Effects of Sin⁻ versions of histone H4 on yeast chromatin structure and function

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Previous studies have identified single amino acid changes within either histone H3 or H4 (Sin⁻ versions) that allow transcription in the absence of the yeast SWI–SNF complex. The histone H4 mutants are competent for nucleosome assembly *in vivo*, and the residues that are altered appear to define a discrete domain on the surface of the histone octamer. We have analyzed the effects of the Sin⁻ versions of histone H4 on transcription and chromatin structure *in vivo*. These histone H4 mutants cause an increased accessibility of nucleosomal DNA to Dam methyltransferase and to micrococcal nuclease. Sin⁻ derivatives of histone H4 also grossly impair the ability of nucleosomes to constrain supercoils *in vivo*. Nucleosome-mediated repression of the *PHO5* gene is severely impaired by these histone H4 mutants; *PHO5* expression is derepressed to 31% of the wild-type induced level. In contrast to the induction caused by nucleosome depletion, full *PHO5* derepression by Sin⁻ versions of histone H4 requires upstream regulatory elements. In addition, Sin⁻ derivatives of histone H4 do not activate expression from *CYC1* or *GAL1* promoters that lack UAS elements. We propose that these Sin⁻ mutations alter histone–DNA contact residues that play key roles in restricting the accessibility of nucleosomal DNA to transcription factors.

Keywords: chromatin/nucleosome/SIN1/SWI–SNF/transcription

Introduction

The fundamental unit of eukaryotic chromatin structure is the nucleosome, which contains 146 bp of DNA wrapped about twice around an octamer of histone proteins (two each of H2A, H2B, H3 and H4). The histone octamer is organized as a central tetramer of histones H3 and H4, flanked by two heterodimers of histones H2A and H2B (Arents *et al.*, 1991). Biochemical experiments have shown that histones can repress transcription *in vitro*, either by blocking accessibility of the general transcription machinery to promoter sequences (Lorch *et al.*, 1987; Laybourn and Kadonaga, 1991; for review, see Paranjape

et al., 1994), or by interfering with the ability of sequence-specific activators to access their binding sites (for review, see Adams and Workman, 1993).

Genetic studies in yeast have supported the view that nucleosomes are global repressors of transcription. For example, depletion of nucleosomes *in vivo* leads to a derepression of a large number of genes, including *CYC1*, *GAL1* and *PHO5* (Han and Grunstein, 1988). In addition, increased expression or deletion of the yeast *SPT11* and *SPT12* genes, which encode histones H2A and H2B, can restore transcription to crippled promoters (Clark-Adams *et al.*, 1988; Fassler and Winston, 1988). Single amino acid changes or deletions in the N-terminal domain of histone H4 can also alleviate repression of the yeast silent mating-type loci (Kayne *et al.*, 1988; Johnson *et al.*, 1992) and disrupt repression by the $\alpha 2$ repressor (Roth *et al.*, 1992).

Single amino acid changes in either histone H3 or H4 have also been identified that alleviate transcriptional defects due to inactivation of the yeast SWI–SNF complex (Kruger *et al.*, 1995). These Sin⁻ (switch-independent) mutations cause single amino acid substitutions in the central, histone fold domains of histones H3 or H4. The regions that contain Sin⁻ mutations are in close proximity to one another on the surface of the histone octamer, and it has been proposed that residues altered by Sin⁻ mutations may define a functional domain (the SIN domain) that behaves formally as a negative regulator of transcription. Three of the five Sin⁻ mutations in histones H3 and H4 alter surface residues that are predicted to contact DNA directly, and one is located directly beneath these surface residues and may also be involved in DNA–histone interactions. One possibility for how Sin⁻ versions of histones alleviate the need for the SWI–SNF complex is that these mutants disrupt DNA–histone interactions which leads to an increased accessibility of transcription factor-binding sites. Alternatively, Sin⁻ versions may destabilize the dimer–tetramer interface of the histone octamer which may also lead to increased factor binding (Kruger *et al.*, 1995). Either one of these perturbations in nucleosome structure may mimic how the SWI–SNF complex remodels chromatin (for review, see Peterson and Tamkun, 1995).

In the present work, we have characterized the effects of Sin⁻ mutations in histone H4 on chromatin structure *in vivo* and on transcriptional repression of the yeast *PHO5* gene. Sin⁻ versions of histone H4 lead to an increased accessibility of nucleosomal DNA to Dam methyltransferase and micrococcal nuclease (MNase), and they also disrupt the ability of nucleosomes to supercoil DNA. These histone H4 mutants also disrupt nucleosome-mediated repression of the *PHO5* gene; surprisingly, maximal *PHO5* derepression requires upstream regulatory sequences. We propose a model in which Sin⁻ versions of histone H4 lead to a change in nucleosome structure that

Table I. Mutant H4 derivatives as the sole source of histone H4 confer a Sin⁻ phenotype

Strain	<i>HO-lacZ</i> activity (Miller units)
<i>SWI1⁺ HHF2⁺</i>	1.04
<i>swi1⁻ HHF2⁺</i>	0.22
<i>swi1⁻ hhf2-7</i>	1.04
<i>swi1⁻ hhf2-8</i>	0.88
<i>swi1⁻ hhf2-13</i>	0.69

Results of liquid β -galactosidase assays of strains with an *HO-lacZ* reporter. All assays were done in triplicate, and the averages are shown. Assay values for each strain varied by <20%. Strains used were CY311, CY635, CY636, CY637 and CY638.

is permissive for the binding of transcriptional activator proteins, but not for components of the general transcription machinery.

Results

In the original identification of Sin⁻ versions of histone H4, yeast strains contained two wild-type histone H4 genes (*HHF1* and *HHF2*) as well as a Sin⁻ version (*hhf2^{sin}*) on a low copy plasmid (Kruger *et al.*, 1995). As one might expect, these *hhf2^{sin}* mutants are partially dominant for the Sin⁻ phenotype (partial relief of the defect in *HO-lacZ* transcription due to inactivation of the SWI–SNF complex). One possibility is that the Sin⁻ phenotype requires a unique combination of wild-type and mutant histone H4. To test this possibility, we constructed a congenic set of *SWI1⁺* and *swi1⁻* yeast strains that express a Sin⁻ version of histone H4 as the sole source of H4 polypeptide. In these strains, both genes encoding histone H4 (*HHF1* and *HHF2*) have been deleted, and the cells contain two additional episomal copies of *HHF2*. The synthesis of a wild-type copy of histone H4 is under the control of the *GAL* promoter (pGAL-H4) while synthesis of one of three different Sin⁻ versions of histone H4 is controlled by the wild-type *HHF2* promoter. When grown in glucose, these strains express only the Sin⁻ version of histone H4 and they are viable, as described previously (Kruger *et al.*, 1995). Table I shows the results of liquid β -galactosidase assays which measure the expression of a chromosomal *HO-lacZ* fusion gene. In a *swi1⁻* mutant, *HO-lacZ* expression is 21% of the wild-type level. In the presence of the *hhf2-7* (R45C), *hhf2-8* (V43I) and *hhf2-13* (R45H) alleles, expression of *HO-lacZ* is restored to 100, 85 and 66% of wild-type levels, respectively. Therefore, nucleosomes that contain only Sin⁻ versions of histone H4 are sufficient to partially restore transcription in the absence of the SWI–SNF complex.

Stoichiometry of the histone octamer is not altered by Sin⁻ versions of histone H4

One mechanism by which Sin⁻ versions of histone H4 may alleviate the requirement for the SWI–SNF complex is by destabilizing interactions between the histone H3–H4 tetramer and the histone H2A–H2B dimers (Kruger *et al.*, 1995). This model predicts that chromatin isolated from cells that express only Sin⁻ versions of histone H4 will be depleted of H2A–H2B dimers. To investigate this possibility, chromatin was prepared from cells that express either wild-type or the R45H version of histone H4

Fig. 1. Stoichiometry of histones in chromatin that contains wild-type or the R45H version of H4. Soluble chromatin isolated from cells that contain either wild-type histone H4 (top panel) or the R45H version (bottom panel) were sedimented on sucrose gradients. Peak fractions were precipitated with acid, separated on 18% SDS–PAGE and stained with silver. The positions of histones H3, H2B, H2A and H4 are indicated to the right of the figure. Purified yeast histone octamers were electrophoresed in lane M.

and fractionated on sucrose gradients. Fractions were precipitated with acid and analyzed by SDS–PAGE and silver staining (Figure 1). Quantitation of the ratios of histones H2A and H2B to histones H3 and H4 indicates that the Sin⁻ version of histone H4 does not change the stoichiometry of the histone octamer. In addition, no changes in histone stoichiometry were observed if nucleosomes were sedimented under a wide range of salt concentrations (data not shown). This indicates that this Sin⁻ version of histone H4 is able to assemble a nucleosome with a normal stoichiometry of histones and, once this octamer is assembled, the histone H3–H4 tetramer/H2A–H2B dimer interface appears to be stable. These experiments do not rule out the possibility that this Sin⁻ version of histone H4 assembles nucleosomes at a lowered efficiency; however, similar amounts of octamers are recovered from equal amounts of wild-type and Sin⁻ yeast nuclei. These results are consistent with the view that Sin⁻ versions of histone H4 do not grossly impair histone–histone interactions.

Sin⁻ versions of histone H4 cause a dramatic change in the structure of bulk chromatin

Three of the five histone H3 and H4 Sin⁻ mutations alter surface residues that are predicted to be in direct contact with nucleosomal DNA. It is possible that Sin⁻ versions of histone H4 disrupt crucial histone–DNA contacts, which leads to an increased accessibility of nucleosomal DNA to transcription factors. To investigate this possibility, we analyzed the accessibility of nucleosomal DNA in isolated nuclei to digestion by MNase and in intact cells to methylation by *Escherichia coli* Dam methyltransferase (Figures 2 and 3).

To examine the accessibility of nucleosomal DNA to nuclease cleavage, nuclei were isolated from strains that

Fig. 2. Effect of a Sin^- version of H4 on chromatin structure. (A) Micrococcal nuclease (MNase) digestion of bulk chromatin. Nuclei from isogenic strains that contain either wild-type (CY438) or the R45H version (C442) of histone H4 were digested with MNase, and the purified DNA products were separated on an agarose gel and stained with ethidium bromide. In each case, the digestion profile is as follows (towards the center of the gel): no MNase, or 142 U of MNase for 7.5, 10, 15 or 20 min. The arrows indicate the ~160 bp nuclease-resistant gaps between MNase products that are indicative of nucleosome protection. (B) Chromatin structure of *HIS3*. Southern blot of gel shown in (A) probed with a 1.7 kb *Bam*HI *HIS3* probe.

express either wild-type or the R45H Sin^- version of histone H4 and treated with MNase. Digested DNAs were purified, electrophoresed on agarose gels, and stained with ethidium bromide to visualize bulk chromatin (Figure 2A). These DNAs were then transferred to nylon membranes and probed with ^{32}P -labeled DNA fragments to analyze

the chromatin structure surrounding the *HIS3* and *PHO5* genes (Figure 2B and data not shown). Comparison of the MNase digestions shows that chromatin that contains the Sin^- version of histone H4 is much more sensitive to MNase digestion than chromatin that contains wild-type histone H4. In the wild-type strain, a nucleosomal ladder consisting of 7–8 repeats could easily be detected, whereas only a dinucleosome repeat could be clearly detected when chromatin contained the Sin^- version (Figure 2A and B). Similar results were obtained in three independent experiments. In addition, the Sin^- version of histone H4 caused an increase in the smearing between the bands generated by MNase digestion, which suggests either a perturbation in nucleosome spacing (i.e. enhanced nucleosome mobility) or an increased accessibility of DNA wrapped onto the histone octamer.

***Sin*⁻ versions of histone H4 alter the accessibility of nucleosomal DNA to Dam methyltransferase**

It is possible that the changes in MNase digestion patterns described above may have occurred during the preparation of nuclei prior to or during nuclease digestion. To test whether Sin^- versions of histone H4 alter accessibility of DNA in a precisely positioned nucleosome in living cells, we assayed DNA methylation by Dam methyltransferase adjacent to the $\alpha 2$ operator of a well-characterized minichromosome, TALS4, in yeast strains that express Dam methyltransferase. In cells that express the $\alpha 2$ repressor (α cells), TALS minichromosomes contain precisely positioned nucleosomes adjacent to the $\alpha 2$ operator. In cells that lack the repressor (a cells), nucleosomes are present but are not as precisely positioned (Roth *et al.*, 1990; Kladde and Simpson, 1994).

To create the TALS4 minichromosome (Kladde *et al.*, 1996), GATC sites were introduced (Higuchi *et al.*, 1988) into the parent plasmid TALS with a translational frequency of about one per helical turn over greater than half of the length of DNA in nucleosome IV (Figure 3A). Previous mapping studies with MNase showed that the introduction of these sites does not affect nucleosome positioning (Kladde and Simpson, 1994). This result was expected since the positioning of nucleosomes next to the $\alpha 2$ operator in α cells is dominant, occurring on a range of DNA sequences (Roth *et al.*, 1990; Shimizu *et al.*, 1991) including a synthetic random sequence (Patterson and Simpson, 1994).

It has been shown previously that the levels of Dam methylation increase with culture density (Fisher-Adams and Grunstein, 1995; Kladde and Simpson, 1996). Thus, cells containing either wild-type histone H4 or the Sin^- versions, V43I or R45H, were grown to late log/stationary phase to ensure that levels of Dam methyltransferase were not limiting and to avoid potential complications due to the slower growth rates of the strains that contain the Sin^- versions of H4. A slower growth rate might decrease the rate at which replication dilutes methylation or a longer cell cycle may permit more time for modification. The levels of Dam methylation at GATC sites in nucleosome IV of TALS4 were assessed by digestion of isolated DNA with *Dpn*I, which cuts dimethylated sites, followed by primer extension (Figure 3A). The minichromosome was also digested with *Eco*RI to provide a unique cleavage site for run-off extension of another primer which serves

as an internal loading control (Figure 3B). In cells that express wild-type histone H4 (lanes 1 and 4), the pattern of methylation is qualitatively identical to the previously observed pattern (Kladde and Simpson, 1994). In α cells (lane 1), GATC sites in the linker and adjacent two helical turns of DNA internal to the nucleosome are highly methylated (i.e. cut by *DpnI*), whereas sites nearer to the nucleosomal pseudodyad are relatively inaccessible. In contrast, in **a** cells (lane 4), the methylation level at the GATC site located near the pseudodyad (1464 map units) was 7.5-fold greater than in α cells, indicating a much higher accessibility to Dam methyltransferase. The high levels of methylation preclude quantification of methylation levels at other sites further from the primer. These results demonstrate that cells containing wild-type H4

have a nucleosome precisely positioned next to the $\alpha 2$ operator in TALS4 in α cells, but not in **a** cells.

In α cells that contain a Sin⁻ version of histone H4 as the sole source of H4, a significant increase in methylation near the pseudodyad of nucleosome IV was observed (compare lanes 2 and 3 with lane 1). The V43I and R45H versions, respectively, led to a 3.5- and 5.0-fold increase in modification at the GATC site at 1464 map units relative to cells that express wild-type histone H4 (Figure 3C). Qualitatively, methylation was also increased at 1454 and 1445 map units in α cells containing the Sin⁻ versions of histone H4 versus wild-type H4. This increased level of methylation was not the result of increased expression of Dam methyltransferase (Figure 3D). Identical changes in Dam methyltransferase accessibility were also observed in cells harvested in early log phase growth (data not shown). Although the Sin⁻ versions of histone H4 lead to dramatic increases in methylation in nucleosome IV in α cells, the level of modification is not as high as that seen in **a** cells, demonstrating that the chromatin that contains Sin⁻ versions of H4 remains organized in α cells. This level of organization is presumably sufficient to maintain repression of **a** cell-specific genes since cells that contain Sin⁻ versions of H4 do not exhibit qualitative defects in mating or in pheromone production (Kruger *et al.*, 1995).

The ability of nucleosomes to supercoil DNA is impaired by Sin⁻ versions of histone H4

When DNA is wrapped 1.8 times around the histone octamer, a single negative supercoil is introduced into a closed circular plasmid (Germond *et al.*, 1975; Worcel *et al.*, 1981; Wang, 1982; Simpson *et al.*, 1985). To test whether Sin⁻ versions of histone H4 affect the ability of nucleosomes to constrain supercoils, we used a rapid isolation procedure to isolate total DNA from cells that express wild-type or the R45H version of histone H4, electrophoresed the DNA on chloroquine-agarose gels and measured the superhelical density of the endogenous 2 μ plasmid by Southern blot analysis (Figure 4). Plasmid

Fig. 3. Dam methylation in the TALS4 minichromosome in strains that contain wild-type and Sin⁻ versions of histone H4. **(A)** Primer extension analysis. The position of nucleosome IV and the location of GATC sites are indicated on the right. Total DNA was isolated from Dam-expressing α and **a** cells containing the indicated versions of histone H4 [*HHF2* (wt); *hhf2-8* (V43I); and *hhf2-13* (R45H)] in TALS4. Samples were digested with *DpnI* (and *EcoRI*, see below), and subjected to primer extension with a primer that anneals to the $\alpha 2$ operator and extends to first encounter a GATC site near the pseudodyad (1464 map units). Due to the high levels of methylation, this is the only site which yields data that are rigorously quantitative. **(B)** The loading control (from the same gel and PhosphorImager exposure as in A) was generated by linearizing TALS4 at the unique *EcoRI* site (1 map unit) followed by primer extension from the MS6b primer which yields the 65 bp product visible at the bottom of the gel. **(C)** Quantification of DAM methylation levels. The amount of methylation (arbitrary units) was determined on a PhosphorImager for the pseudodyad-proximal GATC site at 1464 map units and normalized to the 65 bp control product. Data are plotted as the fold increase in methylation at 1465 map units relative to that obtained in α cells containing wild-type histone H4. **(D)** Analysis of expression of Dam methyltransferase from stationary phase **a** cells. Total RNA was isolated from a parental strain (CY440) without integrated Dam and from Dam-expressing strains that contain wild-type and Sin⁻ versions of histone H4; 20 μ g of total RNA was loaded per lane. **(i)** 18S rRNA visualized by ethidium bromide staining. **(ii)** RNA hybridized with a probe containing the entire Dam-coding region.

Fig. 4. A Sin^- version of histone H4 interferes with the ability of nucleosomes to supercoil DNA. Genomic DNA from isogenic strains containing either wild-type (lane 1) or the R45H (lane 2) version of histone H4 was isolated (see Materials and methods) and electrophoresed on a 0.7% agarose gel in the presence of 0.6 $\mu\text{g/ml}$ chloroquine. DNA was transferred to nylon and blots were probed with a 2.2 kb *EcoRI* fragment from the 2 μ plasmid. The uppermost band is relaxed 2 μ plasmid DNA. Similar results were observed in three independent DNA preparations.

isolated from cells that express wild-type histone H4 contained an average of ~ 18 negative supercoils (Figure 4, lane 1); in contrast, plasmid isolated from cells that express the R45H version of histone H4 consistently showed an increase in the amount of fully relaxed plasmid, and the distribution of supercoiled species had an average of about nine negative supercoils (Figure 4, lane 2). This decrease in superhelical density is consistent with a loss of at least nine nucleosomes from the plasmid, or a dramatic change in the wrapping of DNA onto the histone octamer.

***Sin⁻* versions of histone H4 derepress *PHO5* transcription**

Transcription of the *PHO5* gene is repressed when the growth medium contains high levels of inorganic phosphate and expression is induced at least 20- to 30-fold when cells are starved for phosphate (Oshima, 1982; Rogers *et al.*, 1982). When cells are grown in high phosphate media (repressed state), nucleosomes are precisely positioned over the *PHO5* promoter region (Almer and Horz, 1986) and cover three of the four upstream activation sequence (UAS) elements as well as the TATA box. Upon induction, the chromatin structure of this region is altered such that all four UAS elements and the TATA element become accessible to nuclease digestion (Almer *et al.*, 1986). Transcriptional repression of *PHO5* appears to be due at least in part to these positioned nucleosomes since *PHO5* transcription can be partially derepressed by depleting nucleosomes *in vivo* (Han *et al.*, 1988).

Since nucleosomes appear to play a large role in repression of *PHO5*, we tested whether this repression might be disrupted by Sin^- versions of histone H4. Primer extension was used to measure *PHO5* expression in strains

Fig. 5. Derepression of *PHO5* transcription by Sin^- versions of H4. (A) RNA was isolated from cells that contain either wild-type (CY423), R45C (CY424), V43I (CY425), or the R45H (CY426) version of histone H4, and *PHO5*, *CLN3* and *LYS2* expression was analyzed by primer extension. Cells were grown in high (repressive) or low (inducing) phosphate media. (B) Primer extension analyses were performed on RNA from strains that contained either two copies of *hhf2-7* (lane 2, R45C), two copies of *HHF2* (lane 3, wild-type) or one copy of *HHF2* and one copy of *hhf2-7* (lane 4). Lane 1 is a primer extension control containing no RNA. Strains used were CY424, CY426 and CY539. (C) Primer extension analysis with RNA isolated from isogenic strains containing either wild-type histone H4 (lane 1, CY236), Sin^- versions of histone H4 (lane 3, R45C; lane 4, V43I; lane 5, R45H) or a deletion of *SIN1* (lane 2, CY237).

containing wild-type or Sin^- versions of histone H4 (Figure 5A). Expression of the *CLN3* or *LYS2* genes was used as an internal control for RNA loading. In strains that expressed wild-type histone H4, *PHO5* expression was high when cells were starved for phosphate and very low when cells were grown in high phosphate medium (4% of the induced level; Figure 5A; WT: L versus H phosphate levels). When cells expressed the R45C, R45H or V43I versions of histone H4, the induced level of *PHO5* was normal, but repression of *PHO5* was crippled; expression in high phosphate was 23, 31 and 8%, respectively, of the wild-type induced level (low phosphate). Derepression of *PHO5* was also observed if cells expressed both a wild-type and a Sin^- version of histone H4 (Figure 5B, lane 4). These levels of derepression are almost identical to the activation of *PHO5* transcription that occurs upon nucleosome depletion (24% of the induced level; data not shown; Han and Grunstein, 1988). Nucleosome loss, however, is a lethal event, whereas cells that contain Sin^- versions of histone H4 are viable.

Fig. 6. Derepression of *PHO5-lacZ* fusion genes by Sin⁻ versions of H4. Plasmids were introduced into cells containing either wild-type (CY553) or the R45H (CY554) version of histone H4. Plasmids pMH313, pMH314, pMH324 and pMH329 (Han and Grunstein, 1988) contain the indicated *PHO5* upstream regulatory sequences fused to the *E. coli lacZ* gene. Plasmids pLGA-312-178 (Guarente and Mason, 1983) and pLR1Δ2 (West *et al.*, 1984) contain fusions of the *CYC1* and *GAL1* minimal promoters (UAS⁻) to *lacZ*, respectively. β -Galactosidase activity assays were performed (Stern *et al.*, 1984) on at least six independent transformants and the Miller units were averaged. Values varied by <20%.

Deletion of the *SIN1* gene does not derepress *PHO5* transcription

Since mutations in genes encoding histone H3, histone H4 and *SIN1* can all cause a Sin⁻ phenotype, it is possible that they exert their effects through the same mechanism (Kruger and Herskowitz, 1991; Kruger *et al.*, 1995). If this is the case, then mutations in the *SIN1* gene should also lead to a derepression of *PHO5*. Levels of repressed *PHO5* transcription were measured in yeast strains containing either wild-type histone H4 (CY236), Sin⁻ versions of histone H4 (CY424, CY425, CY426) or a deletion of the *SIN1* gene (CY237) (Figure 5C). Unlike the R45H, R45C and V431 versions of histone H4, deletion of the *SIN1* gene did not derepress *PHO5* transcription; the level of transcription was the same for the wild-type control and *sin1* deletion strains (lanes 1 and 2, respectively). In contrast, *PHO5* was derepressed 12-, 13- and 5-fold by the R45C, R45H and V431 versions of histone H4, respectively (compare lanes 3, 5 and 4 with lanes 1 and 2). These results show that the phenotype of a *sin1* mutant and of Sin⁻ mutations in *HHF2* (histone H4) are not equivalent, and, furthermore, they suggest the possibility that *SIN1* and the *SIN* domain of histone H4 may play distinct roles in chromatin-mediated transcriptional repression.

Full derepression of *PHO5* by Sin⁻ versions of histone H4 requires UAS elements

Derepression of *PHO5* transcription requires four UAS elements, a TATA box at position -52, and an initiator element (Figure 6, top; Rudolph and Hinnen, 1987; Han and Grunstein, 1988; Vogel and Hinnen, 1990). In contrast, induction of *PHO5* by nucleosome depletion requires only the TATA and initiator elements (Han and Grunstein, 1988). To determine the *cis*-acting sequences required for *PHO5* derepression by Sin⁻ versions of histone H4, we analyzed expression from a set of *PHO5-lacZ* fusion reporters (Han and Grunstein, 1988). Reporter pMH313 contains the intact *PHO5* promoter upstream of the bacterial *lacZ* gene; reporter pMH314 contains only two of the four UAS elements; plasmid pMH324 has no UAS elements and pMH329 lacks all *PHO5* promoter elements, including the TATA box (Figure 6). These plasmids were introduced into cells that expressed either wild-type histone H4 or only the R45H version, and the β -galactosidase activity of extracts prepared from cells grown under repressive conditions (high Pi) was determined (Figure 6). In the case of the full-length *PHO5* reporter (pMH313), the R45H version of histone H4 led to a 5.6-fold increase in expression when compared with expression in the strain that contained only wild-type histone H4. When only two

of the UAS elements were deleted (pMH314), expression was derepressed only 2-fold in the strain containing the R45H version of histone H4. This deletion eliminates induced expression in wild-type cells (Han and Grunstein, 1988). Likewise, when all the UAS elements were removed (pMH324), expression was derepressed only 2.8-fold. No depression was observed if both the UAS and TATA sequence elements were deleted (pMH329). Thus, maximal derepression of *PHO5-lacZ* by the R45H version of histone H4 requires both upstream and proximal promoter elements. These results differ dramatically from the effects of nucleosome depletion on expression from these same *PHO5-lacZ* reporters—derepression is nearly identical in the presence and absence of UAS elements (Han and Grunstein, 1988).

In addition to the derepression of *PHO5* expression, nucleosome depletion also activates expression from several other reporters that contain only minimal promoters (Han and Grunstein, 1988). This result led to the suggestion that the transcriptional activation caused by nucleosome depletion is due to an increased accessibility of the general transcription machinery for proximal promoter elements. We tested two UAS-less reporters for activity in cells that express the R45H version of histone H4 (Figure 6, *CYC1* and *GALI* panels). In contrast to nucleosome depletion, the Sin⁻ histone mutant does not derepress these minimal promoter elements (2.6 versus 2.9 units for *CYC1*; 28.1 versus 30.7 units for *GALI*). These results reinforce the view that transcriptional derepression caused by Sin⁻ versions of histone H4 and by nucleosome loss occur by distinct mechanisms.

Discussion

We describe the effects of Sin⁻ versions of histone H4 on yeast chromatin structure and function. These altered histones are competent for assembly into nucleosomes, but they alter nucleosome structure such that nucleosomal DNA is more accessible to cleavage by MNase and to methylation by bacterial Dam methyltransferase. In addition, Sin⁻ versions of histone H4 grossly impair the ability of nucleosomes to constrain negative supercoils. These changes in nucleosome structure have a significant impact on transcriptional regulation. For instance, the requirement for the SWI-SNF complex in *HO* transcription is partially relieved, and repression of the *PHO5* gene is disrupted. In the latter case, the extent of derepression is similar in magnitude to loss of half the nucleosomes from the *PHO5* gene. Surprisingly, maximal derepression of *PHO5* by Sin⁻ versions of histone H4 requires intact *PHO5* upstream regulatory sequences. These results suggest that nucleosomes that contain Sin⁻ versions of histone H4 may be permissive for the binding of transcriptional activator proteins, but that they are still competent to block the binding of the basal transcription machinery to nucleosomal sites.

Are the effects of Sin⁻ versions of histone H4 related to SWI-SNF action?

Sin⁻ versions of histones H3 and H4 were identified originally as mutations that partially alleviated defects in expression of a *HO-lacZ* fusion gene in *swi1*, *swi2* or *swi3* mutants (Sternberg *et al.*, 1987; Kruger *et al.*, 1995).

These observations were instrumental in developing the hypothesis that the SWI-SNF complex functions in transcription by disrupting chromatin (Kruger *et al.*, 1995). One possibility is that Sin⁻ versions of histones create an altered nucleosome structure that mimics SWI-SNF-dependent nucleosome disruption. Alternatively, Sin⁻ histones may disrupt nucleosome structure quite differently, and alleviation of *swi/snf* phenotypes may be an example of non-specific bypass suppression. However, several results indicate a surprising degree of similarity between SWI-SNF action and the properties of nucleosomes that contain Sin⁻ versions of histone H4. For instance, Sin⁻ versions of H4 do not appear to destabilize histone-histone interactions, but do change histone-DNA interactions such that nucleosomal DNA is more accessible to nucleases and Dam methyltransferase. Likewise, the SWI-SNF complex disrupts histone-DNA interactions which leads to an increased accessibility of nucleosomal DNA to transcription factors (Cote *et al.*, 1994; Imbalzano *et al.*, 1994; Kwon *et al.*, 1994), but the complex does not grossly destabilize histone-histone interactions (Cote *et al.*, 1994; J.Cote, C.L.Peterson and J.Workman, unpublished results). SWI-SNF is able to change the topology of minichromosomes *in vitro* (Kwon *et al.*, 1994), and correspondingly Sin⁻ versions of H4 have an equally dramatic effect on minichromosome topology *in vivo*. Finally, SWI-SNF is required for the induced level of expression of several genes in yeast, but it is not required for transcription from minimal promoters or for genes that are expressed at a low constitutive level. Similarly, Sin⁻ versions of H4 appear to enhance the function of one or more upstream activators of *PHO5* under repressing growth conditions, but these altered histones do not affect expression from minimal promoters. These observations suggest the possibility that nucleosomes that contain Sin⁻ versions of H4 may be similar to a disrupted nucleosome structure due to SWI-SNF action. This hypothesis predicts that DNA templates reconstituted with purified Sin⁻ versions of histones will not be disrupted further by SWI-SNF.

Phenotypic differences between nucleosome loss and Sin⁻ versions of histone H4

Many of the phenotypes caused by Sin⁻ versions of histone H4 are similar to those caused by nucleosome depletion. For instance, nucleosome depletion and Sin⁻ versions of H4 both enhance the MNase digestion rate of bulk chromatin and cause a similar derepression of *PHO5* transcription. Furthermore, both nucleosome depletion and Sin⁻ versions of histone H4 lead to a dramatic loss in negative supercoils from minichromosomes. However, several results indicate that the changes in chromatin structure due to Sin⁻ versions of H4 are not equivalent to nucleosome loss. First, in strains that are induced for nucleosome loss, cells rapidly arrest growth at the G₂-M boundary and lose viability. In contrast, strains that contain Sin⁻ versions of H4 as the sole source of H4 protein are viable and, in the case of V43I, the growth rate is indistinguishable from strains containing wild-type H4 (Kruger *et al.*, 1995). Secondly, oligonucleosomes isolated from nuclei of strains containing Sin⁻ versions of H4 have a normal complement of histones and these nucleosomes appear to be stable *in vitro*. We have noticed, however, that isolated oligonucleosomes that contain the R45H

version of H4 do not bind as tightly to hydroxylapatite resin as compared with those from wild-type chromatin, which would be consistent with a loosening of histone–DNA contacts (M.A.Wechsler and C.L.Peterson, unpublished results).

Nucleosome depletion *in vivo* can lead to transcriptional activation of reporter genes that contain only a core promoter element (TATA and initiator elements). In most cases the effect is dramatic, for instance transcriptional activation from a minimal *CYC1* reporter gene is induced 94-fold by nucleosome depletion (Han and Grunstein, 1988). In contrast, a Sin⁻ version of histone H4 does not lead to significant derepression of either a *CYC1* or *GAL1* minimal reporter gene. In the case of *PHO5*, nucleosome depletion derepresses expression equally well in the presence or absence of upstream regulatory elements. Sin⁻ versions of H4, on the other hand, lead to a similar level of derepression but require these regulatory sequences for maximal derepression. These results suggest that Sin⁻ versions of H4 have a more subtle effect on chromatin structure than does nucleosome loss, and that Sin⁻ mutations may enhance the ability of upstream activator proteins to function in a chromatin context, but do not perturb chromatin sufficiently to facilitate promiscuous function of the general transcription machinery. This view is consistent with the fact that Sin⁻ alleles of histone H3 partially alleviate the requirement for the SWI–SNF complex, but do not alleviate the need for upstream activator proteins (i.e. SWI4 and SWI6; Sternberg *et al.*, 1987).

Sin⁻ versions of histone H4 and the structure of the histone octamer

In the Arents and Moudrianakis model of the nucleosome (Arents and Moudrianakis, 1993), the central 12 turns of DNA contact 12 repetitive structural motifs located on the octamer surface. Each histone dimer presents two parallel β -bridge motifs and one paired end of helices (PEH) motif on the octamer surface. These motifs contain either positively charged amino acids, arginine and lysine, or hydroxyl-containing residues that are predicted to contact DNA. The repetitive nature of the DNA–histone contacts on the octamer surface suggests that each contact might contribute equally to nucleosome structure, stability and function. The residues altered by Sin⁻ mutations, however, are tightly clustered on the surface of the histone octamer and may disrupt only a localized region of histone–DNA contacts (Kruger *et al.*, 1995). The R116H (*hht1-1/sin2-1/hht2-1*) and T118I (*hht2-3*) substitutions in histone H3 affect adjacent surface residues that are predicted to interact with nucleosomal DNA (Kruger *et al.*, 1995). Likewise, the R45C (*hhf2-7*) and R45H (*hhf2-13*) substitutions in histone H4 alter a residue in one of the parallel β -bridge motifs that is adjacent to H3 T118 on the octamer surface (Arents and Moudrianakis, 1993; Wolffe, 1994; Kruger *et al.*, 1995). While the V43I (*hhf2-8*) substitution does not affect a surface residue in histone H4, it alters an amino acid that lies just below the β -bridge that contains R45. Thus, Sin⁻ mutations appear to disrupt a clustered set of DNA–histone interactions at two symmetry-related positions on each dimer of the (H3–H4)₂ tetramer. These substitutions may loosen the central DNA wrap around the histone octamer (Wolffe, 1994) or

drive the DNA onto an alternative path. The enhanced accessibility of nucleosomal DNA to MNase digestion and to methylation by Dam methyltransferase is consistent with both of these possibilities. Furthermore, a loosening of the central wrap might give rise to the dramatic changes in minichromosome topology caused by Sin⁻ versions of histone H4, since each mutant octamer may constrain less than one negative supercoil.

Since Sin⁻ mutations alter residues in one of the β -bridge motifs, it is surprising that mutations that alter other β -bridge residues or residues in PEH motifs were not recovered in previous hunts for Sin⁻ alleles of histones (Kruger *et al.*, 1995). Recently, a mutagenic PCR approach to identify Sin⁻ alleles in *HHF2* also identified mutations in residues that altered the β -bridge motif that contains R45 (Z.Han and C.L.Peterson, unpublished). These results reinforce the view that histone–DNA contacts for the central 12 turns of nucleosomal DNA are not functionally equivalent.

Materials and methods

Media, strains and plasmids

Strains are described in Table II. Strain UKY412 is described in Kim *et al.* (1988). Cultures were grown at 30°C in YEPD (2% yeast extract, 1% bacto-peptone, 2% glucose) or synthetic media [6.7 g/l yeast nitrogen base without amino acids (Difco laboratories)] supplemented with amino acids as described (Stern *et al.*, 1984). Synthetic media contained either 2% dextrose (SD media) or 2% galactose, 0.5% sucrose (SGS media) as indicated in the text. For induction of *PHO5* expression, cultures were grown in SGS without tryptophan and uracil, washed with water, and inoculated into either high or low phosphate media prepared as described by Han *et al.* (1988). Plasmids pCP550 and pCP551 contain the *Bam*HI–*Eco*RI fragment of *HHF2* and *hhf2-13* respectively in pRS414 (Sikorski and Hieter, 1989).

Stoichiometry of histones in yeast chromatin

Nuclei were prepared as described (Linder and Thoma, 1994) with the following modifications. Spheroplasts from 2 l of cells at 2.7×10^7 cells/ml were lysed by passage through a Yamato LH-21 homogenizer four times (1000 r.p.m., 4°C). All subsequent manipulations were done at 4°C unless otherwise noted. Lysed spheroplasts were centrifuged twice (5 min, 5700 r.p.m.) in a JA-17 rotor (Beckman). The crude nuclear pellet was harvested (20 min, 16 000 r.p.m.) and the resulting pellet was washed once with NP-40 as described. Soluble chromatin was extracted from the NP-40-washed nuclei as described, except that NP-40 nuclei were resuspended in buffer A [20 mM Tris pH 8.0, 150 mM NaCl, 5 mM KCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF)] to A₂₆₀ 0.1, adjusted to 5 mM CaCl₂, and incubated with 186 U/ml of MNase for 2.5 min at 37°C. Nuclei were pelleted in a microcentrifuge for 20 min at 14 000 r.p.m.. Soluble chromatin in the supernatant was fractionated on a linear 5–15% (w/v) sucrose gradient in 10 mM Tris pH 8.0, 1 mM EDTA, 10 mM NaCl (17.5 h, 20 000 r.p.m.) in an SW-40 rotor (Beckman). Fractions of 0.5 ml were collected, 50 μ g of bovine insulin was added, and protein was precipitated with 20% trichloroacetic acid (final concentration). Samples were separated on 18% SDS–PAGE gels and visualized with Silver Stain Plus (Bio Rad). Histones were quantitated with a Zeineh scanning densitometer SLR-1D/2D.

Micrococcal nuclease digestion of yeast nuclei and analysis of bulk chromatin structure

Nuclei were isolated from 4 l of cells that contained either wild-type (CY438) or R45H (CY442) versions of histone H4 as described above, except that after the NP-40 wash, the nuclei were resuspended in 1 ml of buffer B (100 mM Tris pH 8.0, 50 mM NaCl, 10 mM MgSO₄, 20% glycerol, 3 mM dithiothreitol, 2 mM EDTA, 1 mM PMSF, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 0.097 TIU/ml aprotinin), frozen in liquid nitrogen and stored at –70°C. For MNase digestion, thawed nuclei were collected in a microcentrifuge, resuspended in MNase digestion buffer (1 M sorbitol, 20 mM PIPES pH 6.5, 5 mM CaCl₂, 150 mM NaCl) to

Table II. Yeast strains used

Strain	Genotype
CY236	MATa <i>HO-lacZ ura3-52 leu2</i> ⁼
CY237	MATa <i>HO-lacZ sin1Δ ura3-52 leu2</i> ⁼ , congenic to CY236
CY311	MATa <i>HO-lacZ ade2-101° lys2-801^a trp1-Δ1 Δhhf1::HIS3 Δhhf2::LEU2</i> [pUK499 (URA3 pHHF2)]
UKY412	MATa <i>ade2-101° his3-Δ200 leu2-3,112 lys2-801^a trp1-Δ90 ura3-52 thr⁻ tyr⁻ arg4-1 Δhhf1::HIS3 Δhhf2::LEU2</i> [pUK499 (URA3 pHHF2)]
CY423	isogenic to UKY412 but also contains pUK421 (<i>TRP1 pGAL1-HHF2</i>)
CY424	isogenic to CY423, but pCP326 (<i>URA3 hhf2-7</i>) replaces pUK499
CY425	isogenic to CY423, but pCP327 (<i>URA3 hhf2-8</i>) replaces pUK499
CY426	isogenic to CY423, but pCP328 (<i>URA3 hhf2-13</i>) replaces pUK499
CY439	isogenic to UKY412, but pCP327 (<i>URA3 hhf2-8</i>) replaces pUK499
CY440	MATα isogenic to UKY412
CY441	isogenic to CY440, but pCP327 (<i>URA3 hhf2-8</i>) replaces pUK499
CY442	isogenic to UKY412 but pCP328 (<i>URA3 hhf2-13</i>) replaces pUK499
CY444	isogenic to CY440, but pCP328 (<i>URA3 hhf2-13</i>) replaces pUK499
CY539	isogenic to UKY412 but pCP326 (<i>URA3 hhf2-7</i>) replaces pUK499 and also contains pCP550 (<i>TRP1 HHF2</i>)
CY553	isogenic to UKY412 but pCP550 (<i>TRP1 HHF2</i>) replaces pUK499
CY554	isogenic to UKY412 but pCP551 (<i>TRP1 hhf2-13</i>) replaces pUK499
CY626	isogenic to UKY412 but also contains pCP550 (<i>TRP1 HHF2</i>)
CY635	MATα <i>HO-lacZ swi1Δ::LEU2 ade2-101° lys2-801^a trp1-Δ1 Δhhf1::HIS3 Δhhf2::LEU2</i> [pUK421 (<i>TRP1 pGAL1-HHF2</i>)] [pUK499 (<i>URA3 pHHF2</i>)], congenic to CY311.
CY636	isogenic to CY635, but pCP326 (<i>URA3 hhf2-7</i>) replaces pUK499
CY637	isogenic to CY635, but pCP327 (<i>URA3 hhf2-8</i>) replaces pUK499
CY638	isogenic to CY635, but pCP328 (<i>URA3 hhf2-13</i>) replaces pUK499

a DNA concentration of 580 µg/ml, and digested with 286 U/ml of MNase for 7.5, 10, 15 or 20 min. The reactions were terminated with 0.1 volume of stop solution (0.5 M Tris pH 8.0, 250 mM EDTA, 5% SDS). Samples were treated with 1.7 mg/ml proteinase K at 50°C for 2 h, extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), once with chloroform:isoamyl alcohol (24:1), and ethanol precipitated. DNA was resuspended in TE (10 mM Tris pH 8.0, 1 mM EDTA) and electrophoresed on a 1% agarose-TBE gel. Southern blots were probed with either a random primed ³²P-labeled 1.7 kb *Bam*HI fragment containing *HIS3* or a 764 bp *Apa*I-*Bam*HI fragment containing *PHO5*.

Determination of accessibility to Dam methyltransferase in TALS4

The *dam* methyltransferase gene expressed from a fortuitous *Escherichia coli* sequence was integrated into strains UKY412, CY439, CY440, CY441, CY442 and CY444 at the *LYS2* locus as reported previously (Kladde and Simpson, 1994). Primer extension analysis on TALS4 (Kladde *et al.*, 1996) was performed with the following modifications: cells were grown at 30°C in minimal medium lacking tryptophan until late logarithmic/stationary phase. Total nucleic acid was isolated as described (Holm *et al.*, 1986) with the addition of five phenol:CHCl₃ extractions and a 3 M ammonium acetate precipitation (Gilbert *et al.*, 1992). Samples were resuspended in 50 µl of 1 mM Tris pH 7.4, 0.1 mM EDTA. Samples were digested with *Eco*RI and *Dpn*I under conditions that ensure complete digestion and were then subjected to primer extension analysis as described (Kladde and Simpson, 1994) except that the MS6 primer used for the loading control, MS6b, was shortened at the 5' end by 11 nucleotides.

Northern analysis

For analysis of Dam methyltransferase transcripts, total yeast RNA was prepared by the LiCl isolation method and electrophoresed as described by Rose *et al.* (1990), except that the formaldehyde was omitted and 0.5 µg/ml ethidium bromide was included in the gel and the running buffer. The RNA was transferred to a GeneScreen Plus membrane and probed with a fragment containing the complete Dam-coding region which was amplified by PCR and labeled by random priming.

Superhelical density of plasmid DNA

Total yeast DNA was isolated from 10 ml cultures of log phase cells. Cells were collected (10 min, 4000 r.p.m.) in a J6-B centrifuge (Beckman) and resuspended in 1 ml of 15 mM Tris pH 8, 5 mM EDTA, 1.5% toluene cooled to -20°C. Cells were then washed once with TE and spheroplasts prepared by incubation for 15 min at 37°C in 1 M sorbitol, 100 mM Tris pH 8.0, 20 mM EDTA, 0.1% 2-mercaptoethanol, and 1 mg/ml zymolyase 100T. Spheroplasts were collected in a microcentrifuge (5 min, 5000 r.p.m.) at 4°C, resuspended in 0.3 ml of TE, and lysed with one-tenth volume of 10% SDS at room temperature for 5

min. Insoluble material was precipitated with 0.2 ml of 3 M potassium acetate, and nucleic acids in the supernatant were precipitated with ethanol. The isolated DNA was treated with RNase, ethanol precipitated and electrophoresed in 0.1 M Tris-borate buffer containing 0.6 µg/ml chloroquine at 2.5 V/cm for 40 h. Southern blots were probed with a random primed ³²P-labeled 2.2 kb *Eco*RI fragment containing 2 µ sequences.

Primer extension analysis of PHO5 transcription

Cells containing either wild-type (CY423), V43I (CY424), R45H (CY425), or the R45C (CY426) version of histone H4 were grown for 8 h in YEPD media that contained either high or low phosphate. RNA was isolated from log phase cells and primer extension assays were performed as described (Peterson *et al.*, 1991) using the following primers: *PHO5* 5'-CCTAAGGGAATGGTACCTGC-3'; *CLN3*, 5'-CTAGCGGTAGCATACCTGC-3'; *LYS2*, 5'-GCTCGACTCCATCGACAT-TGC-3'. Autoradiographs were quantitated by PhosphorImager analysis (Molecular Dynamics), and the *PHO5* signals were normalized to the *CLN3* signal to control for RNA loading.

Acknowledgements

We thank M.Grunstein for strains and plasmids, Gregory Viglianti for helpful advice and discussions, Mary-Ann Bjornsti for advice on the supercoiling assay and the rapid DNA isolation procedure for that assay, and Loree Griffin-Burns and other members of the Peterson laboratory for comments on the manuscript. This work was supported by NIH grants GM49650 to C.L.P. and F32 GM16109 to M.A.W. C.L.P. is a scholar of the Leukemia Society of America.

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Received on October 1, 1996; revised on December 17, 1996