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Genetic experiments have identified two structurally similar nucleosomal domains, SIN and LRS, required for transcriptional repression at genes regulated by the SWI/SNF chromatin remodeling complex or for heterochromatic gene silencing, respectively. Each of these domains consists of histone H3 and H4 Loops that form a DNA-binding surface at either superhelical location (SHL) ±2.5 (LRS) or SHL ±0.5 (SIN). Here we show that alterations in the LRS domain do not result in Sin− phenotypes, nor does disruption of the SIN domain lead to loss of ribosomal DNA heterochromatic gene silencing (Lrs− phenotype). Furthermore, whereas disruption of the SIN domain eliminates intramolecular folding of nucleosomal arrays in vitro, alterations in the LRS domain have no effect on chromatin folding in vitro. In contrast to these dissimilarities, we find that the SIN and LRS domains are both required for recruitment of Sir2p and Sir4p to telomeric and silent mating type loci, suggesting that both surfaces can contribute to heterochromatin formation. Our study shows that structurally similar nucleosomal surfaces provide distinct functionalities in vivo and in vitro.

The nucleosome constitutes the central structure of chromatin and comprises two chains each of histones H2A, H2B, H3, and H4, which are assembled into a histone octamer and around which 147 bp of DNA is wrapped ±1.7 times (18). Previous studies identified two nucleosomal surfaces, SIN (switch independent) and LRS, which are important for transcriptional repression in Saccharomyces cerevisiae (13, 24, 33). These domains lie at opposite ends of the crescent-shaped, quasisymmetric H3-H4 heterodimer. Histone residues altered in sin mutants or in five of the lrs mutant alleles can be structurally superimposed by a rotation of 180° around a symmetry axis at superhelical location (SHEL) ±1.5. Thus, the two “ends” of each (H3-H4) “crescent” are structurally equivalent yet organize different regions of the DNA (Fig. 1). Alterations within these two clusters relieve distinct forms of transcriptional repression: the sin mutant alleles partially bypass the need for the SWI/SNF chromatin remodeling complex (13, 24, 33), whereas disruption of the SIN domain eliminates intramolecular folding of nucleosomal arrays in vitro (15, 22). In contrast, mutations within genes encoding histones H3 or H4 lead to single-amino-acid changes that cluster around the nucleosomal dyad axis (SHEL ±0.5) (13, 18). Because interactions between DNA and the histone octamer are strongest at the dyad region of the nucleosome, disruption of histone-DNA contacts in this region may be the rate-limiting step in nucleosomal dissociation and sliding (20). X-ray structures of Sin− mononucleosomes have demonstrated that Sin− versions of histone H4 or H3 do not affect the overall structure of the nucleosome but rather disrupt only a small percentage of histone-DNA contacts (22). These subtle changes in histone-DNA interactions provide a likely explanation for why Sin− mononucleosomes dissociate at significantly lower ionic strength and why they have a lower energy barrier towards temperature-induced nucleosome repositioning (“sliding”) (7, 15, 22). In contrast, nucleosomal arrays reconstituted with Sin− versions of histone H4 do not exhibit altered positioning of nucleosomes, nor do Sin− nucleosomes within these arrays show altered accessibility to restriction enzymes (12). Interestingly, these Sin− nucleosomal arrays show defects in formation of condensed, 30-nm-like fibers. This folding defect mimics the loss of condensation due to removal of the histone N-terminal domains (8, 12). These results have led to two models for how Sin− histones might compensate for inactivation of SWI/SNF in vivo: (i) the enhanced thermal mobility of Sin− mononucleosomes makes chromatin remodeling unnecessary, and (ii) the unfolded state of Sin− nucleosomal arrays may mimic the “remodeled state” of chromatin acted on by SWI/SNF. These models may not be mutually exclusive, as the enhanced mobility of Sin− nucleosomes may inhibit chromatin condensation.

In contrast to the Sin− alterations, little is known about the...
biochemistry of Lrs\textsuperscript{−} histones. The *ls* mutants were identified in an unbiased yeast genetic screen for mutations that alleviate silencing of an RNA polymerase II (Pol II) reporter gene that had been inserted into the ribosomal DNA locus (24). Additionally, the *ls* mutant alleles were found to alleviate transcriptional silencing at telomeres and, to a lesser extent, the silent mating type loci (*HM* loci). Given the striking structural similarity of the LRS and SIN domains, it is attractive to hypothesize that these two surfaces fulfill similar functions. Here we have compared the in vivo and in vitro phenotypes of Lrs\textsuperscript{−} and Sin\textsuperscript{−} histone alterations. We find that *ls* mutants do not exhibit Sin\textsuperscript{−} phenotypes, nor do sin mutants exhibit defects in ribosomal DNA silencing. In contrast to Sin\textsuperscript{−} versions of H4, we find that an Lrs\textsuperscript{−} version of histone H3 does not disrupt
formation of condensed, 30-nm-like fibers in vitro. Our genetic and biochemical studies indicate that these two nucleosomal surfaces have distinct functionalities in vitro and in vivo.

MATERIALS AND METHODS

Strains, media, and plasmids. All yeast strains are described in Table 1. Yeast media were exactly as described previously (31), except that 0.8 mM adenine was added to the synthetic complete (SC) plates lacking uracil (SC-Ura) and incubated overnight at 37°C. Yeast patches were then replica plated onto a Whatman 50 filter placed on a second SC-Ura plate and incubated overnight at 37°C. Relative expression levels of the HO-lacZ reporter gene in each strain were demonstrated using a β-galactosidase filter assay, as described previously (13).

Reverse transcription-PCR (RT-PCR) analysis of HO-lacZ and PHOS expression levels. PHOS transcriptional repression under high-phosphate conditions and HO-lacZ expression were assayed in JPY12 transformed with pDM18 derivatives of histones H3 and H4. Cells were grown to mid-log phase in yeast extract-peptone-dextrose (YPD) medium at 30°C, and 10 ml of cell culture was harvested for RNA extraction with hot acidic phenol. First-strand cDNA synthesis was performed using 2.5 μg total RNA, SuperScript II RNase H− reverse transcriptase (Invitrogen), and 2 pmol each of PHOS, HO-lacZ, or ACTI downstream primers, following the manufacturer’s instructions. Subsequently, semiquantitative, 32P-labeled PCR was performed using 2 μl of the first-strand cDNA reaction, Taq Polymerase (Promega), and gene-specific primer sets to determine the relative levels of PHOS, HO-lacZ, and ACTI mRNA for each mutant strain. After 14 cycles (for ACTI) or 22 cycles (for PHOS and HO-lacZ) of amplification, PCR products were electrophoresed on 10% acrylamide. Signals were quantified using a PhosphorImager and ImageQuant v4.2 (Molecular Dynamics). For quantification, PHOS expression levels were normalized to ACTI expression, and PHOS expression in the wild-type strain was set to 1.0. Data shown in Fig. 2B are the average of four independent experiments with standard deviations. Primer sequences are available upon request.

Recombinant histone and histone octamer preparation. Xenopus laevis histone H3 R83A was cloned from pET3D1-Histone H3 C28S (CP1035) after Quikchange XL site-directed mutagenesis (Stratagene). Clones were confirmed by sequence analysis using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit v3.0 (Perkin Elmer) and transformed into BL21(DE3) cells (Invitrogen).

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Sedimentation velocity analysis. Sedimentation velocity experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge using scanner optics at 260 nm. Samples were equilibrated at 20°C under vacuum for at least 1 h prior to sedimentation at 25,000 g in a 60 Ti rotor. Boundaries were analyzed by the method of van Holde and Weischet (36) using UltraScan version 4.0 for Unix. Data were plotted as boundary fraction (y axis) versus S20,w (sedimentation corrected to water at 20°C) to yield the G(s) distributions (Fig. 3B). Analyses were performed three times, and data are representative of a single experiment.

Silencing assays. Silencing strength in the ribosomal DNA (rDNA) was assessed with the mURA3/HIS3 reporter by serial dilution on SC-His medium to prevent elimination of the rDNA reporter containing 0.1% 5-fluoroorotic acid (5-FOA) to assay down-regulation of rDNA::mURA3. Silencing strength of the telomeric DNA was assayed using the MET15 color assay. Strains to be tested were plated onto SC-Trp plates, and HO-lacZ reporter gene expression was analyzed using a β-galactosidase filter assay. (B) Relative levels of HO-lacZ and ACT1 gene expression in panel A were determined by RT-PCR for an swiΔ strain (CY240) transformed with plasmids expressing either wild-type or Lrs histone H3 or the Sin histone H4-R45C. Four clones of each were grown on SC-Trp plates, and HO-lacZ reporter gene expression was analyzed using a β-galactosidase filter assay. (B) Relative levels of HO-lacZ and ACT1 gene expression in panel A were determined by RT-PCR for an swiΔ strain (CY240) transformed with plasmids expressing either wild-type histone H4, histone H3-R83A, or histone H4-R45C. Similar results were observed for strains expressing other Lrs histones. (C) Lrs alterations do not lead to derepression of PHO5 gene expression in high-phosphate media. JPY12 cells expressing either wild-type, Lrs, or Sin histones as the sole source of histone H3 or H4 were grown to mid-log phase in YEPD medium and harvested for RNA. The level of PHO5 and ACT1 gene expression was analyzed by RT-PCR. PHO5 expression was normalized to ACT1 expression in each strain, with expression levels in the wild-type strain set to 1.0. The bottom panels show raw data from a representative experiment. Quantified data are graphed above and reflect the average of four independent experiments with standard deviations. WT, wild type.

**FIG. 2.** Lrs alterations do not show a Sin phenotype in vivo. (A) Lrs alterations do not suppress swi/swi defects in HO-lacZ transcription. Strains CY232 (SWI) and CY240 (swi1), both containing an HO-lacZ reporter gene, were transformed with plasmids expressing either wild-type or Lrs histone H3 or the Sin histone H4-R45C. Four clones of each were grown on SC-Trp plates, and HO-lacZ reporter gene expression was analyzed using a β-galactosidase filter assay. (B) Relative levels of HO-lacZ and ACT1 gene expression in panel A were determined by RT-PCR for an swiΔ strain (CY240) transformed with plasmids expressing either wild-type histone H4, histone H3-R83A, or histone H4-R45C. Similar results were observed for strains expressing other Lrs histones. (C) Lrs alterations do not lead to derepression of PHO5 gene expression in high-phosphate media. JPY12 cells expressing either wild-type, Lrs, or Sin histones as the sole source of histone H3 or H4 were grown to mid-log phase in YEPD medium and harvested for RNA. The level of PHO5 and ACT1 gene expression was analyzed by RT-PCR. PHO5 expression was normalized to ACT1 expression in each strain, with expression levels in the wild-type strain set to 1.0. The bottom panels show raw data from a representative experiment. Quantified data are graphed above and reflect the average of four independent experiments with standard deviations. WT, wild type.

**Silencing assays.** Silencing strength in the ribosomal DNA (rDNA) was assessed with the mURA3/HIS3 reporter by serial dilution on SC-His medium to prevent elimination of the rDNA reporter containing 0.1% 5-fluoroorotic acid (5-FOA) to assay down-regulation of rDNA::mURA3. Silencing strength of the telomeric DNA was assayed by serial dilution on SC-Ura. Serial dilutions were performed as follows. Cells were scraped from the plates and resuspended in 100 μl of sterile water. The cell suspension was normalized to an A600 reading of 0.5 and then serially diluted in 5-fold or 10-fold increments; 5 μl of each dilution was spotted onto either nonselective or selective agar plates using a 12-channel pipette. Plates were incubated for 2 to 5 days.

**Colony color silencing assays.** rDNA silencing was also assayed using the MET15 color assay. Strains to be tested were plated onto lead (MLA) plates to give approximately 100 to 200 colonies per plate. The plates were incubated at 30°C for 8 days and then photographed. Telomeric silencing was also assayed using the ADE2 color assay. Strains to be tested were plated onto SC-Trp plates to give approximately 100 to 200 colonies per plate. The plates were incubated at 30°C for 3 days and then were incubated at 4°C for 3 days and photographed.
Western blot analysis. Histone H3 K79 dimethylation, H3 K79 trimethylation, total histone H3, Sir2p, and Sir4p levels were analyzed in JPY12 transformed with pDM18 derivatives of histone H3 and H4. Cells were grown to mid-log phase in YEPD medium at 30°C, and 10 ml of cell culture was pelleted, rinsed with TBS (20 mM Tris [pH 7.4], 150 mM NaCl), and resuspended in 150 μl of Laemmli buffer. Each cell lysate was combined with 300 μl of glass beads in a 1.5-ml Eppendorf tube and vortexed at maximal speed for 10 min at 4°C. The cell lysates were then heated for 2 min at 95°C and clarified by centrifugation, and 7 μl of cell lysate was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis with anti-histone H3 dimethyl K79 (Upstate 07-366), anti-histone H3 trimethyl K79 (Abcam 2621), anti-histone H3 (Cell Signal Technology 9715), anti-Sir2p (sc-6666), anti-Sir4p (sc-6671), and anti-TBP (M. Green, University of Massachusetts Medical School) antibodies. Experiments were performed at least twice, and data are representative of a single experiment.

In vitro Dot1 methylation assay. Recombinant Dot1 and yeast nuclear extracts were purified as previously described (37), except that the extracts were made from a dot1Δ derivative of JPY12. Nuclear extract concentrations were determined by measuring the spectrophotometric absorbance at 260 and 280 nm, and concentrations were normalized by dilution in a buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 2 mM MgCl2, 0.1% NP-40, 2.5 mM 2-mercaptoethanol, and 10% glycerol. Assays were carried out by adding 1 μl of the normalized nuclear extract to a reaction containing 1 μl of purified recombinant Dot1p, 2 μCi of S-adenosyl-[methyl-3H]methionine, 50 mM Tris-Cl (pH 7.9), 1 mM EDTA, 0.5 mM EGTA for a total volume of 15 μl. Reactions were incubated at 30°C for 6 h and were quenched by the addition of 5 μl of 5× SDS-PAGE sample buffer.

FIG. 3. Lrs alterations do not disrupt nucleosomal array folding in vitro. Histone octamers reconstituted from recombinant histones H2A, H2B, H4, and either wild-type H3 or H3-R83A were deposited onto 208-11 DNA templates by salt dialysis. (A) R83A Lrs nucleosomes are indistinguishable by native PAGE. Arrays harboring either the wild type or the Lrs R83A version of histone H3 were cleaved with EcoRI, electrophoretically separated on a native 4% PAGE, and stained with ethidium bromide. The mononucleosome (Nuc) and naked DNA (Naked) bands are indicated. (B) R83A Lrs nucleosomal arrays show normal intramolecular, salt-dependent folding as shown by sedimentation velocity analysis of 208-11 arrays in the presence or absence of Mg2+. The G(s) distributions are depicted for the indicated arrays sedimented in either TE (10 mM Tris [pH 8.0], 0.25 mM EDTA) or TE with 1.75 mM MgCl2. S20,w is the sedimentation coefficient corrected to water at 20°C. (C) Intermolecular oligomerization is not altered by the histone H3-R83A mutation. Nucleosomal arrays were incubated in TE with varying concentrations of MgCl2 at room temperature for 15 min, followed by centrifugation in a microcentrifuge at 14,000 × g for 10 min. The percentage of array remaining in the supernatant is plotted as a function of MgCl2 concentration. (D) rDNA silencing of the lrs mutant allele H3-R83A was determined by assaying for growth on SC-Ura to measure expression of the mURA/His reporter and plating on Pb-containing media to assay expression of MET15 reporter, both integrated into the rDNA locus. WT, wild type. R, ratio.
buffer. Samples were separated by SDS-PAGE on 10% gels, and the resulting gels were stained with Coomassie blue. The products were identified by fluorography. Parallel samples were probed by immunoblotting for histone H3 (Abcam 1791).

Chromatin immunoprecipitations (ChIP), Sir2p, Sir4p, and Rap1p binding was analyzed in JPY12 transformed with pDM18 derivatives of histone H3 and H4. Cells were grown to mid-log phase in YEPD medium. Chromatin was immunoprecipitated as described by Kuo and Allis (14) using whole cell lysate from 1 x 10^7 cells and 10 µl of polyclonal antibody against Sir2p (Santa Cruz sc-6666). Sir4p (Santa Cruz sc-6671), or Rap1p (M. Grunstein, University of California at Los Angeles; or Santa Cruz sc-20167). The recovered DNA was subjected to semiquantitative, 32P-labeled PCR to determine the relative amount of precipitated DNA. After 25 cycles of amplification, PCR products were electrophoresed on 10% acrylamide, and signals were quantified using a PhosphorImager and ImageQuant v4.2 (Molecular Dynamics). Quantification reflects the amount of precipitated DNA relative to the total input DNA (relative immunoprecipitation [IP]). Each immunoprecipitation was normalized relative to the IP observed for the nonspecific PHO5 locus. Each experiment was repeated three times, and the data shown are representative of a single experiment. In addition, for each experiment a titration of the input DNA (1:10, 1:50, and 1:100) was included in the PCR quantification to ensure that the PCRs were in the linear range. Primer sets used for PCR quantification are as follows: HMLα upstream (5'-AGT TTG CCG CAC GGA CCT ATT TGG-3') and downstream (5'-TAA GAT GCT GCC GCA CAA CTC TC-3'); HMRα upstream (5'-GTC CAA GGT ATG AGC TTA ATC TTC-3') and downstream (5'-CGG AAT CGA GAA TCT TCG TAA TGG-3'); PHO5 upstream (5'-GAA TCT TCG TAA TGG-3') and downstream (5'-AGT TTT CGG CAC GGA CTT ATT TGG-3'). Primer sets for chromosome VI-R have been described previously (21).

Quantitative mating assays. Mating efficiency was assayed in JPY12 transformed with pDM18 derivatives of histone H3 and H4. The mating efficiency of each strain was determined using a quantitative mating assay. Briefly, each mutant strain (MATA) and the mating-type tester strain CY385 (MATα) were grown to mid-log phase in SC-Trp medium at 37°C. Next, 2 x 10^6 cells of each mutant strain were mixed with 1 x 10^6 cells of the tester strain in 5 ml of YEPD medium. Cells were briefly centrifuged and incubated at 30°C for 4 to 7 h. Cells were then resuspended and sonicated gently for 10 s to disrupt clumps, diluted in fresh medium, and plated on SC plates to titer diploid cells and on SC-Lys to titer total cells. The mating efficiency is expressed as the titer of diploid cells divided by the titer of total cells. Data shown are the averages of three experiments with standard errors.

Crystal structure images. SIN and LRS histone mutants were mapped onto the yeast nucleosomal structure (Brookhaven PDB 1ID3I) (18) using Pymol (4).

RESULTS

lrs mutants do not have a Sin− phenotype. The LRS and SIN nucleosomal domains are structurally similar, and alterations in either of these surfaces leads to defects in transcriptional repression. However, despite their loss of silencing phenotypes, lrs mutant alleles show no defect in growth either in the presence or absence of wild-type histones, suggesting that their defect is specific to silenced, heterochromatin-like regions of the genome. In contrast, sin mutant alleles do affect cell growth and viability when the Sin− versions are the sole source of histone H3 or H4. H3-E105K, H3-T118I, H3-R116H, and H4-R45C are all inviable as the sole source of histones, whereas H4-V43I and H4-R45H are viable but grow slowly when expressed alone (unpublished results; see also references 16 and 38). Despite the different requirements of intact LRS and SIN domains for yeast cell viability, we wished to test whether each domain contributed to transcriptional silencing by the same mechanism. First, we tested whether lrs mutant alleles exhibit a Sin− phenotype.

In yeast that lack the SWI/SNF chromatin-remodeling complex, an HO-LacZ reporter gene is not expressed; however, expression can be partially rescued in the presence of a sin mutant histone allele (13, 33). Using this reporter gene, the lrs mutant alleles were tested for expression of HO-LacZ in an swi1 mutant that inactivates SWI/SNF. Whereas expression of the H4 R45C sin mutant allele led to significant expression of HO-lacZ in the swi1 mutant, expression of several lrs mutant alleles had no effect (Fig. 2A and B). As expected, expression of Lrs− or Sin− histones had no effect on HO-lacZ expression in the presence of an intact SWI/SNF complex (Fig. 2A). Furthermore, expression of Lrs− histones does not alleviate the slow-growth phenotype of the swi1 mutant, whereas expression of the H4-R45C or H4-R45H Sin− version leads to more robust growth on both plates and in liquid media (data not shown). Thus, unlike sin mutant alleles, Lrs− histones do not bypass the SWI/SNF requirement for HO-lacZ expression or for wild-type growth rates.

To provide an additional measurement of the Sin− phenotype, we analyzed expression of PHO5. Previously, we showed that sin mutant alleles lead to partial derepression of PHO5 in high-phosphate media, suggesting that the SIN domain contributes to nucleosome-mediated repression of basal transcription (38). To test whether lrs mutant alleles have a similar phenotype, PHO5 expression was monitored by RT-PCR (Fig. 2C). Whereas expression of a sin mutant allele (H4-R45C) led to approximately fourfold higher levels of PHO5 expression, strains containing lrs mutant alleles did not significantly derepress PHO5 (Fig. 2C). These results demonstrate that alterations within the LRS domain do not lead to Sin− phenotypes in vivo.

The LRS domain is not required for nucleosomal array formation in vitro. Previously we showed that nucleosomal arrays reconstituted with recombinant Sin− versions of histone H4 are unable to condense into 30-nm-like fibers in vitro (12). To test whether Lrs− histones also disrupt chromatin folding, we prepared recombinant histone octamers that harbor wild-type histone H3 or an Lrs− version (H3-R83A), and each of these octamers was used to assemble model nucleosomal arrays (Fig. 3). The previously reported Lrs allele H3-R83A (24) was later shown by resequencing to represent an H3-R83G allele. Therefore, we tested the rDNA-silencing phenotype of H3-R83A for loss of silencing of both rDNA reporters; in fact, H3-R83A displays a similar loss of rDNA silencing to H3-A75V, our control lrs mutant allele (Fig. 3D). Our analysis focused on the H3-R83A Lrs− version, as this arginine residue is structurally equivalent to H4-R45, which is altered in sin mutant alleles (Fig. 1). A DNA template composed of 11 copies of a 208-bp SS rRNA gene isolated from the sea urchin Lytechinus variegatus (the 208-11 template) was used to generate a positioned array of 11 nucleosomes after in vitro salt dialysis reconstitution (5). As observed previously for Sin− histones (12), the Lrs− version of H3 had no effect on histone octamer assembly (data not shown).

The folding of nucleosomal arrays into 30-nm-like fibers in vitro requires that arrays be fully saturated with nucleosomes (e.g., 11 nucleosomes per array) (29). As an initial means of monitoring the efficiency of nucleosomal array assembly, we digested the reconstituted arrays with EcoRI. Because each SS ribosomal DNA repeat in the 208-11 array template is bordered by EcoRI restriction sites, EcoRI cleavage releases either a 208-bp free DNA fragment or a mononucleosome that can be identified by its slower mobility after native gel electrophoresis. A fully saturated nucleosomal array typically yields...
Recombinant wild-type and Lrs\textsuperscript{\textsuperscript{-}} histone octamers yielded similar levels of nucleosome density at nearly identical ratios of octamers to the 5S ribosomal DNA repeat (ratios of 1.0 to 1.4), indicating that the Lrs\textsuperscript{\textsuperscript{-}} version of H4 does not disrupt nucleosome assembly (Fig. 3A).

In low-ionic-strength buffers, such as Tris-EDTA (TE), 208-11 arrays exist as extended, flexible fibers that sediment in the analytical ultracentrifuge as a nearly homogeneous distribution of ~27S-28S species (17, 29). Previously we showed that arrays reconstituted with Sin\textsuperscript{\textsuperscript{-}} histones sediment slightly slower in TE buffer compared to wild-type arrays, and this observation led us to suggest that Sin\textsuperscript{\textsuperscript{-}} arrays may be more extended in low-salt conditions (12). In contrast, the sedimentation of the Lrs\textsuperscript{\textsuperscript{-}} nucleosomal arrays was identical to that of a wild-type array (Fig. 3B, open symbols).

When divalent cations (Mg\textsuperscript{\textsuperscript{2+}}) are introduced, these model nucleosomal arrays form a heterogeneous, faster-sedimenting species with a 30S-55S distribution (Fig. 3B) (29); formation of the 55S species is consistent with formation of a compact, 30-nm-like chromatin fiber. Saturated Sin\textsuperscript{\textsuperscript{-}} nucleosomal arrays sediment at only ~30S in Mg\textsuperscript{\textsuperscript{2+}}-containing buffer, reflecting a complete absence of salt-dependent folding (12). In contrast to the Sin\textsuperscript{\textsuperscript{-}} arrays, nucleosomal arrays reconstituted with the H3-R83A Lrs\textsuperscript{\textsuperscript{-}} histone are fully competent for formation of compact, 30-nm-like fibers exhibiting a typical 30S-55S distribution (Fig. 3B, closed circles).

In addition to these intramolecular folding reactions, higher concentrations of divalent cations can induce reversible oligomerization of nucleosomal arrays (28). Intermolecular oligomerization generates large (>1,000S) defined structures that are believed to mimic the fiber-fiber interactions that stabilize...
higher order chromosomal domains. Sin− histones do not disrupt the oligomerization of model 5S nucleosomal arrays (12), and likewise the Lrs− version, H4-R83A, has no effect on formation of these higher order structures (Fig. 3C). Thus, even though Lrs− and Sin− nucleosomes have lost a similar number of histone-DNA contacts, only Sin− histones selectively disrupt the intramolecular folding of nucleosomal arrays in vitro.

Sin− mutants do not have a loss of ribosomal DNA-silencing (LRS) phenotype. Although lrs mutant alleles do not show Sin− phenotypes in vivo or in vitro, we also investigated whether sin mutant alleles show an Lrs− phenotype. lrs mutant alleles were identified using two RNA polymerase II-transcribed reporter genes inserted within the ribosomal DNA locus, the MET15 reporter in the NTS2 region, and the mURA3 reporter (with a minimal TRP1 promoter) in the 5′ region of the 35S rRNA gene (24). The lrs mutant alleles lead to a loss of ribosomal DNA silencing, meaning that strains harboring these mutant histones do not grow well on 5-FOA media, reflecting a loss of silencing of URA3, and similarly the
colonies display a lighter tan color, in contrast to wild-type colonies on MLA (lead) plates due to loss of MET15 silencing. We tested the ability of sin mutants to form repressive chromatin at the ribosomal DNA using the above-mentioned reporter system. The viable H4 sin mutant alleles, V43I and R45H, display a slight increase in ribosomal DNA silencing, giving a slightly darker color than the wild type on lead plates and showing enhanced growth on 5-FOA as measured by serial dilution (Fig. 4). Similarly, the inviable sin mutant alleles, H4-R45C, H3-T118I, H3-E105K, and H3-R116H, display wild-type ribosomal DNA-silencing phenotypes. H3-E105K displays a dominant slow-growth phenotype and hence displays smaller colonies overall. The small, darker colonies present on the MLA plates are due to loss of the MET15 reporter by recombination. The same small-colony phenotype is also observed in the telomeric silencing reporter strains (Fig. 5). In contrast, the control lrs mutants are a lighter color compared to the wild-type strain on lead-containing plates (Fig. 4A) and grow less well compared to the wild type on plates that contain 5-FOA (Fig. 4B). Thus, disruption of the SIN domain does not lead to an Lrs phenotype, reinforcing the view that the SIN and LRS nucleosomal surfaces are functionally distinct.

Alterations in both the SIN and LRS domains disrupt telomeric silencing. The LRS surface is also important for both telomeric and, to a lesser extent, silent mating-type locus silencing (24). Although alterations of the SIN surface do not display a loss of ribosomal DNA silencing, it is possible that they could share other silencing defects. The mechanism of ribosomal DNA silencing differs from that of telomeric or HM silencing in that SIR3 and SIR4 are not required. Also, the ribosomal DNA, while inhibitory to Pol II transcription, is very active in Pol I and Pol III transcription, indicating that some components of the mechanisms of silencing for the ribosomal DNA are distinct (1, 3, 27). The sin mutant alleles were tested for telomeric silencing defects using subtelomeric URA3 and ADE2 reporters. In wild-type yeast, the majority of such cells repress subtelomeric reporters, thereby permitting cells to grow on 5-FOA and displaying red or pink colonies with white sectors (10). However, cells defective in telomeric silencing express URA3 and are sensitive to 5-FOA, and colonies have a white or light pink color when grown on nonselective media with limiting adenine. Both sin and lrs mutant alleles display defects in telomeric silencing (Fig. 5). In the presence of wild-type histones, only one of the H3 sin mutant alleles showed a strong defect in telomeric silencing. In contrast, the H4 sin mutant alleles showed a strong silencing defect when the Sin/H11002 histones were expressed as the sole source of H4. The lrs mutant alleles, with the exception of H3-K79R, also show a

**FIG. 6.** Sin and Lrs alterations do not generally disrupt H3-K79 methylation. (A) JPY12 cells expressing either wild-type, Lrs-, or Sin- histones as the sole source of histone H3 or H4 were grown to mid-log phase in YEPD medium. Cells were lysed in Laemmli buffer and lysates were analyzed by SDS-PAGE, followed by Western blot analysis with antibodies raised against histone H3 dimethyl K79 and TBP (loading control; upper panel) or histone H3 trimethyl K79 and total histone H3 (loading control; lower panel). (B) Soluble chromatin was isolated from wild-type (JPY12), dot1Δ (EMHY234), and a series of dot1Δ Lrs strains and incubated with recombinant Dot1p and [3H]S-adenosyl-methionine. Reaction products were separated by SDS-PAGE, and methylated histone H3 was detected by fluorography (upper panel). Histone H3 was detected by immunoblot analysis to normalize input levels of histone proteins in each reaction (lower panel).
loss of telomeric silencing (Fig. 5; see also reference 24). Thus, the SIN and LRS domains are both required for telomeric gene silencing.

The SIN and LRS domains are not generally required for H3-K79 methylation. A current view is that methylation of H3-K79 distinguishes euchromatin from heterochromatin by preventing Sir protein association in euchromatic regions (37). Inactivation of Dot1p, the H3-K79 methylase, is thought to cause Sir proteins to delocalize from the silenced regions and redistribute throughout the genome (37), leading to a disruption of silencing. Given that H3-K79 is within the LRS domain, we tested the simple hypothesis that \( \text{lrs} \) mutant alleles disrupt silencing because they cripple the binding or activity of the Dot1p methylase. Likewise, it is also a formal possibility that the SIN domain is required for Dot1p function. First, we used Western blot analysis to monitor the levels of H3-K79 di- and trimethylation in bulk chromatin from cells that express wild-type, \( \text{lrs} \)/\( \text{H11002} \), or \( \text{sin} \)/\( \text{H11002} \) histones as the sole source of histone protein. Figure 6 demonstrates that most \( \text{lrs} \) mutant alleles are not defective in H3-K79 di- and trimethylation in bulk chromatin from cells that express wild-type, \( \text{lrs} \), or \( \text{sin} \) histones as the sole source of histone H3 or H4 grown to mid-log phase in YEPD medium and then processed for Western immunoblot analysis (A) or chromatin immunoprecipitation (B to E). (A) Western blot analysis shows that \( \text{lrs} \) and \( \text{sin} \) histone alterations do not affect Sir2p protein levels. (B to D) ChIP analysis of Sir2p or Sir4p recruitment to the telomere, 500 bp (B) and 70 bp (C) from the end of the right arm of chromosome VI and to the nonspecific PHO5 promoter (D). An additional JPY12 strain containing an \( \text{sir4} \) deletion (\( \text{sir4} \Delta \)), which abolishes both Sir2p and Sir4p telomeric binding (21), was used to determine background signals in this assay. Quantification shown below the panels indicates the percent immunoprecipitated telomeric DNA (IP/Input) normalized to the percent IP from the nonspecific PHO5 locus (D) to normalize for IP efficiency. The normalized value for the wild-type strain was set at 1.0. Quantification below panel D indicates the percent immunoprecipitated PHO5 DNA (IP/Input), with the value for wild-type cells set at 1.0. (E) Rap1p binding 70 bp from the end of the right arm of chromosome VI was analyzed using antibodies to Rap1p. Similar results were observed in an independent experiment. Chr., chromosome.

As an additional test for whether \( \text{lrs} \) mutant alleles disrupt Dot1p function, we also performed in vitro labeling analyses. Soluble chromatin was isolated from \( \text{dot1} \Delta \) cells harboring either wild-type or \( \text{lrs} \) histones and incubated with recombinant Dot1p and radioactive S-adenosyl-methionine. Reaction products were separated by SDS-PAGE, and methylated histone H3 was detected by fluorography. Consistent with the bulk chromatin analysis, only a small subset of \( \text{lrs} \) mutant alleles eliminated Dot1p-dependent methylation (Fig. 6B). The results agree with the immunoblotting results, except that one mutant that was immunoreactive in vivo did not show in vitro labeling for unknown reasons (V81A). Overall, these results are consistent with previous reports showing that an H3-K79A substitution has a more severe effect on Sir protein occupancy of silenced regions than loss of Dot1p (23, 37). These data support the view that the SIN and LRS domains do not function solely by influencing Dot1p binding or by controlling methylation of H3-K79.

LRS and SIN domains are required for Sir2p and Sir4p binding to telomeric chromatin. To further investigate the role of the LRS and SIN domains in telomeric silencing, we mon-
TABLE 2. LRS and SIN domains function with Sir1p to silence HM loci

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>% Mating efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPY12</td>
<td>H3 WT</td>
<td>100</td>
</tr>
<tr>
<td>CY1109</td>
<td>H3 WT sir1Δ</td>
<td>4.4 ± 0.9</td>
</tr>
<tr>
<td>CY1113</td>
<td>H3 WT sir2Δ</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>CY1045</td>
<td>H3-K79E</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>CY1110</td>
<td>H3-K79E sir1Δ</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>CY1044</td>
<td>H3-K79R</td>
<td>94.0 ± 8.9</td>
</tr>
<tr>
<td>CY1111</td>
<td>H3-K79R sir1Δ</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>CY1047</td>
<td>H3-R83A</td>
<td>68.2 ± 2.5</td>
</tr>
<tr>
<td>CY1112</td>
<td>H3-R83A sir1Δ</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>CY1169</td>
<td>H4-R45H</td>
<td>28.2 ± 6.2</td>
</tr>
<tr>
<td>CY1278</td>
<td>H4-R45H sir1Δ</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>CY1171</td>
<td>H4-V43I</td>
<td>75.6 ± 4.5</td>
</tr>
<tr>
<td>CY1279</td>
<td>H4-V43I sir1Δ</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

*a* WT, wild type. Mating efficiency was normalized to the H3 WT strain (set at 100%).

FIG. 8. Sin− and Lrs− histones do not disrupt Sir2p and Sir4p binding to silent HML and HMR chromatin. Binding of Sir2p and Sir4p to the silent mating-type loci HML and HMR and the nonspecific PHO5 promoter in JPY12 cells harboring either wild-type, Lrs− (A to C), or Sin− (D to F) histones as the sole source of histone H3 or H4 was examined using ChIP analysis, as described in the legend to Fig. 7. An asterisk designates mutations that have previously been shown to disrupt mating (24, 35).
Sir2p or Sir4p closely parallel the observed defects in telomeric silencing.

**LRS and SIN domains function with Sir1p to recruit Sir2p and Sir4p to HM chromatin.** An intact LRS domain is required for full silencing of an RNA polymerase II reporter gene that is inserted within the HML silent mating type locus (24). However, except for H3-K79E and H3-L82S, most lrs mutant alleles do not appear to strongly derepress the native HM loci, as the mutants mate normally and show wild-type levels of Sir2p and Sir4p recruitment to the HML and HMR loci (Table 2; Fig. 8A and B) (24). Likewise, alterations within the SIN domain do not lead to dramatic defects in mating or Sir protein recruitment to HM loci, although the H4-R45H allele does show a reproducible defect in these assays (Table 2; Fig. 8D and E). Why should the LRS and SIN domains be required for Sir protein recruitment at telomeres but not at HM loci? A major difference between telomeric and HM silencing is that Sir1p is required specifically at the HM loci for the recruitment and stabilization of Sir2p, Sir3p, and Sir4p (25, 31). If the LRS and SIN domains provide an additional binding surface for assembly of Sir proteins on chromatin, we entertained the possibility that Sir1p might play a partially redundant role with these nucleosomal surfaces. To test this hypothesis, we monitored mating and Sir protein recruitment in sir1Δ strains that harbored wild-type, Lrs−, or Sin− histones.

In the absence of Sir1p, both the LRS and SIN domains are essential for mating and for Sir2p and Sir4p recruitment. In an sir1Δ strain, cells mate at ~4.5% efficiency when wild-type histones are present, but expression of Lrs− or Sin− histones cripples mating to undetectable levels (Table 2). Likewise, in the absence of Sir1p, recruitment of Sir2p and Sir4p to HML in strains containing histone H3 Lrs− alterations compared to strains containing wild-type histone H3. (B) Binding of Sir2p and Sir4p to HML in strains containing Sin− histone alterations compared to wild-type histone H3 and the H3 R83A Lrs− alteration. As a control for nonspecific binding, Sir2p and Sir4p binding to the PHO5 promoter is also shown. Sir2p and Sir4p binding to HML was normalized to the amount of binding observed for the nonspecific PHO5 locus as described in the legend to Fig. 7. WT, wild type.

**FIG. 9.** In the absence of Sir1p, Sin− and Lrs− histones abolish Sir2p and Sir4p binding to silent HML chromatin. (A) JPY12 cells containing the indicated SIR gene deletions and harboring either wild-type, Lrs−, or Sin− histones as the sole source of histone H3 or H4 were grown to mid-log phase in YEPD medium and processed for ChIP analysis, as described in the legend to Fig. 7. (A) Binding of Sir2p and Sir4p to HML in strains containing histone H3 Lrs− alterations compared to strains containing wild-type histone H3. (B) Binding of Sir2p and Sir4p to HML in strains containing Sin− histone alterations compared to wild-type histone H3 and the H3 R83A Lrs− alteration. As a control for nonspecific binding, Sir2p and Sir4p binding to the PHO5 promoter is also shown. Sir2p and Sir4p binding to HML was normalized to the amount of binding observed for the nonspecific PHO5 locus as described in the legend to Fig. 7. WT, wild type.
the lrs and sin mutants equally (Fig. 10). Additionally, the telomeric silencing defect shared by sin and lrs mutants is only slightly reduced by overexpression of SIR2.

**DISCUSSION**

The sin and lrs mutant alleles of histone genes were both identified in unbiased screens for specific phenotypes, and no overlap was seen between the two collections of alleles (13, 24). However, in both cases, the mutations alter a cluster of amino acid residues that define two structurally similar DNA-histone contact surfaces. Thus, it seemed likely that loss of the DNA-histone contact at SHL ±2.5 (lrs mutant alleles) or loss of contact at SHL ±0.5 (sin mutant alleles) might yield similar changes in chromatin structure in vitro and in vivo. However, we found that lrs mutants do not show a Sin− phenotype in vivo, nor do sin mutants show loss of ribosomal DNA silencing. In vitro these two nucleosomal surfaces also show distinct properties. Although both Sin− and Lrs− histones are fully competent for assembly of nucleosomes, only Sin− nucleosomes disrupt the salt-dependent formation of compact, 30-nm-like fibers (12). This disparity suggests that unlike the SIN surface, the LRS surface is not important for formation of this particular higher order chromatin structure. This latter observation also illustrates that loss of a single DNA-histone contact does not lead to an obligatory defect in chromatin folding. Our study reinforces the view that each histone-DNA contact site is functionally distinct and, moreover, that the nucleosome can be divided into distinct surfaces that exert different functions.

The SIN and LRS surfaces are required for telomeric and HM silencing. Although only lrs mutants disrupt gene silencing at the ribosomal DNA locus, both the SIN and LRS domains are required for silencing of a reporter gene that is integrated close to a telomere. Furthermore, amino acid substitutions within either the SIN or LRS domain disrupted the binding of Sir2p and Sir4p at telomeric chromatin. Additionally, both LRS and SIN surfaces are important for HM loci silencing and Sir2p and Sir4p binding to HM chromatin. Their defects are decidedly less dramatic at the HM loci relative to telomeres, potentially reflecting the redundancy of factors responsible for recruiting the Sir2/Sir3/Sir4 complex to the HM loci (25). The synergistic effect of an sir1Δ and both lrs and sin mutant alleles in both HM loci silencing and binding of Sir2p and Sir4p suggests that both nucleosomal surfaces participate in pathways parallel to SIR1 and upstream of Sir2/Sir3/Sir4 recruitment to the HM loci.

These two nucleosomal surfaces highlight the differences and similarities between the two types of silenced regions of the genome, namely ribosomal DNA and telomeric/HM loci. Telomeric and HM silencing share similar requirements for trans-acting factors, namely Sir2, Sir3, Sir4, and Rap1. HM silencing also requires Sir1p. In contrast, ribosomal DNA si-
lencing does not require the trans-acting Sir1p, Sir3p, or Sir4p but instead depends on Sir2p and other subunits of the RENT complex (34). These different protein requirements may reflect a fundamental difference between ribosomal DNA silencing and the other two forms of silencing (26). While the differences are many, the commonalities are the absolute requirement for Sir2p and the LRS surface.

There are at least three ways that Sin− or Lrs− histone might disrupt heterochromatin formation: (i) there can be defects in the ability to organize the DNA, (ii) there can be defects in the ability to associate with other nucleosomes, or (iii) there can be defects in the ability to associate with a trans-acting factor. An Lrs− version of histone H3 is fully competent to organize DNA into nucleosomes in vitro, and arrays of these Lrs− nucleosomes can undergo normal salt-dependent condensation. These observations argue against models (i) and (ii) and suggest the possibility that the LRS surface may interact with a key trans-acting factor that influences Sir2p binding. While it is tempting to hypothesize that the LRS surface is an Sir2p binding site, overexpression of Sir2p does not specifically alleviate the ribosomal DNA-silencing phenotype of lrs mutant alleles, and little suppression is seen for the telomeric silencing phenotype. Additionally, lrs mutant alleles display only small defects in HM loci silencing despite an absolute requirement for Sir2p. Although the lack of a specific effect of SIR2 overexpression on LRS mutants is not unequivocal, it does suggest that there might be some other factor that binds to the LRS surface which itself influences or directly promotes Sir2p binding.

Although sir mutant alleles alter key histone-DNA contacts at the nucleosomal dyad, Sin− histone octamers organize DNA into nucleosomes that are nearly identical in structure to canonical nucleosomes (22). Sin− mononucleosomes do show an enhanced propensity to slide along DNA in cis at lower temperatures than wild-type mononucleosomes (33 to 42°C versus 43°C) (7, 22), although Sin− nucleosomal arrays do not show changes in nucleosome positioning or DNA accessibility even after extended incubation at 37°C (12). What is quite clear is that Sin− nucleosomal arrays are unable to condense into 30-nm-like fibers in vitro (12). Thus, a simple model for the role of the SIN domain in silencing proposes that the optimal substrate for Sir2/Sir3/Sir4, but perhaps not the RENT complex, is a folded nucleosomal array. The RENT complex may be competent to bind nucleosomes in or out of the context of higher order chromatin. Alternatively, an ordered, compact structure may not be required for ribosomal DNA silencing as it is for both telomeric and HM loci silencing. In conclusion, our study reinforces the view that although the nucleosome is one complex made up of several histone fold motifs, it has functionally distinct surfaces that exert unique functions.

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