Histone octamer function in vivo: mutations in the dimer–tetramer interfaces disrupt both gene activation and repression

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Introduction

The fundamental unit of chromatin organization, the histone core complex, is maintained as an octamer by two types of protein–protein interactions (Eickbush and Moudrianakis, 1978). One class is the strong interaction between H3 and H4 within the tetramer, and between H2A and H2B within the dimer subunits. The second class is the weaker interaction between the [H3–H4]2 tetramer and the two [H2A–H2B] dimer subunits. These dimer–tetramer interactions are of considerable interest because they are proposed to play important roles in many aspects of chromosome function.

DNA replication is one such candidate function. Chromatin assembly in vivo is thought to occur through the stepwise deposition of histone subunits onto newly replicated DNA. Biochemical experiments have shown that [H3–H4]2 tetramers are deposited first during assembly, and then nucleosome formation is completed with the later addition of [H2A–H2B] dimers (Worc al et al., 1978; Smith et al., 1984). Several in vitro studies with replication-coupled chromatin assembly systems are consistent with this two-step model (Dilworth et al., 1987; Almouzni et al., 1990). It has also been suggested that pre-existing histone octamers may dissociate into tetramers and dimers during replication (Jackson, 1990). Thus, these interactions could be significant both for the assembly of new nucleosomes, and for the rearrangement of old nucleosomes during replication.

Several lines of evidence suggest that RNA transcription is also likely to depend on functional histone dimer–tetramer interactions. In vivo, histone [H2A–H2B] dimers are in dynamic exchange in the chromatin, part of which appears to depend on RNA polymerase activity (Jackson, 1990). Biochemical and physical analyses of nucleosomes from chromatin partially enriched for actively transcribing genes have indicated an altered structure consistent with the depletion of one of the [H2A–H2B] dimers (Baer and Rhodes, 1983; Locklear et al., 1990). Strong support for a role for dimer–tetramer interactions in transcription is also provided by genetic experiments in Saccharomyces cerevisiae where normal transcription depends on the balanced gene dosage of the histone gene sets. Changes in the normal ratio of the histone H2A–H2B gene sets relative to the H3–H4 gene sets, either too high or too low, can affect the selection of specific gene promoters (Clark-Adams et al., 1988) and bypass the need for positive transcriptional activation complexes, presumably by disrupting normal chromatin architecture (Hirschhorn et al., 1992). Recently, bypass mutants in transcriptional activation were identified in histones H3 and H4, and one class of such mutants were in the histone fold facing the [H2A–H2B] interface of the octamer (Kruger et al., 1995). A variety of studies in vitro are consistent with these results. For example, nucleosomes lacking one [H2A–H2B] dimer interact with RNA polymerase more strongly than the complete nucleosome core (Gonzalez et al., 1987) and are transcribed more efficiently (Gonzalez and Palacian, 1989). In addition, the histone-binding protein nucleoplasm facilitates the binding of the transcription factors GAL4-AH, USF and SP1 to nucleosomal DNA through the sequential displacement of [H2A–H2B] dimers followed by H3–H4 tetramer displacement onto competing DNA (Chen et al., 1994).
thesis, changes in the dosage of the H2A–H2B gene sets in S.cerevisiae have been reported to alter the chromatin over certain chromosomal regions (Norris et al., 1988).

Finally the altered stoichiometry of the core histones produced by imbalanced expression of the octamer sub-units also impairs the fidelity of mitotic chromosome transmission, suggesting possible roles for these dimer–tetramer interactions in chromosome behavior during mitosis (Meeks-Wagner and Hartwell, 1986; Smith and Stirling, 1988).

All of these results suggest that interactions between dimer and tetramer subunits of the protein core of the nucleosome may be critical for normal nuclear functions. Here we report the results of the first direct genetic analysis of these interactions and their roles in gene expression. Based on the X-ray crystal model of the histone octamer, we perturbed the dimer–tetramer interface with site-directed mutations and examined the effects of these mutations on transcription, cell cycle progression and general viability in the budding yeast S.cerevisiae. The phenotypes of these mutants show that disruption of the normal dimer–tetramer interactions can have both strong positive and negative effects on gene transcription, and define a new role for chromatin structure in the regulation of G1 progression in the cell division cycle.

Results

Selection of tyrosines for mutagenesis

In choosing sites for directed mutations that would specifically alter the dimer–tetramer interfaces of the histone octamer, we focused on tyrosine residues in H4 based on the results of both solution and crystallographic studies. Early findings from solution physicochemical studies (Eickbush and Moudrianakis, 1978) demonstrated that the core histone octamer in solution behaves as a tripartite thermodynamic entity in reversible equilibrium with its subunits, one [H3–H4], tetramer and two [H2A–H2B] dimers. Analysis of the properties of this equilibrium led directly to the proposal that the centrally located tetramer interacts with the two flanking dimers via a limited number of contacts that include essential tyrosines. The reversible modulation of these interfacial contacts was proposed to be responsible for the functional cycles of chromatin. The importance of tyrosine contacts for histone octamer structure has since been supported by a wide variety of biophysical studies involving both spectroscopic analysis (Butler and Olins, 1982; Michalski-Scribe et al., 1982) and chemical modifications (Chan and Piette, 1982; Kleinschmidt and Martinson, 1984; Zweidler, 1992).

The tripartite organization of the histone octamer and the involvement of tyrosines in the integrity of the structure were confirmed by crystallographic studies (Arents et al., 1991; Arents and Moudrianakis, 1993) that also identified a common architectural motif among the four core histones, termed the histone fold (Arents and Moudrianakis, 1993). The core octamer of the nucleosome contains four structural subunits, in three thermodynamic domains, creating a left-hand superhelical protein ramp (Arents et al., 1991). Proceeding into the octamer structure along the superhelical axis, the order of dimer subunits is [H2A1–H2B1], [H31–H41], [H32–H42] and [H2A2–H2B2]. Figure 1A shows a view of the histone octamer, looking down the superhelical axis, with the molecular 2-fold axis horizontal. In this view, the [H2A2–H2B2] dimer subunit (blue) and the [H32–H42] dimer subunit (H3 green and H4 white) comprise most of the visible structure, with a portion of H3 (green) also visible in the lower left quarter of the structure.

The interfaces between the centrally located tetramer and the flanking dimers are formed from both fold and non-fold elements and contain a number of tyrosine residues in two distinct groupings. In the first group, tyrosine 72 (Y72) and tyrosine 88 (Y88) of H4 interact with one of the flanking [H2A–H2B] dimers, while tyrosine 98 (Y98) interacts with the other. This is illustrated in Figure 1B where the [H2A2–H2B2] dimer has been removed, exposing Y98 (black) of H4 which interacts with that dimer. On the other hand, Y72 (yellow) and Y88 (red) of H4 are only partially visible because they are in contact with the remaining [H2A1–H2B1] dimer in the figure. The back view shown in Figure 1C reveals the interactions of the other H4 subunit. In this view, it can be seen that removal of the [H2A2–H2B2] dimer has exposed Y72 (yellow) and Y88 (red) of H4 where they would normally make contact with that dimer. The Y98 residue of H4 is not visible in this view because it is in contact with the remaining [H2A1–H2B1] dimer and covered by that dimer. The symmetry of these interactions is illustrated in Figure 1D in which just the [H3–H4] tetramer is shown. Thus, one of the major features of the dimer–tetramer interactions in the histone octamer is that each of the H4 molecules in the [H3–H4] tetramer touches both [H2A–H2B] dimer subunits across the dimer–tetramer interfaces.

Further details of these interactions are depicted in the ribbon representation shown in Figure 1E. In this view of the octamer, the [H31–H41] dimer subunit has been removed from the model for clarity, leaving the dimers [H2A1–H2B1], [H32–H42] and [H2A2–H2B2]. The two groupings of interactions described above are apparent. In the first group, where [H2A2–H2B2] contacts the tetramer, the histone fold part of H4 interacts with the histone fold part of H2B to form a four-helix bundle. Although a number of residues from both H2B and H4 contribute to this interface, one of the most striking features of the interface is the large hydrophobic domain generated by the contacts between three tyrosines: Y72 and Y88 from H4, and Y83 from H2B. These three tyrosines form a cluster in which the planes of the side chains are approximately perpendicular to each other. In the second group, the contact between H4 and [H2A1–H2B1] arises from a non-fold, extended chain arm of H4 running roughly parallel to a non-fold, extended chain arm of H2A. Most of these contacts arise from main chain interactions. Within this interface, however, Y98 of H4 is exceptional due to the insertion of the large tyrosyl ring into a cleft in the dimer surface. Symmetrical interactions occur with the other [H31–H41] dimer subunit not shown in this representation. Thus, there are two distinct classes of interactions between histone H4 and the [H2A–H2B] dimers, and both biophysical and structural studies have identified tyrosine residues as key participants in each type of interaction.
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Fig. 1. Tyrosine residues in the dimer–tetramer interfaces of the core histone octamer. (A) View of the histone octamer looking down the superhelical axis with the molecular 2-fold axis horizontal from left to right. (B) Same view as (A), but with the second dimer subunit, [H2A2–H2B2], removed to expose the buried interface. (C) A view of the hexamer substructure model shown in (B), created by rotating 90° around the vertical axis, looking down the molecular 2-fold axis from the ‘back’. (D) Same view as (C) with the other dimer subunit, [H2A1–H2B1], also removed leaving just the [H3–H4]2 tetramer. For (A–D), histone H4 is white, histone H3 is green and the H2A–H2B dimer subunits are blue. Histone H4 Tyr72 is yellow, Tyr88 is red and Tyr98 is black. (E) A ribbon representation illustrating the relationship of the two [H2A–H2B] dimer subunits with the [H3–H4]2 dimer subunit. For (A–D), histone H4 is white, histone H3 is green and the H2A–H2B dimer subunits are blue. Histone H4 Tyr72 is yellow, Tyr88 is red and Tyr98 is black. Interactions symmetrical with those shown for the [H3–H4]2 dimer subunit in the panel are formed by the [H3–H4]2 dimer subunit as well. The [H2A1–H2B1] dimer is dark blue, the [H3–H4]2 dimer is white and the [H2A2–H2B2] dimer is light blue. The H4 tyrosines Y72, Y88 and Y98 (white) and the H2B tyrosine Y83 (blue) are modeled with the tyrosyl ring shown.

Construction and properties of mutants

Based on these structural considerations, we constructed mutant histone H4 alleles with amino acid substitutions at positions 72, 88 and 98. In the initial series of constructs, the tyrosine codons at these positions were changed to glycine codons, creating mutant alleles hhf1-36 (Y72G), hhf1-37 (Y88G) and hhf1-38 (Y98G). The wild-type gene and each of these new mutant alleles were integrated at the LEU2 locus on chromosome III, providing the only source of histone H4 in their respective cells (see Materials and methods). These ectopic integrations produced a set of strains that were isogenic except for their histone H4 alleles.

As can be seen in Figure 2, all three mutants have
does not support cell growth. This result is consistent with the overall chromatin structure of the viable H4 tyrosine mutants.

Fig. 2. Conditional growth of histone H4 tyrosine mutants. Growth is shown on YPD plates for wild-type and four histone H4 mutant strains after incubation for 5 days at 28°C or 37°C. All the strains were isogenic except for the histone H4 alleles: WT (HHT1), Y98W (hhf1-38), Y98H (hhf1-37), Y88G (hhf1-39) and Y72G (hhf1-36).

Fig. 3. Micrococcal nuclease digestion of chromatin. The ethidium bromide staining of the nuclease-digested chromatin is shown for wild-type (HHT1) and Y72G (hhf1-36) mutant strains, both for cells grown at 28°C and after 3 h at 37°C. Similar nucleosome ladders can be observed for the mutant, the wild-type strain and the mutant at 37°C.

The result for hhf1-36, which has the most severe phenotype, is shown in Figure 3. At both permissive and restrictive temperatures no differences were detected in the oligonucleosome ladders produced by partial micrococcal nuclease digestion. Similar results were obtained for hhf1-37 and hhf1-38 (data not shown). These results indicate that there is no gross defect in the general organization of nucleosomes in the H4 tyrosine mutants at either the permissive or restrictive temperature, and are similar to those obtained for the overall chromatin structure of histone H2A and H2B gene dosage mutants (Norris et al., 1988).

HTA1–HTB1 synthetic dosage phenotypes

The pattern of viability of the single and double glycine substitution mutants was consistent with the disruption of interactions between histone H4 and the [H2A–H2B] dimers. We reasoned that if point mutations in the H4 tyrosines perturb these interactions, then altered dosage of the H2A and H2B genes might significantly affect the growth phenotype of the mutants. To test the effect of reduced H2A and H2B gene dosage, we deleted the HTA1–HTB1 gene set encoding these proteins (Hereford et al., 1979) by one-step gene disruption (see Materials and methods). Decreased dosage of HTA1–HTB1 did not alter the temperature-dependent growth characteristics of either the wild-type or mutant H4 strains (data not shown). To test the effect of overexpression of histones H2A and H2B, strains expressing hhf1-36 (Y72G), hhf1-37 (Y88G) or hhf1-39 (Y98H) were transformed with a high copy YEp24-derived plasmid expressing the HTA1–HTB1 gene set and examined for their growth at restrictive and semi-permissive temperatures. Overexpression of HTA1–HTB1 had no detectable effect on hhf1-37 (Y88G) at either 34°C or 37°C (data not shown). However, as can be seen in Figure 4, at a semi-permissive temperature of 34°C,
Increased HTA1–HTB1 gene dosage strongly inhibited the growth of hhf1-36 (Y72G) cells, compared with expression of YEp24 alone. A slight inhibition was also detected with hhf1-39 (Y98H) cells (Figure 4). These synthetic dosage phenotypes of high-copy HTA1–HTB1 suggest a functional genetic interaction between H2A–H2B dimers and the Y72G and Y98H mutant histone H4 proteins (Kroll et al., 1996).

**H4 tyrosine mutants are Spt**

Alterations in the relative ratio of the H3–H4 and H2A–H2B gene dosage pairs can have strong effects on a variety of cell functions, including mitotic chromosome transmission (Meeks-Wagner and Hartwell, 1986; Smith and Stirling, 1988) and mRNA transcription (Clark-Adams et al., 1988; Hirschhorn et al., 1992). These effects of histone gene stoichiometry are presumably mediated by changes in the composition of the chromatin through alterations in dimer–tetramer interactions. If the molecular defect in the H4 tyrosine mutants is in dimer–tetramer interactions, then these mutants should exhibit the same phenotypes as strains with altered gene ratios. To test this prediction, we examined the ability of the mutations to suppress Ty1 solo δ insertions. Insertions of Ty1 solo δ elements in the 5' regions of the HIS4 (his4-912δ) or LYS2 (lys2-128δ) genes cause alterations in their transcription initiation start sites leading to non-functional transcripts. Mutations in SPT genes suppress these defects by restoring transcription of the affected genes (Simchen et al., 1984; Winston et al., 1984; Fassler and Winston, 1988). Either overproduction or underproduction of histone dimer gene sets produces an Spt' phenotype and restores gene expression (Clark-Adams et al., 1988).

As shown in Figure 5, hhf1-36 (Y72G), hhf1-37 (Y88G) and hhf1-39 (Y98H), but not hhf1-40 (Y98W) or wild-type HHF1, were able to suppress his4-912δ and lys2-128δ mutations and support colony formation on SDC-HIS and SDC-lys plates. All three alleles were strong suppressors of his4-912δ and weaker suppressors of lys2-128δ. The hhf1-39 (Y98H) allele was the weakest suppressor of the three, and growth in medium lacking lysine was not observed until at least 4 days of incubation. Thus, substitution mutations in these H4 tyrosines each result in cells that display Spt' phenotypes.

**Histone H4 mutants are Sin**

A number of histone mutations, including altered stoichiometry of the dimer–tetramer gene sets, suppress the loss of transcriptional activation caused by mutations in genes encoding the SWI–SNF complex proteins, rendering regulated target genes SWI–SNF independent (Sin') (Happel et al., 1991; Malone et al., 1991; Hirschhorn et al., 1992; Kruger et al., 1995). Since the H4 tyrosine mutations targeted the dimer–tetramer interface, we anticipated that they might have Sin' phenotypes.

The SUC2 gene encodes the enzyme invertase necessary for utilization of sucrose and raffinose as carbon sources. The transcriptional activation of SUC2 depends on the function of the SWI–SNF complex to remodel the repressive chromatin structure at the SUC2 promoter (Hirschhorn et al., 1992). Mutants in SWI–SNF complex genes, such as snf2 or snf5, fail to remodel promoter chromatin structure and do not activate SUC2 transcription. Deletion of the histone HTA1–HTB1 locus generates an active chromatin structure over the SUC2 promoter, thus bypassing the requirement for SWI–SNF complex function and activating transcription in swi and snf mutants (Hirschhorn et al., 1992). We tested the tyrosine histone H4 mutants for Sin' phenotypes by knocking out SNF2 in the mutants using a snf2::URA3 disruption allele. As shown in Figure 6A, none of the H4 alleles suppressed the growth defect of the snf2 disruption on raffinose plates. Consistent with these results, there was also no increase in SUC2 mRNA levels in the double mutants (Figure 6B).

We next examined the effect of the histone mutations.
Histone H4 mutations suppress the inositol auxotrophy due to a snf2::URA3 disruption. (A) Growth of the wild-type H4 and mutant strains is shown for media lacking inositol or supplemented with 100 μM of myo-inositol. All the strains carried the snf2::URA3 disruption. Wild-type HHT1 and hhf1-40 (Y98W) cells required inositol to grow, whereas hhf1-36 (Y72G), hhf1-37 (Y88G) and hhf1-39 (Y98H) cells could grow without inositol in the medium. (B) mRNA levels of SUC2 in the wild-type and histone H4 Tyr mutant cells grown under repressive (2% glucose) or inducing (0.02% glucose) conditions analyzed by Northern blot. Northern blots were probed with SUC2 and ACT1 probes.

Histone H4 mutants do not suppress the defect in SUC2 transcription due to a snf2::URA3 disruption. (A) Wild-type and histone H4 Tyr mutant strains, in SNF2 and snf2 backgrounds, were grown on complete media and then replica-plated onto media containing glucose or raffinose as carbon sources. Although all the strains were able to grow in the medium supplemented with glucose, only the SNF2 strains grew in the raffinose medium. (B) mRNA levels of SUC2 in the wild-type and histone H4 Tyr mutant cells grown under repressive (2% glucose) or inducing (0.02% glucose) conditions analyzed by Northern blot. Northern blots were probed with SUC2 and ACT1 probes.

Histone H4 mutants do not relieve the requirement for INO2. Mutants on INO1 gene expression since it also requires the function of the SWI–SNF complex for activation (Peterson et al., 1991). In contrast to the results at SUC2, hhf1-36 (Y72G) and hhf1-37 (Y88G) relieve the growth defect of snf2 mutants on Ino – plates (Figure 7A). The hhf1-39 (Y98H) allele also relieves repression, but more weakly (Figure 7A). Consistent with its Spt+ phenotype, no suppression was observed for hhf1-40 (Y98W). These results were confirmed by Northern blot analysis of INO1 mRNA levels (Figure 7B). In the SNF2 wild-type strain, the induction of the INO1 mRNA by low inositol was ~14-fold (lanes 1 and 2). The isogenic snf2 mutant shows 1994; Ashburner and Lopes, 1995). As shown in Figure 8, the hhf1-37 allele did not suppress an ino2::TRP1 disruption for growth on media lacking inositol. Identical results were obtained with the other mutant alleles of H4 (data not shown). Consistent with this growth pattern, the levels of INO1 mRNA in ino2::TRP1 mutants were low both in wild-type and in hhf1-37 cells (data not shown).

Histone H4 mutants alter chromatin structure at the INO1 locus. We next examined the chromatin structure of the INO1 locus in wild-type histone and H4 mutant strains, in both wild-type SNF2 and snf2 mutant backgrounds. An autoradiograph of the results of a micrococcal nuclease footprint of the locus is shown in Figure 9A. In wild-type cells, the most significant difference between repressing...
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Fig. 9. Histone H4 mutations alter the chromatin structure at the INO1 locus. (A) Autoradiograph of an indirect end-labeled blot of chromatin digests. Nuclei were partially digested with micrococcal nuclease and then purified DNA was digested to completion with BglII. The blot was probed with a radiolabeled AflIII–BglII fragment of 295 bp. The nuclease digestion patterns of chromatin are presented in lanes 1–15 as sets of three digests for each strain and growth condition. These are keyed at the top of the figure: inositol ‘+’ indicates medium with 10 mM inositol and ‘−’ indicates medium lacking inositol. SNF2 ‘+’ indicates wild-type SNF2 and ‘−’ indicates the snf2::URA3 knockout allele; HHF1 ‘+’ indicates wild-type HHF1 and ‘−’ indicates the hhf1-37 allele. The MNase ramps indicate increasing concentrations of enzyme in the digests. Lane M shows the position of restriction fragments that serve as internal molecular weight markers; the coordinates of the marker-cut sites are labeled relative to the BglII site located within the INO1 coding sequence. Lane D shows the micrococcal nuclease digestion pattern of purified DNA. A diagram of the locus is shown at the left of the panel scaled to the coordinates of the autoradiograph image. The locations of the INO1 gene and the YJL151C open reading frame are shown by the open boxes. The sequence features are labeled as follows: ‘Y’ for a 30 bp oligopyrimidine tract; ‘T’ for putative TATA box elements; ‘R’ for a URS1 negative regulatory sites; and ‘A’ for INO1 UAS promoter elements. The micrococcal nuclease-cut sites are labeled A–O along the right side of the panel. (B) Summary of changes in the chromatin structure at the INO1 locus. Micrococcal nuclease digestion sites labeled A–O above the lines correspond to the bands labeled in (A). The relative intensity of the sites is reflected in the size of the arrows, with the larger arrows representing greater sensitivity to the nuclease. Line (a): a diagram of the locus representing the linear DNA sequence. Features are keyed as in (A). Line (b): downward arrows represent the cut sites in naked DNA, while upward arrows represent sites in the chromatin of either HHF1 SNF2 cells under high inositol repressing conditions or HHF1 snf2::URA3 cells. Line (c): the upward arrows represent sites in the chromatin of HHF1 SNF2 cells induced by the absence of inositol. Line (d): the upward arrows represent sites in the chromatin for hhf1-36 and hhf1-37 histone H4 mutants, either hhf1 SNF2 or hhf1 snf2::URA3 both for repressing and inducing medium. Lane (e): the DNA coordinates relative to the BglII site within the INO1 coding region. The positions of the four internal marker fragments are shown in larger type face.

(lanes 1–3) and inducing conditions (lanes 4–6) occurs at nuclease site G. Site G is one of the few digestion sites within the locus that is specific to chromatin templates, and it becomes inaccessible to nuclease only under repressing conditions (arrow, lane 3). In addition to this change, there is also a quantitative increase in the sensitivity of nuclease site B under inducing conditions (arrow, lane 6). Both these sites are functionally significant. Site G encompasses an upstream URS1 and a UASINO site known to be required for gene regulation both in vivo and in vitro (Lopes et al., 1991; Ambroziak and Henry, 1994; Swift and McGraw, 1995). Site B covers the start site for INO1 mRNA initiation, and a change in the chromatin structure of this region is consistent with a proposed positive downstream element suggested by genetic analysis (Swift and McGraw, 1995).

The snf2::URA3 gene disruption blocks the formation of the active chromatin configuration at INO1. In the absence of Snf2p, the chromatin structure of the locus maintains the repressed state even under inducing conditions (lanes 7–9); site G remains accessible to nuclease (arrow, lane 9) and site B becomes relatively insensitive.
The effect of the hhf1-37 allele on this structure is shown in lanes 13–15. Consistent with its Sin' phenotype, the hhf1-37 mutant establishes the active chromatin state at INO1 even in the snf2::URA3 disruption background. In particular, in the hhf1-37 snf2::URA3 double mutant, site G now becomes resistant to micrococcal nuclease digestion, even though it is sensitive in the snf2::URA3 single mutant. Although only weakly apparent in Figure 9A, we conclude that site B also becomes more sensitive to digestion in the hhf1 snf2 double mutant on the basis of additional experiments (data not shown). The results of micrococcal nuclease footprinting of hhf1-36 chromatin were identical to those obtained for the hhf1-37 mutants (data not shown).

In addition to these alterations that correlate with SNF2 function, we also detected histone-dependent changes that may be unrelated to normal mechanisms of gene activation. In both hhf1-37 SNF2 (lanes 10–12) and hhf1-37 snf2::URA3 (lanes 13–15) strains, site N becomes strongly hypersensitive (arrows, lanes 11 and 14). This site is also hypersensitive in hhf1-37 strains under repressing conditions (data not shown). Site N lies in the upstream promoter region of YJL151C, an open reading frame of unknown function. YJL151C encodes a 0.6 kb mRNA and its transcription is regulated coordinately with INO1 (Hirsch and Henry, 1986; Dean-Johnson and Henry, 1989). However, the prominent digestion of site N appears to be specific for the histone mutants and does not correlate with inducing or repressing growth conditions. Thus, although the H4 tyrosine mutants do not have a global disruption of micrococcal nucleas ladders (Figure 3), the results presented here show that they do have at least two classes of chromatin defects: (i) defective interactions with SWI–SNF function and probably other chromatin regulatory proteins, and (ii) localized mutant-specific structural changes.

**CDC phenotype of the mutants**

The lethality of hhf1-38 and hhf1-41 plus the Ts’ phenotypes of the other H4 tyrosine mutants show that the histone dimer–tetramer interactions play essential roles in the cell. To begin to understand these essential pathways, we examined the Ts’ phenotypes of the conditional mutants in greater detail. In principal, perturbing dimer–tetramer interactions might be expected to cause defects in at least two different major pathways: RNA transcription and DNA replication. Therefore, we asked whether the normal kinetics of cell division cycle (CDC) progression were affected in the tyrosine mutants at the restrictive temperature. Defects in DNA replication might be expected to cause a cell division cycle arrest at the G2–M boundary by activating the DNA damage checkpoint regulatory system (Hartwell and Weinert, 1989; Weinert et al., 1994). Alternatively, defects in transcription might lead to either a general growth arrest, or blocks at specific points in the division cycle that might depend on the proper expression of particular genes.

To assess the cell cycle pattern of the mutants, liquid cultures were grown at the permissive temperature until early log phase and then shifted to 37°C. Periodically after the temperature shift, cells were fixed and their cell cycle distribution assayed by flow cytometry. The DNA histograms for these measurements are shown in Figure 10.

As expected, the growth rate of the hhf1-40 (Y98W) mutant was wild-type and the cell cycle distribution of the population was identical at the permissive and restrictive temperatures. On the other hand, cells expressing hhf1-37 (Y88G) or hhf1-39 (Y98H) grew very slowly at the restrictive temperature. However, the DNA histograms did not show any accumulation of cells in any specific stage of the cell cycle. In contrast, hhf1-36 (Y72G) cells rapidly arrested cell division at 37°C with a characteristic terminal morphology consisting of unbudded cells with a single nucleus and 1C DNA content.

The G1 arrest of hhf1-36 (Y72G) was confirmed using synchronous cultures. Early G1 cells were isolated by centrifugal elutriation and split into two cultures, one grown at 28°C and the other at 37°C. As can be seen in Figure 11A, at the restrictive temperature hhf1-36 cells arrested in the first division cycle with unreplicated DNA. Cells remained tightly arrested even after 5 h at 37°C, at which time the cells maintained at 28°C were approximately at the end of the second division cycle. An isogenic control strain expressing the wild-type HHF1 gene divided synchronously at both the permissive and restrictive temperatures (Figure 11B). These results indicate that the rate-limiting growth step in the H4 tyrosine replacement mutants is not likely to be in DNA replication. Rather, the division cycle phenotypes suggest a more global defect in gene expression for hhf1-37 and hhf1-39 mutants, and a specific G1 arrest in the case of hhf1-36.

The H4-dependent step in hhf1-36 cells was mapped relative to 'Start,' the G1–S phase transition, through reciprocal shift experiments using α-factor and the restrictive temperature. First, early G1 cells were selected by centrifugal elutriation, arrested at 37°C for 2.5 h, and then returned to 28°C. As shown in Figure 12, cells shifted into medium containing α-factor remained arrested in G1,
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while those shifted into medium without α-factor resumed progression through the division cycle. In the complementary experiment, early G1 cells were first arrested with α-factor at 28°C for 2.5 h. They were then either shifted to 37°C or kept at 28°C, and washed free of α-factor. Cells at 28°C resumed normal cell cycle progression when the α-factor was removed, while cells shifted to 37°C remained arrested in G1 (Figure 12). These results show that the G1 histone H4-dependent step defined by hhf1-36 and the α-factor-dependent step at Start are interdependent.

**G1 cyclin gene expression in hhf1-36**

This interdependence of histone H4 function and Start suggested that the expression of one or more regulatory components of the G1–S phase transition might be blocked in hhf1-36 cells. We first examined the expression of the G1 cyclin genes CLN1, CLN2 and CLN3, because the proteins produced are functionally unstable and hence a failure in their transcription would result in a rapid first cycle cell division arrest (Richardson et al., 1989; Cross, 1990; Wittenberg et al., 1990). Northern analysis was used to quantify mRNA in cells synchronized by centrifugal elutriation and grown at either 28 or 37°C for 3 h (Figure 13). While the mRNA levels of CLN1 and CLN2 peaked at ~2 h for cells grown at 28°C, they were virtually absent in cells grown at 37°C. The levels of CLN3 mRNA were also lower in the cells at 37°C than those at 28°C. These results suggested that the G1 arrest of hhf1-36 cells was due to the reduced expression of the G1 cyclin genes. We confirmed this hypothesis by transforming these cells with a plasmid expressing a dominant mutant allele of the CLN2 gene (CLN2-4) that encodes a hyperstable protein. The CLN2-4 allele (generously provided by Drs Lanker and Wittenberg) encodes a carboxy-terminal truncation of Cln2p that is missing sequences that target rapid degradation (Rogers et al., 1986). Expression of CLN2-4 suppresses the G1 arrest phenotype of hhf1-36, and synchronized hhf1-36 CLN2-4 cells are able to complete the G1–S phase transition at the restrictive temperature (Figure 14). Similar results were obtained with CLN2 under the control of the GAL1 promoter; hhf1-36 pGAL-CLN2 cells grown at 37°C in the presence of galactose failed to arrest in G1, while those grown in raffinose

arrested as expected (data not shown). However, CLN2-4 is not sufficient to suppress hhf1-36 for viability at 37°C. At the restrictive temperature they progress slowly through the cell division cycle and stop dividing after a few cycles without a unique terminal morphology. Thus, it is likely that hhf1-36 cells have additional defects in essential functions besides their failure to activate transcription of the G1 cyclin genes.

Since the transcriptional activation of the CLN1 and CLN2 genes as well as the post-translational activity of CLN3 depend on activation of the SWI4 and SWI6 genes (Nasmyth and Dirick, 1991; Ogas et al., 1991), we measured SWI4 and SWI6 mRNA levels in hhf1-36 cells. As shown in Figure 15, both SWI4 and SWI6 mRNA levels were greatly reduced in exponentially growing
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![Graph](image)

**Fig. 13.** Northern analysis of hhf1-36 (Y72G) cells. The mRNA levels of the CLN1, CLN2, CLN3, ACT1 and TUB2 are shown for cells synchronized by centrifugal elutriation and grown at 28 or 37°C for the indicated periods of time.

![Graph](image)

**Fig. 15.** SWI4 and SWI6 transcription is down-regulated in the hhf1-36 (Y72G) cells grown at 37°C. Total RNA was prepared from exponentially dividing cultures grown at 28 or 37°C.

The衅ne-directed mutational analysis reported here shows that the three tyrosine residues at positions 72, 88 and 98 in the H4 histone fold are essential for function. The single substitution allele hhf1-38 (Y98G) and the double substitution allele hhf1-41 (Y72G+Y88G), are both lethal. Single substitution mutants with either hhf1-36 (Y72G), hhf1-37 (Y88G) or hhf1-39 (Y98H) are temperature sensitive, showing defects in transcription and cell proliferation. These tyrosines were selected for mutation because of their predicted roles in mediating interactions between histone H4 and the [H2A–H2B] dimer subunits. Several lines of genetic evidence are consistent with this prediction. First, the pattern of lethal tyrosine substitutions is consistent with the physical structure of the octamer and the interactions of each H4 with each dimer. Second, the mutants have the Spt– and Sin– phenotypes expected for altered dimer–tetramer interactions (Clark-Adams et al., 1988; Hirschhorn et al., 1992; Kruger et al., 1995). Third, overexpression of histones H2A and H2B causes a synthetic dosage lethality in the case of hhf1-36 (Y72G) and growth inhibition in the case of hhf1-39 (Y98H), suggesting a genetic interaction between [H2A–H2B] dimer subunits and the mutant H4 proteins. One interpretation of this dosage lethality is that histone [H3–H4]2 tetramers might interact with [H2A–H2B] dimers to form a proportion of aberrant nucleosomes when the H4 tyrosines in the dimer–tetramer interface are mutated. An increased dosage of [H2A–H2B] dimers then might drive an increased proportion of complexes into functionally defective nucleosomes.

The phenotypes of the H4 tyrosine substitution mutants reveal striking consequences for gene transcription, both positive and negative. Previous studies have shown that the N-terminal domains of histones H3 and H4 may have both positive and negative roles in gene regulation (Durrin et al., 1991; Mann and Grunstein, 1992; Hecht et al., 1995). However, mutations in the histone fold domains have only derepressed gene expression, presumably by disrupting a repressive chromatin structure (Hirschhorn et al., 1995; Kruger et al., 1995). The positive transcriptional phenotypes seen with the mutants reported here are in agreement with these previous studies and are consistent with the model of SWI, SNF and SIN gene function (Winston and Carlson, 1992; Peterson and Tarkun, 1995). For example, at INO1 the tyrosine substitution mutants bypass the normal requirement for the SWI–SNF complex in remodeling chromatin structure, but do not relieve the requirement for the gene-specific transcriptional activators INO2 and INO4.

The negative transcriptional effects of hhf1-36 are unusual and important in that they repress the expression of genes critical for cell division cycle control during G1. The phenotypes of hhf1-36 cells are similar to those of mutants in the CDC68 (SPT16) gene (Rowley et al., 1991; Lycan et al., 1994). Like hhf1-36, cdc68 mutations suppress the transcription defects of Ty1 δ-insertions at his4-9128 and lys2-1288 (Malone et al., 1991). This Spt– phenotype is likely to be the result of altered histone
dimer–tetramer stoichiometry because of the reduced transcription of HTA1 and HTB1 in cdc68 cells (Xu et al., 1995). At the restrictive temperature, cdc68 mutants also arrest in late G1 as a consequence of low levels of CLN1, CLN2, CLN3, SWI4, and SWI6 mRNA (Rowley et al., 1991; Lycan et al., 1994). For both cdc68 and hhf1-36 mutants, expression of dominant CLN2 alleles suppresses the G1 arrest but not the Ts phenotype, suggesting that the transcription of other essential genes is also defective. It is unlikely that hhf1-36 acts indirectly through CDC68 expression since cdc68 mutants show a marked decrease in ACT1 mRNA at the restrictive temperature (Rowley et al., 1991) while hhf1-36 mutants do not (Figure 13). However, given the strong genetic links between CDC68 function and chromatin structure (Xu et al., 1993; Lycan et al., 1994), one target of Cdc68p might be the regulation of histone dimer–tetramer interactions. In such a case, the effect of cdc68 mutations and hhf1-36 could have similar consequences for chromatin structure, producing similar phenotypes in the mutants.

The transcriptional activation of CLN1 and CLN2 is thought to be the rate-limiting step in the entry of cells into the division cycle (Tyet et al., 1993; Dirick et al., 1995). The activation of CLN2 is complex and is mediated through two cis-acting upstream activation sites (UAS1 and UAS2) in the promoter of the gene (Cross et al., 1994; Stuart and Wittenberg, 1994). Transcriptional activation through these UAS elements depends directly on SWI4 in at least two ways: (i) as a complex with Swi4p and Swi6p to form the transcription activation complex SBF which binds to multiple Swi4–Swi6 cell cycle box (SCB) DNA elements located within UAS1 (Nasmith and Dirick, 1991; Ogas et al., 1991); and (ii) in a less well understood mechanism involving UAS2 that is independent of SCB sites (Cross et al., 1994; Stuart and Wittenberg, 1994). Expression of SWI4 and SWI6, in turn, can be stimulated by the activity of the G1 Cln/Cdc28p kinase (Taba et al., 1991; Dirick et al., 1992). Cellular regulation of CLN2 transcription is controlled by parallel trans-acting pathways. One pathway involves CLN3 (Stuart and Wittenberg, 1995), apparently through the activation of SBF bound at the CLN2 promoter by the Cln3p/Cdc28p kinase (Dirick et al., 1995; Koch et al., 1996). A less well understood parallel pathways involves SIT4 and BCK2 (Di Como et al., 1995). Because of these interlocking regulatory links, we do not currently know the primary transcriptional defects in hhf1-36 chromatin. In principle, the loss of CLN1, 2 and 3 and SWI4 and 6 gene transcription in hhf1-36 mutants could result from either a direct repression through the chromatin structure at the promoter of one or more of the genes involved, or an indirect effect through the derepression of an unknown transcriptional repressor. At present we cannot distinguish between these two models. Nevertheless, the important role of chromatin structure in the control of G1 cyclin expression is clearly established by the hhf1-36 mutant.

It is striking that the histones also participate in the regulation of the G2–M cell cycle transition. Using a screen for synthetic lethal mutants, Ma et al. (1996) recently uncovered genetic interactions between the N-terminal domains of histones H3 and H4 and regulators of Swi1p, the kinase responsible for the phosphorylation of Cdc28p that controls a morphogenetic checkpoint at the G2–M transition (Lew and Reed, 1995). For our finding that histone dimer–tetramer subunit interactions also affect the G1–S transition, through expression of the G1 cyclins, suggests that the regulatory components of the cell division cycle may have evolved to depend on multiple aspects of chromatin structure. The immediate challenges for future experiments will be to identify the primary targets of the hhf1-36 chromatin defect from among the interlocking genes in the cyclin regulatory circuit, and to discriminate between direct and indirect mechanisms of repression.

Materials and methods

Strains and plasmids

Escherichia coli strains JM109 (recA1 supE44 endA1 hisd17 gyrA96 relA1 thi-1 (λc18-proAB) F′ (traD36 proAB lacZΔM15) and BHI 71-18 (mutS thi supE λc18-proAB mutS-Tn10 F′ [proAB+ lacZΔM15]) were used in the oligonucleotide-directed mutagenesis. Strain J221 [lacY leu28 Δ GryE5 recA1 hisd17 Δ(cis)3] was used for transformation of ligations requiring selection for the LEU2 gene. Strain DH5α [supE44 ΔlacZ169 (800 lacZΔM15) hisd17 recA1 endA1 gyrA96 thi-1 relA1) was used for all other bacterial transformations.

The genotypes of the yeast strains used in this work are shown in Table 1. Strain MX4-22A was used as parental strain to construct the histone H4 mutant alleles by ectopic integration at the LEU2 locus. Strain MSY784 was used for testing Spt+ phenotypes and was obtained by crossing A970-3A with MSY423. S283C (MATa gal1-1 mal1) was used for α-factor production. Plasmid pMS329 is a CEN4/ARS1/URA3/SGP1 vector containing the wild type HHT1 and HHT1 genes (Megee et al., 1990). Plasmid pMS347 is a LEU2/CEN4/ARS1 vector containing HHT1 and a unique BamHI site for the insertion of fragments carrying derivatives of HHT1 (Megee et al., 1990). Mutated histone H4 BamHI fragments produced by oligonucleotide-directed mutagenesis were subcloned into pMS347 to create plasmids pMS337 (HHF1), pMS363 (hhf1-36), pMS364 (hhf1-37), pMS365 (hhf1-38), pMS366 (hhf1-39), pMS367 (hhf1-40) and pMS368 (hhf1-41). Plasmid TRT1 was kindly provided previously by L. Hereford (Hereford et al., 1979).

Media

YPD, YNB, SC, inositol and 5-FOA media were made as described by Rose et al. (1990) and MV medium was prepared as described by Hereford et al. (1981). Plates for testing carbon source requirements included antimycin A (Sigma) at a final concentration of 1 μg/ml (Carlson et al., 1981). For INO1 expression in liquid culture, cells were grown in YNB medium (induced) or YNB plus 100 μM inositol (repressed).

Oligonucleotide-directed mutagenesis

The Altered SitesTM in vitro mutagenesis system (Promega) was used to produce the mutant alleles of histone H4. A 405 bp BamHI fragment bearing wild-type copy 1 histone H4 was cloned into the phagemid vector pALTer-1. Mutant H4 sequences were confirmed by DNA sequencing.

Ectopic integrations

Histone H4 alleles. The 2.1 kb EcoRI–HindIII fragments containing wild-type HHT1 and mutant alleles of HHF1 were subcloned from pMS347–derived plasmids into the integrating vector pRS305 between the EcoRI and HindIII sites. The resulting plasmids were linearized with HpaI to target the integration to the LEU2 locus and used to transform yeast strains MX4-22A and MSY784. Integration was verified by Southern blot analysis.

CLN2 alleles. Plasmids pUC18-HIS2-CLN2-4 and YIpG2- GAL::CLN2 were a gift from S. Lanker and C. Wittenberg. A 3.7 kb SalI–EcoRI fragment from pUC18-HIS2-CLN2-4 containing the CLN2-4 allele was cloned between the SalI and EcoRI sites of pRS306, and the resulting plasmid was transformed into yeast strain MSY781. For the GAL::CLN2 integration, an EcoRI–BamHI fragment from YIpG2-CLN2 was transferred to pRS306. Integration was targeted to the URA3 locus by digestion of the plasmid DNA with ClaI and then used to transform MSY781. Integration was verified by Southern blot analysis.
Gene disruptions

SNF2. A 1.1 kb fragment spanning bases 3054–4145 of the SNF2 gene was amplified by PCR and cloned between the BamHI and XhoI sites of the integrating plasmid pH5306. The plasmid was linearized by digestion with NheI that cuts in the SNF2 fragment to target the knockout to the SNF2 gene. Integration was verified by Southern blot analysis.

INO2. A linear 1.9 kb Smal–SalI fragment from pMN118 (Nikoloff and Henry, 1994), provided by P.McGraw and S.Henry, containing TRP1 as selectable marker was used to disrupt the INO2 locus. Disruption was verified by Southern blot.

HT1–HTB1. A 3.2 kb EcoRI–HpaI linear fragment from pUC9–HT1–HTB1 (Nikoloff and Osley, 1987), provided by M.A.Osley, was used to transform strains MSY623, MSY625, MSY626 and MSY781. Ura− transformants were subjected to Southern blot analysis to confirm deletion of the HT1–HTB1 locus.

Synchronized cultures

Cells in early G1 were collected by elutriation (Gordon and Elliot, 1977) and split into two equal fractions. One was resuspended in fresh medium at 28°C and the other at 37°C. Samples of 5×10^6 cells were removed from both cultures at 30 min intervals and analyzed for DNA content and cell morphology to monitor the cell cycle progression. DNA content was measured by flow cytometry after propidium iodide staining, and morphology was assessed by microscopic examination of cells stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) (Smith, 1991). For reciprocal-shift experiments, early G1 cells were collected by elutriation and resuspended at 3×10^6 cells/ml in fresh MV medium containing α-factor and grown until cells in a control culture without α-factor reached the end of S phase. Cells were then collected by filtration, washed twice with MV medium, and resuspended in fresh MV medium pre-warmed at either 28 or 37°C. In the reciprocal experiment, cell cultures arrested at the restrictive temperature (37°C) were split into two parts, shifted to the permissive temperature (28°C), and α-factor was added to one of the cultures.

RNA analysis

Total RNA was extracted from cells grown to a density of 5×10^6–1×10^8 cells/ml. Ten μg of total RNA were electrophoresed in a formaldehyde-agarose gel (1% agarose) and then transferred to a nitrocellulose membrane. Nucleic acid probes for hybridization were: SUC2, a 772 bp fragment amplified by PCR; INO1, a 0.6 kb BgII–PvuII fragment from plasmid pH318 (Hirsch and Henry, 1986) provided by S.Henry; CLN1, a 1.2 kb EcoRI–PvuII fragment from plasmid pGAL-CLN1 (a gift from F.Cross); CLN2, a 1.6 kb BamHI–XhoI fragment from plasmid YIPG2 (a gift from C.Wittenberg); CLN3, a 1.8 kb BamHI fragment from plasmid pW16 (a gift from F.Cross); SUC4, a 718 bp fragment amplified by PCR and cloned into pUC19 vector; and SUC2, a 1.2 kb EcoRI–SalI fragment from plasmid pMN118 (Nikoloff and Henry, 1994), provided by P.McGraw and S.Henry, containing TRP1 as selectable marker was used to disrupt the INO2 locus. Disruption was verified by Southern blot.

Micrococcal nuclease digestion

Chromatin was prepared from wild-type and hlf1-36 (Y72G) cells in early log-phase growth. Nuclei were isolated as described previously (Nelson and Fangman, 1979) using Zymolyase (ICN Biomedicals, Inc), 2 mg per 6×10^6 cells for spheroplasting. Cells were lysed in 18% Ficoll, 0.02 M PIPES (pH 6.3), 0.5 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF) with five strokes in a Dounce homogenizer. Nuclei were digested with micrococcal nuclease (Sigma) in 1.1 M sorbitol, 0.02 M PIPES (pH 6.3), 0.5 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF) with five strokes in a Dounce homogenizer. Nuclei were digested with micrococcal nuclease (Sigma) in 1.1 M sorbitol, 0.02 M PIPES (pH 6.3), 0.5 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF) with five strokes in a Dounce homogenizer. Nuclei were digested with micrococcal nuclease (Sigma) in 1.1 M sorbitol, 0.02 M PIPES (pH 6.3), 0.5 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF) with five strokes in a Dounce homogenizer. Nuclei were digested with micrococcal nuclease (Sigma) in 1.1 M sorbitol, 0.02 M PIPES (pH 6.3), 0.5 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF) with five strokes in a Dounce homogenizer.
from the treated nuclei were then cut to completion with BglII, and resolved by electrophoresis through a 1.5% agarose gel. Naked DNA control samples were prepared by digesting purified whole genomic DNA with micrococcal nuclease at 3 U/ml in SPC adjusted to 10 mM CaCl$_2$. Internal molecular weight markers for the INO1 region were purified from digestions of plasmid DNA with BglII and other restriction endonucleases cutting in the 5’ upstream DNA. These comprised the 879 bp BglII–SacI, 651 bp BglII–SpI, 441 bp BglII–SpI1, 295 bp BglII–AffIII and 100 bp BglII–Chl fragments. Gels were blotted and hybridized with either the 100 bp BglII–Chl or the 295 bp BglII–AffIII fragments to visualize the micrococcal nuclease digestion fragments produced from the locus.

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Mutational analysis of histone octamer function


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