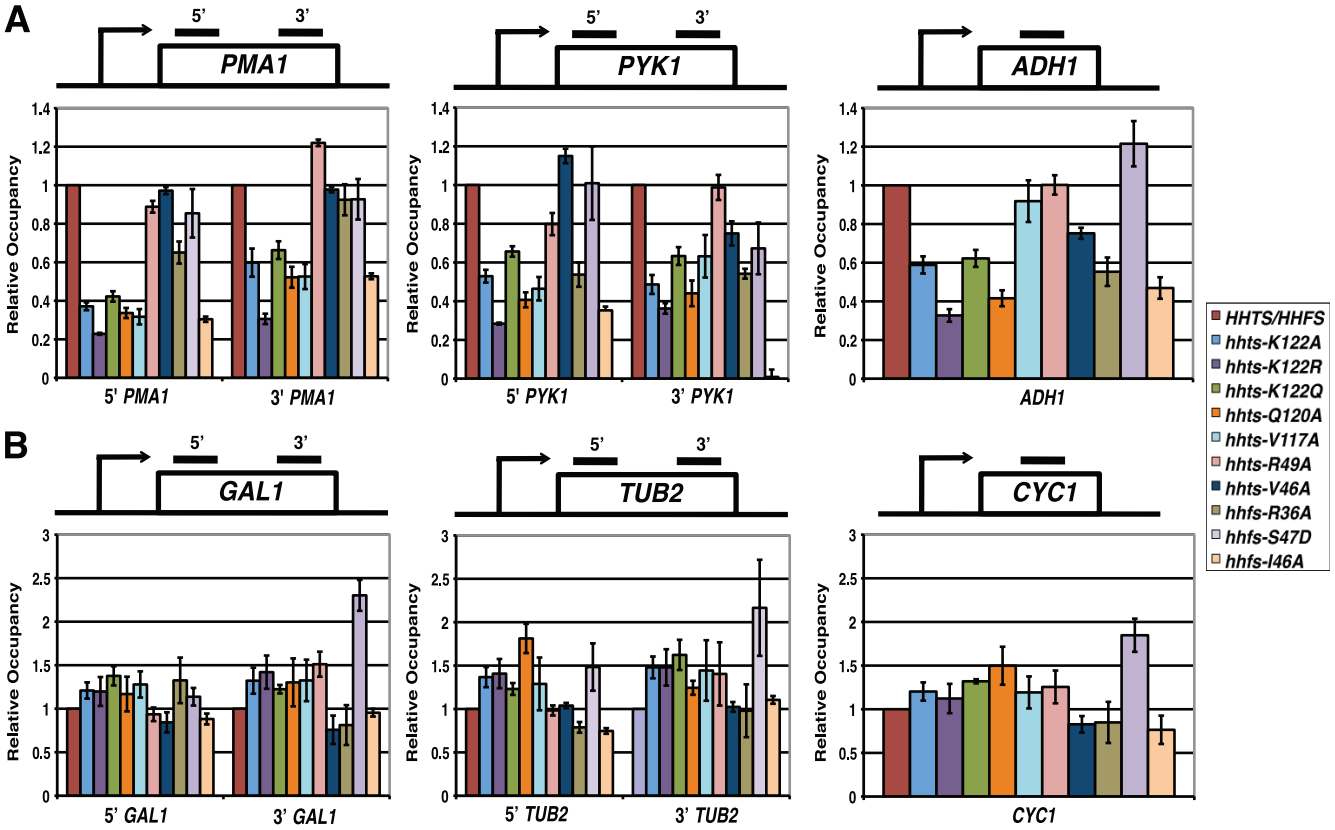

FIG. 9. Effect of histone mutants on histone H3 occupancy over the coding regions of a subset of yeast genes. (A) Histone H3 ChIP analysis was performed on chromatin prepared from (*hht1-hhf1*)Δ strains expressing *HHTS-HHFS* alleles (JDY86, YJ927, and YJ928) or the indicated histone mutant alleles (JDY86 derivative, YJ925, YJ926, and YJ930 to YJ946) that were grown in YPD at 30°C. Histone H3 occupancy was measured within the coding region of three highly transcribed genes: *PMA1*, *PYK1*, and *ADH1*. The regions assayed by qPCR are marked with the black bars in the diagram provided for each gene. All values are normalized to a control region in chromosome V and represent the means ± SEM of three experiments. Histone H3 occupancy at each genomic location determined for the strains expressing wild-type histone H3 and H4 was arbitrarily set to 1. (B) Histone H3 occupancy at three lowly transcribed genes, *GAL1*, *TUB2*, and *CYC1*, was determined as described for panel A.

grown in galactose (*GAL1* on) but not glucose (*GAL1* off), directly demonstrating that the effect of these mutants is transcription dependent.

An alternative possibility is that the rate or magnitude of chromatin remodeling at the *SER3* promoter that is required for *SER3* activation is enhanced in these histone mutants. If this were the case, nucleosome occupancy at the *SER3* promoter should be restored in the absence of the chromatin remodeling factor and the sequence-specific activator that is responsible for its recruitment. However, we have found that the deletion of the *SER3* UAS, which severely impairs *SER3* expression in the absence of *SRG1* transcription, does not restore nucleosome occupancy to the *SER3* promoter (33 and data not shown). Although we cannot completely discount this possibility, these results are more consistent with the histone mutations being defective for *SRG1* transcription-dependent deposition of nucleosomes across the *SER3* promoter.

Interestingly, changes of six of the eight histone residues required for *SER3* repression do not permit cryptic intragenic transcription, which also is controlled by transcription-dependent chromatin architecture (14, 47, 60). Based on our ChIP studies of other lowly transcribed genes, the absence of cryptic intragenic transcription is likely attributable to the low transcription frequency of the *FLO8*, *STE11*, and *SYF1* genes that were used in this assay (37). The fact that histone H3 V46A and R49A mutants permit cryptic initiation is likely a consequence of the reduced di- and trimethylation of H3 K36 that we observe in these mutants. The Set2-dependent methylation of histone H3 K36 has been shown previously to play an important role in preventing cryptic intragenic transcription (22–24, 77). However, evidence from this work and others strongly suggests that the role of H3 V46 and R49 in promoting K36 methylation is distinct from their role in *SER3* repression. First, the alteration of these two residues does not alter nucleosome occupancy across lowly transcribed genes. Second, the deletion of the Set2 methyltransferase that is responsible for histone H3 K36 methylation has no effect on *SER3* gene expression (33). Third, substitutions of H3 K36 have little to no effect on *SER3* gene expression (data not shown; also see Table S2 at http://www.pitt.edu/~martens/supplemental_data/). Therefore, H3 V46 and R49 may have multiple roles in maintaining nucleosome integrity during transcription depending on the target gene, i.e., an indirect role by facilitating the Set2-dependent meth-

ylation of lysine 36 on histone H3 or a more direct role in transcription-coupled histone deposition. It will be interesting to determine how histone H3 R49 and V46 functionally relate to other histone residues needed for proper H3 K36 methylation, including the N-terminal tail; histone H4 K44; three H2A residues, I112, L116, and L117; and two H3 residues, R52 and N108 (22–24, 77).

Several of the histone residues that strongly derepress *SER3* cluster at the nucleosome dyad when substituted. Histone residues H3 K122, Q120, and V117 are located within the L2 loop of histone H3, which is juxtaposed to the L1 loop of histone H4 that includes I46 and S47, to form the L1L2 region of the $(H3\text{-}H4)_2$ tetramer that organizes the central two turns of nucleosomal DNA (61). Recent single-molecule studies have indicated that this region makes the largest contribution to the DNA-histone interactions within a nucleosome (34). Several structural features are likely to contribute to the strength of this binding (62). First, the overall structure of the L1L2 loop region at the nucleosome dyad puts it in close proximity to DNA, allowing an extensive series of hydrogen bonds between the main peptide chain amides and the phosphate backbone of DNA. Second, histone H4 R45 extends into the minor groove, where it makes contact with the DNA backbone and also stabilizes the position of H3 T118, which also contacts the DNA backbone. Third, histone H3 R116 forms a salt bridge with H3 D123 and a hydrogen bond with the backbone carbonyl of T118, further stabilizing the L1L2 loop. Interactions between Q120, K121, and K122 protect this salt bridge from solvent and also coordinate a chloride ion. Genetic studies also have indicated the functional significance of this region. In creating the histone mutant library, Dai et al. noted that of the surprisingly small number of alanine substitutions in histone H3 and H4 that cause lethality, many cluster within this L1L2 loop region, including H4 R45, H3 T118, and H3 R116 (20). As noted earlier, other amino acid substitutions of these three residues also were identified as *sin* mutants (36, 38, 54, 57).

How might this class of histone residues affect the transcription-coupled nucleosome occupancy of highly transcribed genes? One possibility is that mutations that alter any one of these eight residues reduce the affinity of the histone octamer for DNA. In one scenario, these mutations increase the mobility of nucleosomes similarly to what has been shown for *sin* mutations located at the nucleosome dyad (29). Nucleosomes containing these histone mutants would be properly reassembled during *SRG1* transcription, but then they are mobilized away from the *SER3* promoter. However, this is not likely to be the case, as only one of our histone mutants, H3 V117A, confers a *sin* phenotype. Moreover, our nucleosome-scanning experiments gave no indication of nucleosome mobility at other locations, including more nucleosomes positioned at the open reading frames of *AIM9* and *SER3* that flank *SRG1* (Fig. 5) and the repressed *GAL1* promoter (see Fig. S2 at http://www.pitt.edu/~martens/supplemental_data/). A more likely scenario is that a reduction in DNA affinity slows nucleosome reassembly after the passage of RNA Pol II. This could account for our contrasting observations between lowly and highly transcribed regions of the genome. At lowly transcribed genes, a nucleosome will have sufficient time to reassemble prior to the passage of the next RNA Pol II, so the density of nucleosomes will not be affected. However, at highly transcribed genes, nucleosomes will be only partially assembled before being disassembled by the next RNA Pol II molecule, resulting in reduced nucleosome occupancy at these genes.

These eight histone residues may contribute to the overall histone-DNA affinity by distinct mechanisms. The alteration of the residues within the L1L2 region at the nucleosome dyad (H3 K122, H3 Q120, H3 V117, H4 I46, and H4 S47) may disrupt this structure, resulting in a reduced number of contacts between histone and DNA backbone in this region. Alternatively, these residues may be affecting a critical function of the essential residues in this region, such as the threonine at position 118 in histone H3 and the arginine at position 45 of histone H4. Interestingly, several *in vitro* studies investigating *sin* mutations, in particular those involving H3 T118 and H4 R45, have provided evidence to support their role in histone-DNA affinity (38, 57, 70). However, with the exception of V117A, substitutions of H3 K122, H3 Q120, H4 I46A, and H4 S47 do not confer a similar *sin* phenotype, suggesting that any role these residues play in DNA affinity is either more moderate or distinct from those of H3 T118 and H4 R45. The other three mutations, histone H3 R49, H3 V46, and H4 R36, are unlikely to affect the L1L2 loop region at the nucleosome dyad but rather may disrupt histone-DNA interactions at the DNA entry/exit within the nucleosome, where H3 R49 also is positioned within the minor groove of DNA.

Several studies have indicated that chromatin at highly transcribed genes is more dynamic than at lowly transcribed genes and therefore is more likely to be dependent on histone chaperones, such as Spt6 and FACT, for rapid nucleosome reassembly (21, 41, 42, 45, 50, 53, 55, 56, 80, 82, 92). Because our eight histone residues also are required for highly transcribed genes but not lowly transcribed genes, an alternative model is that at least a subset of these eight histone residues disrupt the recruitment and/or function of histone chaperones that are directly involved in the transcription-dependent nucleosome assembly. Possible candidates include Spt6, its partner Spn1(Iws1), FACT, or Spt2(Sin1), which all are required for *SER3* repression. Spt6 and Spt16 (a subunit of FACT) have been shown previously to interact with both histone H3 and with assembled nucleosomes (5, 7, 31, 43, 68, 73). *In vitro* and *in vivo* experiments have provided evidence to support a histone chaperone role for these two factors in promoting the assembly of nucleosomes (1, 15, 41, 44, 47, 68). Spn1 recently has been shown to regulate the binding of Spt6 to nucleosomes *in vitro* (68). Spt2 binds DNA nonspecifically and also is required for transcription-coupled nucleosome assembly (72). In one possible scenario, the L1L2 loop may provide a binding surface for Spt6 or Spt16 to facilitate the reassembly of histones after the passage of RNA Pol II. Alternatively, the L1L2 loop may indirectly affect Spt6 by interacting with or regulating the activity of Spn1. Recent structural studies have identified the L1L2 region as part of the binding interface between histone H3/H4 and another histone chaperone, Asf1 (2, 28). Regarding possible interactions with Spt2, histone H3 R49 and V46 are more likely candidates, as this factor has shown affinity for four-way DNA junctions, a structure similar to what has been shown at the DNA entry/exit points of the nucleosome (54, 62).

In summary, we have provided evidence that at least eight histone residues, five in histone H3 and three in histone H4,

are required for normal transcription-coupled nucleosome occupancy specifically at highly transcribed genes. We also have shown a distinct role for two of these residues, histone H3 R49 and V46, in promoting the Set2-dependent methylation of histone H3 K36. Further analysis of this new class of histone mutants is likely to provide answers to questions and will enhance our understanding of transcription-coupled nucleosome dynamics.

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