Yeast histone H3 and H4 amino termini are important for nucleosome assembly in vivo and in vitro: redundant and position-independent functions in assembly but not in gene regulation

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The hydrophilic amino-terminal sequences of histones H3 and H4 extend from the highly structured nucleosome core. Here we examine the importance of the amino termini and their position in the nucleosome with regard to both nucleosome assembly and gene regulation. Despite previous conclusions based on nonphysiological nucleosome reconstitution experiments, we find that the histone amino termini are important for nucleosome assembly in vivo and in vitro. Deletion of both tails, a lethal event, alters micrococcal nuclease-generated nucleosomal ladders, plasmid superhelicity in whole cells, and nucleosome assembly in cell extracts. The H3 and H4 amino-terminal tails have redundant functions in this regard because the presence of either tail allows assembly and cellular viability. Moreover, the tails need not be attached to their native carboxy-terminal core. Their exchange re-establishes both cellular viability and nucleosome assembly. In contrast, the regulation of *GAL1* and the silent mating loci by the H3 and H4 tails is highly disrupted by exchange of the histone amino termini.

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The nucleosome consists of a central histone H3–H4 tetramer and two H2A–H2B dimers around which ~146 bp of DNA are wrapped. The core of this particle contains the structured α -helical carboxy-terminal portion of each histone (Richmond et al. 1984; Arents et al. 1991), whereas the basic unstructured amino-terminal regions extend from the core where they may be cleaved by trypsin (Weintraub and Lente 1974; Whitlock and Simpson 1977; van Holde 1989). The extended flexible nature of the histone tails and their ability to be modified post-translationally to change their charge (van Holde 1989) suggest that they have dynamic functions, possibly in regulating nucleosome assembly and transcription. Genetic evidence suggests the involvement of the histone amino termini in an essential function. Whereas deletion of either the H2A or H2B tail creates a viable strain, truncation of both H2A/H2B amino termini is lethal (Wallis et al. 1983; Schuster et al. 1986). Similar results have been reported for the H3/H4 amino termini

(Kayne et al. 1988; Morgan et al. 1991; Mann and Grunstein 1992). These experiments suggest that the amino termini of each dimer pair have certain redundant functions required for viability. Whether or not these include nucleosome assembly has been unclear. Histones truncated by trypsin can undergo assembly of relatively stable nucleosome particles in vitro, albeit under nonphysiological conditions during dialysis from high salt (Whitlock and Stein 1978; Dong et al. 1990; Hayes et al. 1991). This finding suggests that the histone amino termini are not required for core particle assembly. However, these data must be interpreted cautiously in evaluating physiological function. Diacetylation of histone H4 lysines K5 and K12 has been correlated with nucleosomal assembly in organisms as varied as *Tetrahymena*, *Drosophila*, and humans (Sobel et al. 1995). Also, chromatin assembly onto replicating DNA in cell-free systems requires subunits p150 and p60 of the assembly factor CAF-1 that interact in vitro with newly synthesized histones H3 and H4. Acetylation of the H4 tail may provide a signal to allow proper targeting and subsequent assembly of newly synthesized histones into nucleo-

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somes (Kaufman et al. 1995). Therefore, the importance of the histone amino termini in nucleosome assembly remains undetermined.

In analyzing the function of the histone amino termini in gene regulation, it is important to distinguish between the functions of H2A/H2B and H3/H4. No distinct functions have yet been ascribed to the H2A or H2B amino termini (Kayne et al. 1988; Durrin et al. 1991; Thompson et al. 1994). Even an exhaustive search for mutations in H2A that affect *SUC2* transcription (Hirschhorn et al. 1995) has uncovered only mutations in or very near the structured hydrophobic core (van Holde 1989). In contrast, deletion and mutagenesis experiments have shown that different portions of the histone H3 and H4 amino-terminal tails are required for repression and activation of a number of yeast loci. For example, the H3 and H4 amino termini have opposite effects on *GAL1* regulation (Durrin et al. 1991; Mann and Grunstein 1992). H3 amino-terminal deletions hyperactivate *GAL1* through the *GAL1* upstream activating sequence (UAS), whereas H4 amino-terminal lesions decrease *GAL1* activation by affecting the *GAL1* TATA region (Fisher-Adams and Grunstein 1995; Wan et al. 1995). The H3 and H4 amino termini are also required for the repression of genes adjacent to yeast telomeres and the silent *HM* mating loci (Kayne et al. 1988; Aparicio et al. 1991; Thompson et al. 1994). Recently, it has been shown that the silencing domains of H3 and H4 interact in vitro with the silencing information regulators SIR3 and SIR4, whose function is specific to the telomeres and *HM* loci (Hecht et al. 1995). These data suggest that the histone H3 and H4 amino termini possess domain structures that may serve as the binding sites for different protein factors to regulate transcription in vivo.

Because the H3 and H4 amino termini may undergo interactions outside of the nucleosomal core, which are required for nucleosome assembly and gene regulation, we asked the following questions in this paper: Are the H3 and H4 amino termini required for nucleosome assembly in whole cells and in vitro? Is this the redundant essential function shared by the H3 and H4 tails? Finally, do the flexible extended tails of H3 and H4 function as discrete units in either assembly or transcriptional regulation when attached to another histone carboxy-terminal core? To address these questions, the H3 and H4 amino-terminal tails were deleted or exchanged to create chimeric histone H3 and H4 proteins. Their effects on nucleosome assembly and gene activity are described below.

Results

Deletion of both the histone H3/H4 amino termini is lethal, but their exchange restores cellular viability

We constructed a series of strains in which H3 and H4 amino-terminal tails were deleted or exchanged as described in Materials and methods. The expression of the mutant histone genes (Fig. 1) is driven by their native promoter on a *CEN4/ARS1/TRP1* vector (plasmid B) in a

GALACTOSE → GLUCOSE

Figure 1. Strategy for the expression of mutant histones in yeast. RMY102 and GFY3001 both contain plasmid A [pRM102; a *CEN4/ARS1/URA3* plasmid that bears *HHT2* (encoding H3) fused to the *GAL10* promoter and *HHF2* (encoding H4) fused to the *GAL1* promoter; Mann and Grunstein 1992; Fisher-Adams and Grunstein 1995]. Their endogenous copies of H3 and H4 genes have been deleted and replaced by *LEU2* and *HIS3* markers. These two strains, which are dependent on galactose for wild-type histone H3 and H4 synthesis, were used for subsequent introduction of plasmid B [a *CEN4/ARS1/TRP1* plasmid that bears chimeric histone H3 (H3*) or chimeric H4 (*H4) under the control of their wild-type histone promoter]. Shifting the strains from medium containing galactose to that containing glucose repressed wild-type H3 and H4 synthesis and made it possible to rapidly determine whether or not the chimeric histones sustained growth (Kayne et al. 1988).

strain (RMY102) containing plasmid A (a *CEN4/ARS1/URA3* vector) carrying wild-type histone H3 and H4 genes under *GAL10* and *GAL1* promoter control (Mann and Grunstein 1992). Viability was tested in these strains after replica-plating from medium containing galactose to that with glucose to repress wild-type H3 and H4 synthesis from the *GAL* promoters in plasmid A. After shifting to glucose, only plasmid B histone genes with nonlethal mutations can support cellular growth. Lethality was confirmed not only by the inability of cells to grow on glucose but also by their concurrent inability to lose plasmid A in medium containing 5-fluoro-orotic acid (5-FOA) that selects against *URA3* expression from plasmid A (Boeke et al. 1987).

The deletions and chimeric fusions of amino- and carboxy-terminal portions of H3 and H4 are shown in Figure 2. We chose to delete residues 4–30 from H3 and 4–28 from H4 because these constructs leave intact the very amino-terminal residues that may be important for protein stability (Gonda et al. 1989) yet delete most of the hydrophilic extended amino terminus in which the internal acetylated lysine residues are found. As described earlier (Kayne et al. 1988; Morgan et al. 1991; Mann and Grunstein 1992), deletion of the H3 amino terminus (Fig.

	Strain	H3 Construct	H4 Construct	Viability	Doubling time (min)
a)	RMV200	N C	N C	Viable	93
b)	RMV430	N C	N C	Viable	135
c)	GFY428	N C	N C	Viable	246
d)	XLY101	N C	N C	Inviable	N/A*
e)	XLY401a	N C	N C	Viable	264
f)	XLY411	N C	N C	Inviable	N/A*
g)	XLY406	N C	N C	Inviable	N/A*

Figure 2. H3 and H4 amino-terminal deletions and chimeras. (Open bars) H3 protein sequence. (Solid bars) H4 sequence. (N) Amino-terminal; (C) carboxy-terminal end of histone protein. Numbers above the bars indicate the residues of each histone protein sequence. Viability was tested after plating on glucose to repress wild-type histone synthesis and allow expression of mutant histone genes from their native promoters as described in Materials and methods. Doubling time was determined as described previously (Durrin et al. 1991). (N/A*) Not applicable.

2, line b) or that of H4 (Fig. 2, line c) allows cellular viability. Cell doubling times were 135 and 246 min, respectively, as compared with 93 min for the wild-type control strain (Fig. 2, line a). If both H3 and H4 (i.e., H3/H4) amino termini were deleted in the same strain (XLY101; Fig. 2, line d), lethality occurred, also confirming previous observations (Morgan et al. 1991). However, viability was restored when both chimeric histones H3N-H4C/H4N-H3C were expressed (XLY401a; Fig. 2, line e). This strain had a similar doubling time (264 min) as that (GFY428) carrying the H4 amino-terminal deletion H4 Δ 4-28 (246 min). In contrast, neither the H3N-H4C nor the H4N-H3C chimera was able to restore viability when coexpressed with a deletion of the H3 (Fig. 2, line f) or H4 amino terminus (Fig. 2, line g). Therefore, it is the actual exchange of the H3 and H4 amino termini and not merely the presence of either chimera that allows viability.

Deletion of the H3/H4 amino termini causes arrest in G₂ similar to that caused by H4 and nucleosome depletion

In an attempt to understand why H3/H4 amino-terminal deletions cause lethality, we examined their effects on cell growth and the cell cycle. When XLY101 cells synchronized in G₁ by α -factor were released from arrest and treated with glucose, they underwent approximately two divisions after which further growth was fully inhibited (Fig. 3). Truncation of the H3/H4 amino termini resulted in irreversible damage. After growth in glucose for 3 hr, <50% of the cells were viable when regrown in galactose-containing medium to resume synthesis of wild-type H3/H4 histones. Lethality increased to ~100% af-

ter 20 hr (Fig. 3). We also observed, by fluorescence-activated cell sorter (FACS) analysis, that expression of truncated H3/H4 for 3 hr in glucose resulted in preferential G₂ buildup, with 80% of these arrested cells having a 2N quantity of DNA (Fig. 4A) and a single DAPI-stained nucleus (Fig. 3, inset).

A block in G₂ is also a phenotype associated with H4 depletion. This is seen in UKY403 when the H4 gene under control of the *GAL1* promoter is repressed in glucose as replication continues (Kim et al. 1988). However, H4 depletion in UKY403 results in a more synchronous arrest. After 2 hr, ~95% of the cells contained a 2N quantity of DNA and a single DAPI-stained nucleus (Fig. 4A). The average DNA amount per cell increases somewhat upon longer glucose treatment, reaching a 3N quantity of DNA by 10–20 hr (Fig. 4B). This is more evident when cellular DNA content is measured as opposed to the use of selected channels representing G₁ and G₂ as described earlier (Kim et al. 1988). For XLY101, the DNA content of G₂ cells, which constitutes ~80% of the cell population, also reached ~3N. The minor G₁ cell fraction reached ~1.5N. G₂-arrested UKY403 and the G₂ fraction of XLY101 had a similar rate of increase of DNA content upon longer glucose treatment. We conclude that loss of the H3/H4 amino termini and H4 depletion both cause G₂ arrest; however, the effects of H4 depletion are more rapid and complete than those caused by loss of the H3/H4 tails.

Deletion of both the histone H3/H4 amino termini disrupts nucleosome assembly in vivo

Because H3/H4 amino-terminal deletion causes G₂ arrest in a large fraction of glucose-treated XLY101 cells,

Figure 3. Deletion of both the H3/H4 amino termini causes irreversible cell cycle-specific arrest. (□) Viability of α factor-synchronized XLY101 cells containing truncated H3 and H4 amino termini after various times of incubation in glucose was measured as described in Materials and methods. (●) Growth of α -factor-synchronized XLY101 cells after various times of incubation in glucose was determined as described in Materials and methods. After transfer to glucose-containing medium the cells underwent approximately two rounds of division before full arrest. (Inset) DAPI-stained glucose-arrested XLY101 cells in G₂ that cannot segregate chromosomal DNA, found at the mother-bud neck.

reminiscent of histone H4 depletion in UKY403 (Kim et al. 1988), we asked whether nucleosome loss occurs under these conditions in XLY101 cells. Two assays, micrococcal nuclease (MNase) digestion and plasmid superhelicity, have been used previously to measure nucleosome loss in UKY403 (Kim et al. 1988). MNase treatment of permeabilized cells cleaves linker DNA between nucleosomes, generating nucleosomal ladders. In galactose, all strains containing different mutant histone constructs showed polynucleosomal ladders similar to those of wild-type cells, extending to some eight to nine bands (Fig. 5A, groups 1–6). This is expected because all strains containing truncated histones also synthesize large quantities of wild-type histones regulated through the *GAL* promoter in galactose. However, shifting each strain to glucose allows production only of the mutant histones and produces an MNase-generated pattern dependent on those altered histones. Under these conditions, deletion of either the H3 or H4 amino terminus did not destroy the nucleosomal ladders [Fig. 5B, cf. groups 3 and 4 with group 1 (wild type)]. In contrast, deletion of both the H3 and H4 amino termini (Fig. 5B, group 5) resulted in a very high level of background digestion and loss of the MNase-generated banding pattern. However, the nucleosomal ladders were restored when both H3/H4 amino termini were exchanged in the same cell (Fig. 5B, group 2). These data show that deletion of the H3/H4 amino termini causes changes in MNase-generated nucleosomal ladders resembling those resulting from nucleosome loss in UKY403 (Fig. 5B, group 6; Kim et al. 1988) or the loss of nucleosome positioning and that exchanging the H3/H4 amino termini restores the nucleosomal ladders.

Superhelical density measures the number of nucleosomes in a plasmid, as a nucleosome induces a single

superhelical turn in a covalently closed plasmid (Worcel et al. 1981; Wang 1982). On shifting UKY403 cells from galactose (G) to glucose (D), there is a large decrease in superhelical density of endogenous 2 μ plasmids, suggesting the loss of >50% of nucleosomes as DNA replication continues in the absence of new histone H4 synthesis (Kim et al. 1988; repeated in Fig. 6, lanes 6G, and 6D). Whereas deletion of either the H3 or H4 amino termini alone had very little effect on plasmid topology (Fig. 6, lanes 3,4), the preferential synthesis of both truncated H3 and H4 histones caused a large decrease in superhelical density (Fig. 6, lanes 5G and 5D) similar to that seen on H4 depletion in UKY403 cells.

We then investigated the extent to which the exchange of H3/H4 amino termini affects plasmid superhelical density. Exchanging the amino termini of H3 and H4 resulted in a pattern of 2 μ plasmid superhelical density similar to that seen in strains carrying wild-type histones (Fig. 6, cf. lanes 1 and 2). We conclude that deletion of the H3/H4 amino termini causes loss of nucleosomal ladders and decreases plasmid superhelical density in a manner similar to that caused by H4 and nucleosome depletion. Exchanging the H3/H4 amino termini corrects these defects considerably.

It should be noted that plasmid superhelicity does not change when comparing growth in galactose and glucose of cells in which the H4 gene is under control of its wild-type promoter (Kim et al. 1988). Also, differences in histone levels that may result from the use of the *GAL* promoter as opposed to the wild-type H3–H4 divergent promoter do not appear to alter superhelical density significantly (Fig. 6, cf. lanes 1, 5G, and 6G). Differences in superhelicity are likely attributable to nucleosome loss rather than G₂ arrest (Wan et al. 1992) and cell death, as nucleosomes can be reassembled in terminally arrested

A

Figure 4. Flow cytometry analysis of cells containing truncated H3/H4 amino termini (XLY101) and those undergoing nucleosome depletion (UKY403). (A) (Left) Flow cytometric analysis of log phase asynchronous cells of strain XLY101 and UKY403 in galactose containing medium. (Right) α -factor-synchronized XLY101 and UKY403 cells were released into glucose and samples were taken at different time points for flow cytometric analysis (shown at right). This demonstrates similar doubling of DNA content in both strains arrested at G_2 at ~ 3 hr. XLY101 cells also contain a minor cell fraction (20%) arrested in G_1 . (B) Quantification of cellular DNA during glucose arrest demonstrates similar increases in the DNA content of G_2 -arrested XLY101 and UKY403 cells. G_1 DNA content of α -factor-arrested cells was designated as 1N. Cellular DNA content during glucose treatment was compared with the G_1 DNA content and plotted against time. G_1 and G_2 cell populations [XLY101 (a) and XLY101 (b), respectively] were analyzed separately to quantitate cellular DNA amount.

B

UKY403 cells (Kim et al. 1988) and in XLY101 cells (data not shown) upon restoring wild-type histone synthesis in galactose. Therefore, deletions of the H3/H4 amino termini are likely to result in nucleosome depletion.

The H3 and H4 amino termini are important for nucleosome assembly in vitro

We asked here whether the H3 and H4 amino termini are required for nucleosome assembly in vitro. This was assessed in a cell-free system that supports chromatin assembly. Yeast whole-cell extracts (Schultz et al. 1991; T. Harkness and M. Schultz, both unpubl.) were prepared from the strains harboring the different histone H3 and H4 amino-terminal tail mutations. Assembly reactions were performed by incubating an aliquot of whole-cell extract with a relaxed, internally labeled plasmid. The

DNA was then analyzed by agarose gel electrophoresis to determine the level of supercoiling and, therefore, the extent of nucleosome assembly (Germond et al. 1975; Laskey and Earnshaw 1980). When the strains were grown in galactose media (Fig. 7, lanes 1–6), the bulk of the relaxed input plasmid was recovered in a highly supercoiled state in all cases, without much accumulation of intermediate topoisomers. This pattern of supercoiling indicates efficient nucleosome assembly. Whereas the six independent extracts from strains grown in galactose supported very similar levels of supercoiling, there were substantial differences among the strains when cells were grown in glucose. Extract from UKY403 (GAL-H4) cells shifted to glucose to deplete H4 synthesis was unable to supercoil the plasmid probe (Fig. 7, cf. lanes 8 and 2). Assembly was also impaired in the extract obtained from XLY101 in which truncated H3/H4 are

Figure 5. Loss of MNase generated nucleosomal ladders by H3/H4 amino-terminal deletion. (Group 1) XLY100; (group 2) XLY104; (group 3) XLY102; (group 4) XLY103; (group 5) XLY101; (group 6) UKY403. These strains express wild-type H3 and H4 histones from plasmid A in galactose (A) or mutant histones from plasmid B in glucose (B) as described in Fig. 1 and the bottom of B. Strains were grown logarithmically in galactose or in glucose containing medium. XLY101, containing truncated H3/H4 and UKY403 in which the sole H4 gene is under *GAL* promoter control are not viable in glucose. Therefore, these cells were switched from galactose- to glucose-containing medium for 6 hr and then sampled for MNase analysis (see Materials and methods). The concentrations of MNase used in the four lanes of each group are: 0, 0.0125, 0.025, and 0.05 units/μl. The left-most column of each gel contains the 123-bp marker ladder.

made (Fig. 7, cf. lanes 12 and 6), as indicated by the increased intensity of bands in the region of the gel that resolves intermediate topoisomers. However, when H3/H4 amino termini were exchanged in the same cell, the production of intermediate topoisomers was reduced significantly (Fig. 7, lane 11; cf. lane 12). Similarly, when the shift to glucose allowed expression of only one amino-terminal deletion mutant (the other histone partner is full-length), high levels of supercoiling were observed (Fig. 7, lanes 9,10).

To verify these qualitative observations, we performed protein titration and time course experiments (Fig. 8) using extracts from RMY200 (wild type), XLY101 (both H3 and H4 amino termini truncated), and XLY401a (H3 and H4 amino termini exchanged) cells grown in glucose. Signals from the intermediate topoisomers and the highly supercoiled products (Int and S in Fig. 7) were determined and quantitated by PhosphorImager analysis. Assembly is expressed as the ratio of supercoiled/intermediate products. Although this ratio magnifies differ-

ences between the extracts (because increasing supercoiling decreases the level of intermediates), it does take into account possible loading differences between the lanes. Because the proportion of open circular/relaxed product did not change significantly with increasing amount of protein and decreased with longer incubation (not shown), a higher assembly value corresponds to more efficient assembly. It is clear from the protein titration curve (Fig. 8A) that the extracts from the wild-type strain and that in which the H3 and H4 amino termini were exchanged have higher assembly activity than the extract whose H3 and H4 amino termini were both deleted. This is true even at 300 μg of protein per reaction, which is at least twofold higher than the amount required for saturation of the supercoiling activity. The time course of assembly (Fig. 8B) is similar for extracts from the wild-type strain and that whose H3 and H4 amino termini were exchanged. By comparison, at 45 min, assembly is significantly impaired in the extract containing deletions at both the H3 and H4 amino termini, and its level barely increases during a 3-hr reaction. Therefore, when both histone amino termini are truncated, nucleosome assembly is deficient all through the reaction. The time course experiment also suggests that although assembly was impaired significantly when

Figure 6. Superhelicity of endogenous 2μ plasmid DNA demonstrates the decrease in superhelical density caused by H3/H4 amino-terminal deletion. (Lane 1) RMY200; (lane 2) XLY401a; (lane 3) RMY430; (lane 4) GFY428; (lanes 5G,5D) XLY101; and (lanes 6G,6D) UKY403. The histone constructs used are as shown at the top. Strains were grown as described in Fig. 5 and probed for endogenous 2μ circle DNA separated on agarose gels containing 10 μg/ml of chloroquine as described in Materials and methods. Strains of lanes 1–4 were grown in glucose. Lanes 5G and 5D (as well as 6G and 6D) demonstrate the results of the shift from galactose (G) to glucose (D). The band near the top of each lane represents nicked plasmid DNA.

Figure 7. H3/H4 amino-terminal deletion causes a defect in nucleosome assembly in vitro. The strains used are as in Fig. 6. Cells were grown either in galactose- (lanes 1–6) or in glucose- (lanes 7–12) containing media before the preparation of whole-cell extracts for in vitro nucleosome assembly. UKY403 (*GAL-H4*) and XLY101 (*GAL-H3/GAL-H4*; *H3Δ4-30/H4Δ4-28*) are not viable in glucose, so cells were switched from galactose (*GAL-H4*: lane 2; *GAL-H3/GAL-H4*: lane 6) to glucose (*H4* depletion: lane 8; *H3Δ4-30/H4Δ4-28*: lane 12) for 6 hr to repress wild-type histone synthesis and then used for extract preparation. Chromatin assembly reactions were performed as described in Materials and methods. (P) Input plasmid; (O,R) open circular and relaxed plasmid; (Int) intermediate levels of supercoiling; (S) highly supercoiled plasmid.

the H3 and H4 amino termini were both deleted, assembly did not decrease with time. Therefore, the nucleosomes that do assemble with truncated H3 and H4 are likely to be stable. This observation is in agreement with previous in vitro data that deletion of all four histone amino termini has little effect on nucleosome stability in vitro over a wide range of conditions (Whitlock and Stein 1978; Dong et al. 1990; Hayes et al. 1991). We therefore believe it unlikely that the presence or absence of nucleosome particles in the assays described reflects solely the effects of H3 and H4 amino-terminal deletions on nucleosome stability. It is interesting to note that deletion of both histone H3 and H4 amino termini has a lesser effect on nucleosome assembly in vitro than in vivo. This may be attributable to direct as well as indirect effects on assembly in vivo that are not present in vitro. We conclude that the H3 and H4 amino termini are important for nucleosome assembly in vitro in a redundant and position-independent manner that is similar to that seen for nucleosomal assays in vivo.

Exchange of the H3/H4 amino termini cannot establish normal nucleosomal positioning and transcription at GAL1

Because H3/H4 amino termini that have been exchanged can support nucleosome assembly, we asked whether

the tails may have discrete, transposable functions in *GAL1* chromatin structure and gene regulation. Chromatin structure was inferred from the access of *Escherichia coli dam* methylase at the *GAL1* TATA element. The H4 amino terminus is required to maintain nucleosomal positioning at the $\alpha 2$ operator (Roth et al. 1992) and at the *GAL1* TATA element (Fisher-Adams and Grunstein 1995). *dam* methylase can modify yeast genomic GATC (*Sau3AI*) sites if its access is not impeded by nucleosomes (Kladde and Simpson 1994). Methylation may be detected by three restriction enzyme isoschizomers: *DpnI*, which cleaves only methylated GATC sequences; *MboI*, which cleaves only unmethylated DNA; and *Sau3AI*, which cuts both. Near the *GAL1* TATA element (15 bp upstream) there is a GATC site that is adjacent to a nucleosome (Fig. 9A) and that is accessible

Figure 8. Quantitation of chromatin assembly in vitro: protein titration and time course. In vitro nucleosome assembly reactions were performed with the following strains: (W.T. H3/H4; □) RMY200; (H3N-H4C/H4N-H3C; ○) XLY401a; and (*GAL-H3/GAL-H4*, *H3Δ4-30/H4Δ4-28*; ■) XLY101. Extracts were the same as in Fig. 7 (cells harvested after growth in glucose), and supercoiling reactions were performed as in Fig. 7, except that either the amount of protein per reaction was varied (A) or the duration of the reaction was varied (B). In A the reactions were stopped after 90 min; and in B the reactions all contained 150 μg of extract. The abundance of intermediate topoisomers and supercoiled products was quantitated by PhosphorImager analysis. (Assembly) The ratio of supercoiled/intermediates.

Figure 9. Transposed H3 and H4 amino-terminal tails cannot function normally to regulate *GAL1* chromatin structure, uninduced transcription, and induced transcription. (A) Schematic representation of the chromatin structure at the *GAL1* promoter. (○) Nucleosomes. (UAS) Upstream activating sequence. (B) Methylation assay. The expression of ectopically produced *dam* methylase was used to assay its accessibility to a GATC sequence 15 bp upstream of the TATA element (Fisher-Adams and Grunstein 1995) in strains wild type and mutant for histones H3 and H4, grown in glucose to repress the *GAL1* gene. *DpnI*, *MboI*, and *Sau3A* are isoschizomers. (D) *DpnI* cuts only methylated (GATC) DNA; (M) *MboI* cuts only unmethylated DNA; (S) *Sau3A* cuts both. The probe used is a PCR product described in Materials and methods. β -galactosidase assay: Uninduced and induced *GAL1* promoter activity was measured using the *lacZ* reporter gene attached to the *GAL1* promoter on a high-copy-number plasmid (pRY131; West et al. 1984). The activities shown are the averages of at least three separate determinations as described (Mann and Grunstein 1992). Variation was <20%. (*GAL1-LacZ*^{*}) Units of β -galactosidase. (Raff) Medium containing 2% raffinose. (Gal) Medium containing 2% galactose. (REL) Relative level of enzyme activity compared to wild-type strain.

to bacterial *dam* methylase in vivo in wild-type cells (Fig. 9B, line a; Fisher-Adams and Grunstein 1995). We wished to determine whether exchanging the H3 and H4 amino termini allowed normal access of *dam* at this site. The data presented here confirm the effect of H4 amino-terminal deletion on reducing *dam* accessibility (cf. *DpnI* and *MboI* cleavage in Fig. 9B, line d) and those showing that H3 amino-terminal deletion has little effect on access by *dam* (Fig. 9B, line b). We found that exchanging the H3 and H4 amino termini caused the GATC sequence to become inaccessible to *dam*, in a manner similar to that observed for deletion of the H4 amino terminus alone (Fig. 9B, lines f,d). Moreover, moving the H3 amino terminus onto the H4 carboxyl terminus did not substitute for the absence of the H4 tail (Fig.

9B, line e). A duplicate H4 amino terminus fused to the H3 carboxyl terminus did cause a moderate decrease in *dam* methylase accessibility (Fig. 9B, line c), suggesting that an extra H4 tail in the wrong place may disturb nucleosomal positioning. We conclude that neither the H3 nor H4 amino termini placed out of context can restore nucleosome positioning at a site adjacent to the *GAL1* TATA element.

To determine whether the exchanged H3 and H4 amino-terminal tails can function to regulate *GAL1* expression, we measured both induced and uninduced *GAL1* promoter activity using *GAL1-lacZ* reporter fusions and assaying for β -galactosidase. When the H4 amino terminus is deleted, *GAL1-lacZ* induction by galactose is reduced from 838 to 39 units of activity, a

20-fold decrease that confirms previous data (Durrin et al. 1991; Fig. 9B, line d). The H3 amino terminus fused to the H4 carboxy-terminal region does not increase *GAL1* activation (Fig. 9B, line e). Exchange of the H3/H4 amino termini (Fig. 9B, line f) also caused a sizable (sevenfold) decrease in *GAL1* activation [114 units]. Consistent with earlier findings (Mann and Grunstein 1992), deletion of the H3 amino terminus caused an ~3.2-fold increase in *GAL1* induction in galactose when compared with the isogenic wild-type control (Fig. 9B, cf. lines b and a). We observed a similar level of *GAL1* hyperactivation (3.6-fold; Fig. 9B, line c) when the H4 amino terminus was fused to the H3 carboxyl terminus in a wild-type H4 background. Therefore, it is possible that the partial alleviation in *GAL1* induction that occurs when the H3/H4 amino termini are exchanged is attributable to the competing effects of H4 and H3 tail mutations on *GAL1*. Consequently, as there appears to be some alleviation of defective *GAL1* induction when the H3/H4 amino termini are exchanged, the evidence suggests that neither H3 or H4 amino terminus functions efficiently in regulating *GAL1* when transposed.

H3 and H4 amino-terminal deletions also result in the derepression of uninduced (basal) *GAL1* activity in neutral carbon sources such as raffinose, which allow the promoter to be neither induced nor repressed (Mann and Grunstein 1992; F. Lenfant and M. Grunstein, unpubl.). The individual H3 and H4 amino-terminal deletions caused an ~3- to 10-fold increase in basal expression, although the fold activity must be interpreted with caution because of the low levels of β -galactosidase activity in the wild-type strain (Fig. 9B, cf. lines b and d with line a). The exchange of the H3/H4 amino termini in the same strain did not restore the repression of basal activity (Fig. 9B, line f). We also found that the fusion of either the H3 or H4 amino terminus to the other carboxyl terminus does not compensate for the loss of an amino terminus in repressing basal expression (Fig. 9B, lines c,e). Therefore, when transposed, the H3 and H4 amino termini cannot function to repress basal expression of the *GAL1* promoter.

Exchanged H3/H4 amino termini cannot repress the silent HM mating loci

Genetic studies have shown that the amino termini of both H3 and H4 are required for repression of the silent mating loci (*HML α* and *HMRa*) and telomeric repression of adjacent genes (Kayne et al. 1988; Aparicio et al. 1991; Thompson et al. 1994). Because genetic and biochemical data suggest that the amino termini of H3 and H4 interact with *trans*-acting SIR3 and SIR4 repressors to allow silencing (Johnson et al. 1990, 1992; Hecht et al. 1995), we wished to determine whether the exchanged H3/H4 amino termini would still repress the silent mating loci. Deletion of the H3 amino-terminal residues 4–30 decreases mating efficiency in a *MATa* strain (a measure of *HML α* derepression) approximately twofold (Fig. 10, line b). In contrast, deletion of the H4 amino-terminal residues 4–28 decreases mating by approximately seven or-

ders of magnitude (Fig. 10, line g). These results confirm those published previously (Kayne et al. 1988; Mann and Grunstein 1992). The presence of the H4 amino terminus fused to the carboxyl terminus of H3 (Fig. 10, line c) or the H3 amino terminus at H4 (Fig. 10, line h) does not cause a very different change in mating efficiency from that seen when the H3 or H4 amino terminus is individually deleted. When the amino-terminal regions were exchanged between H3 and H4, mating was still decreased by approximately seven orders of magnitude (Fig. 10, line i). Therefore, the H3/H4 amino termini, when transposed, cannot function to repress *HML*.

We then asked whether the transposed H4 amino terminus could function to rescue the mating deficiency resulting from a less extreme H4 lesion than the deletion of the entire H4 amino terminus. A single substitution of lysine at position 16 (H4 K₁₆Q) strongly decreases mating of *MATa* cells (Fig. 10, line d) confirming previous work (Johnson et al. 1990; Megee et al. 1990; Park and Szostak 1990). When the H4 amino-terminal tail is fused to the H3 carboxyl terminus in a genetic background carrying H4 K₁₆Q, mating of *MATa* cells was still reduced by about seven orders of magnitude (Fig. 10, line f). This level is similar to that seen in the control experiment in which the H4 K₁₆Q mutation is combined with the H3 amino-terminal deletion (Fig. 10, line e). Therefore, the H4 amino terminus when transposed to the H3 carboxyl terminus cannot function to restore silencing of *HML* when it is derepressed by a less extreme H4 mutation (K₁₆Q).

Silencing at *HMR* is less susceptible to histone mutations because it contains levels of redundant repression not found at *HML* (Thompson et al. 1994). This explains the less extreme effects by histone mutations on *HMR* silencing as seen in Fig. 9, lines d and g. However, we observe at *HMRa* that the presence of the H3 amino terminus on the H4 carboxyl terminus resulted in the complete loss of silencing at *HMRa* (Fig. 10, cf. lines g and h); moreover, the transposed H4 tail cannot rescue the silencing defects of a less extreme H4 mutation (H4 K₁₆Q) in its effects on *HMRa* (Fig. 10, line f). Therefore, the H3/H4 amino termini cannot be exchanged to repress either of the silent *HM* mating loci.

Discussion

The histone H3/H4 amino termini have redundant functions in nucleosome assembly in vivo and in vitro

Deletion of either the histone H3 or H4 amino termini allows viability. However, the simultaneous deletion of both the H3 and H4 amino termini is lethal and their exchange, which results in two different H3/H4 chimeric proteins, enables cellular growth. These data suggest that the H3 and H4 tails possess redundant but essential functions. The phenotypic consequences of loss of both the H3 and H4 tails are similar to those resulting from nucleosome depletion caused by repressing H4 synthesis. In both cases, G₂ arrest occurs although it is more synchronous, more rapid, and affects more cells upon the

	H3 Construct	H4 Construct	MAT a Strain	Mating efficiency	MAT α Strain	Mating efficiency
a)	N C	N C	RMY200	1	GFY3000	1
b)	N C	N C	RMY430	0.5	GFY3430	0.5
c)	N C	N C	XLY405a	0.1	XLY405 α	0.1
d)	N C	N C	XLY409a	8.6×10^{-7}	XLY409 α	0.7
e)	N C	N C	XLY410a	$< 1.8 \times 10^{-7}$	XLY410 α	2.0×10^{-5}
f)	N C	N C	XLY407a	$\leq 4.0 \times 10^{-7}$	XLY407 α	6.9×10^{-5}
g)	N C	N C	GFY428	2.2×10^{-7}	GFY34428	1.5×10^{-3}
h)	N C	N C	XLY404a	$< 2.5 \times 10^{-7}$	XLY404 α	6.3×10^{-6}
i)	N C	N C	XLY401a	$\leq 2.2 \times 10^{-7}$	XLY401 α	1.9×10^{-6}

Figure 10. Transposed H3 and H4 amino-terminal tails cannot repress the silent mating loci. Mating efficiency was determined by the quantitative mating assay as described previously (Johnson et al. 1992) with D585–11C (*MATa lys1*) as the *MATa* tester strain and D587–4b (*MAT α his1*) as the *MAT α* tester strain. (gln16) A substitution mutation from lysine to glutamine at histone H4 position 16.

prevention of new H4 synthesis. It is likely that G₂ arrest results from the absence of normal nucleosome assembly. Both micrococcal nuclease-generated nucleosomal ladders and superhelical density measurements are indicative of nucleosome assembly in whole cells. These demonstrate similar effects resulting from H4 depletion and the loss of the H3/H4 amino termini. We have also found that assembly of nucleosomes lacking their H3 and H4 tails is defective in vitro. Interestingly, exchange of the H3 and H4 amino termini restores nucleosome assembly in vivo and in vitro. Although we cannot exclude the possibility that the H3/H4 amino termini are also involved in other essential functions, our data using whole cells and cell extracts argue strongly that the H3 and H4 amino termini are necessary for the efficient assembly of stable nucleosomal particles in living cells.

This conclusion is in contrast with that of previous work, showing that stable nucleosomes can be assembled in vitro using all four core histones whose amino termini were removed by trypsin treatment (Whitlock et al. 1978; Dong et al. 1990; Hayes et al. 1991). However, the conditions for this assembly were very different from those used in cell extracts. For example, assembly occurred as DNA and histones dissolved in >1 M NaCl and 5 M urea were dialyzed to remove salts and denaturant slowly over a period of several hours. For rapid assembly under physiological conditions that generate proper nucleosome spacing it is necessary to use ATP and assembly factors. These include nucleoplasmin (Laskey et al. 1978) and N1/N2 (Kleinschmidt and Franke 1982; Dil-

worth et al. 1987; Kleinschmidt et al. 1990), which are H2A/H2B and H3/H4 specific, respectively, and are abundant in the oocyte of *Xenopus laevis*; NAP-1, which is present in eukaryotic cells, including yeast cells (Ishimi and Kikuchi 1991; Fujii-Nakata et al. 1992); and replication-dependent chromatin assembly factor (CAF-1) from the nuclei of human cells (Smith and Stillman 1991a,b). The yeast whole-cell extract used in our experiments assembles nucleosomes in an ATP-dependent manner and must contain the required nucleosome assembly factors. Under these conditions, it is possible that the histone amino termini interact with one or more of the assembly factors to allow physiological assembly. Because CAF-1 subunits appear to require the acetylated histone H4 amino terminus for assembly in human cell extracts (Kaufman et al. 1995), it is conceivable that one of the yeast assembly factors (perhaps NAP1) requires either the H3 or H4 amino terminus in a redundant manner to target the H3/H4 tetramer to chromosomal DNA for assembly.

The native location of the H3/H4 amino termini is important for nucleosome positioning and gene regulation

Although the histone H3/H4 amino termini may be exchanged to allow nucleosome assembly, their transposition does not permit proper nucleosome positioning or gene regulation at *GAL1*. One clue for this may lie in the cross-linking of His-18 at the H4 amino terminus to the

sharply bent region of nucleosomal DNA (Ebralidse et al. 1988). It is possible that this interaction between DNA and residues surrounding His-18 is crucial for positioning and that positioning is dependent on the exact location of the H4 tail with regard to the DNA path. In support of this possibility, H4 tail deletions that include His-18 change nucleosome position at the TATA region, reducing *GAL1* activation in vivo (Fisher-Adams and Grunstein 1995; Wan et al. 1995).

In the case of *HM* silencing, we observe that amino-terminal exchange causes a synergistic effect on mating, decreasing mating much more than deletion of either H3 or H4 amino terminus alone. This is comparable to the situation when mutations are present at both the H3 and H4 amino termini (Thompson et al. 1994). We have shown previously that the *trans*-regulators SIR3 and SIR4, which enable silencing of the *HM* loci, interact with the H3 and H4 amino termini in vitro (Hecht et al. 1995). This may explain why the presence of the H3 amino terminus fused to the H4 carboxyl terminus decreases mating of *MAT α* cells more than the mere absence of the H4 amino terminus alone. If SIR3 and SIR4 silence by affecting the interaction of the histone amino termini with underlying nucleosomal DNA, then the extra H3 amino terminus may interact nonfunctionally with factors necessary for silencing.

Both the *GAL1* regulation and *HM* silencing data suggest that the native location of the H3 and H4 amino termini in relation to DNA and other protein factors are important for their role in gene regulation. Proteins such as SIR3 and SIR4 may cause repression by interacting with the histone tails not merely to provide a docking site but to mediate a change in nucleosomal structure. It is possible that transcription factors may interact in a similar manner with histone tails to enable gene activation (Durrin et al. 1991). Why nucleosome assembly is less dependent on the native H3/H4 amino-terminal positions is uncertain. However, it may be explained if the assembly factors that chaperone histones H3 and H4 to chromatin interact with the amino termini of the H3/H4 tetramer before nucleosome assembly.

Materials and methods

Plasmids

The chimeric histones in pYXL401, pYXL404, pYXL405, pYXL406, pYXL407, pYXL411 were generated by recombinant PCR (Higuchi 1990). The following plasmids have been used as templates for recombinant PCR: pRM200, pRM430 (Mann and Grunstein 1992), and pLJ438 (Johnson et al. 1990). DNA sequences of chimeric histones generated were confirmed by dideoxy sequencing using Sequenase (U.S. Biochemical Corp., Cleveland, OH). pYXL409 was constructed by substitution of the wild-type H4 *Bam*HI–*Eco*RI fragment in pRM200 (Mann and Grunstein 1992), with a *Bam*HI–*Eco*RI fragment from pLJ912 (Johnson et al. 1990) containing an H4 mutation (H4 K₁₆Q). pYXL410 was created by replacing the wild-type H3 *Bam*HI–*Sal*I fragment in pYXL409 with the H3 Δ 4–30 *Bam*HI–*Sal*I fragment from pRM430 (Mann and Grunstein 1992). pGF21 was made by replacing the *Bam*HI–*Eco*RI fragment of pRM430

containing wild-type H4 with one containing H4 Δ 4–28 from pPK613 [Kayne et al. 1988].

Strain constructions

As shown in Figure 1 and Table 1, both RMY102 and GFY3001 contain plasmid A: pRM102, a *CEN4/ARS1/URA3* plasmid that bears *HHT2* (encoding H3 copy 2) fused to the *GAL10* promoter, and *HHF2* (encoding H4 copy 2) fused to the *GAL1* promoter (Mann and Grunstein 1992; Fisher-Adams and Grunstein 1995). These two strains, which are dependent on galactose for wild-type histone H3 and H4 synthesis, were transformed with plasmids containing chimeric H3 or H4 genes under the control of their native histone promoter (Fig. 1, plasmid B). Shifting the strains to glucose allowed repression of wild-type H3 and H4 synthesis and growth dependence on the chimeric histone proteins (Kayne et al. 1988). If a strain was viable in glucose, subsequent loss of pRM102 by intrinsic mitotic instability completed the strain construction.

Synchronization, flow cytometry, and DAPI staining

Synchronization of *MAT α* cells with α -factor (Kim et al. 1988), flow cytometry (Nash et al. 1988), and DAPI staining (Han and Grunstein 1987) were done as described. The cells were analyzed for DNA content with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Thirty thousand events were acquired using C30/FACScan Research software (Becton Dickinson) and analyzed on a HP 9000 series model 310 computer.

Cellular growth and viability

The growth rates of all the strains except XLY101 were determined by spectrophotometric means as described previously (Durrin et al. 1991). Upon longer glucose incubation, the size of the XLY101 increased approximately twofold. Therefore, cellular growth in glucose was measured by counting cell numbers in a hemocytometer at different time intervals. The viability of XLY101 cells after growth on glucose was measured by replating cells from glucose onto galactose medium and counting viable colonies after 2 days of incubation at 30°C.

MNase analysis

MNase analysis of yeast chromatin in NP-40-treated spheroplasted cells was performed essentially as described by Fisher-Adams and Grunstein (1995). Cells were grown in 500 ml of galactose or glucose-containing medium to an OD₆₀₀ = 1.0, harvested, washed with water (including carbon source, 2% galactose or glucose), and treated with pretreatment buffer (4 ml/g cells) for 15 min at 30°C. Cells were pelleted at 4000 rpm for 5 min then spheroplasted in S buffer with 3 mg Zymolyase/gram of cells at 30°C. The efficiency of spheroplasting was monitored by diluting the cells 20-fold in water to lyse spheroplasts. Spheroplasts were pelleted in a Beckman centrifuge (4000 rpm for 5 min at 4°C) and were resuspended in digestion buffer (4 ml/gram of cells). Samples were kept on ice for subsequent procedures. For each digest, 475 μ l of buffer containing spheroplasts was added to 25 μ l of solution containing MNase (Worthington) [0, 0.25, 0.5, 1 U/ μ l, i.e., from 0 to 25 units per digestion] at 30°C for 10 min, 50 μ l stop solution was added (10 min on ice) to terminate each digestion. Twenty microliters of 20 mg/ml of proteinase K was added and incubated overnight at 37°C. Each sample was extracted twice with phenol–chloroform (1:1). DNA was precipitated with ethanol, resuspended in 200 μ l

Table 1. Strains used in this study

Strain	Genotype
UKY403	<i>MATa ade2-101(och) his3-Δ200 leu2-3,-112 lys2-801(amb) trp1-Δ901 ura3-52 GAL⁺ thr-tyr-arg4-1 hhf1::HIS3 hhf2::LEU2</i> , plus pUK421 (<i>TRP1, GAL1-HHF2</i>) [Kim et al. 1988]
RMV200	<i>MATa ade2-101(och) his3-Δ200 lys2-801(amb) trp1-Δ901 ura3-52 hht1, hhf1::LEU2, hht2, hhf2::HIS3</i> plus pRM200 (<i>CEN4 ARS1 TRP1, HHT2, HHF2</i>) [Mann and Grunstein 1992]
RMV102	isogenic to RMV200 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] [Mann and Grunstein 1992]
RMV430	isogenic to RMV200 plus pRM430 [<i>CEN4 ARS1 TRP1, hht2Δ4-30, HHF2</i>] [Mann and Grunstein 1992]
GFY3000	<i>MATa ade2-101 his3-Δ201 leu2-3,-112 trp1-Δ901 ura3-52 lys2-801:dam⁺:LYS2 hht1, hhf1::LEU2 hht2-hhf2::HIS3</i> plus pRM200 [<i>CEN4 ARS1 TRP1, HHT2, HHF2</i>] [Fisher-Adams and Grunstein 1995]
GFY3001	isogenic to GFY3000 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] [Fisher-Adams and Grunstein 1995]
GFY3430	isogenic to GFY3000 plus pRM430 [<i>CEN4 ARS1 TRP1, hht2Δ4-30, HHF2</i>] [Fisher-Adams and Grunstein 1995]
GFY34428	isogenic to GFY3000 plus pGF29 [<i>CEN4 ARS1 TRP1, HHT2, hhf2Δ4-28</i>] [Fisher-Adams and Grunstein 1995]
GFY428	isogenic to RMV200 plus pGF29 [<i>CEN4 ARS1 TRP1, HHT2, hhf2Δ4-28</i>]
XLY100	isogenic to RMV200 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] and pRM200 [<i>CEN4 ARS1 TRP1, HHT2, HHF2</i>]
XLY101	isogenic to RMV200 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] and pGF21 [<i>CEN4 ARS1 TRP1, hht2Δ4-30, hhf2Δ4-28</i>]
XLY102	isogenic to RMV200 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] and pRM430 [<i>CEN4 ARS1 TRP1, hht2Δ4-30, HHF2</i>]
XLY103	isogenic to RMV200 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] and pGF29 [<i>CEN4 ARS1 TRP1, HHT2, hhf2Δ4-28</i>]
XLY104	isogenic to RMV200 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] and pYXL401 [<i>CEN4 ARS1 TRP1, H3N(1-30)H4C(29-102), H4N(1-28)H3C(31-135)</i>]
XLY401a	isogenic to RMV200 plus pYXL401 [<i>CEN4 ARS1 TRP1, H3N(1-30)H4C(29-102), H4N(1-28)H3C(31-135)</i>]
XLY404a	isogenic to RMV200 plus pYXL404 [<i>CEN4 ARS1 TRP1, HHT2, H3N(1-30)H4C(29-102)</i>]
XLY405a	isogenic to RMV200 plus pYXL405 [<i>CEN4 ARS1 TRP1, HHF2, H4N(1-28)H3C(31-135)</i>]
XLY406	isogenic to RMV200 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] and pYXL405 [<i>CEN4 ARS1 TRP1 H4N(1-28)H3C(31-135), hhf2Δ4-19</i>]
XLY407a	isogenic to RMV200 plus pYXL407 [<i>CEN4 ARS1 TRP1, H4N(1-28)H3C(31-135), hhf2 gln16</i>]
XLY409a	isogenic to RMV200 plus pYXL409 [<i>CEN4 ARS1 TRP1, HHT2, hhf2 gln16</i>]
XLY410a	isogenic to RMV200 plus pYXL410 [<i>CEN4 ARS1 TRP1, hht2Δ4-30, hhf2 gln16</i>]
XLY411	isogenic to RMV200 plus pRM102 [<i>CEN4 ARS1 URA3, P(GAL10)-HHT2, P(GAL1)-HHF2</i>] and pYXL411 [<i>CEN4 ARS1 TRP1 hht2Δ4-30, H3N(1-30)H4C(29-102)</i>]
XLY401α	isogenic to GFY3000 plus pYXL401 [<i>CEN4 ARS1 TRP1, H3N(1-30)H4C(29-102), H4N(1-28)H3C(31-135)</i>]
XLY404α	isogenic to GFY3000 plus pYXL404 [<i>CEN4 ARS1 TRP1, HHT2, H3N(1-30)H4C(29-102)</i>]
XLY405α	isogenic to GFY3000 plus pYXL405 [<i>CEN4 ARS1 TRP1, HHF2, H4N(1-28)H3C(31-135)</i>]
XLY407α	isogenic to GFY3000 plus pYXL407 [<i>CEN4 ARS1 TRP1, H4N(1-28)H3C(31-135), hhf2 gln16</i>]
XLY409α	isogenic to GFY3000 plus pYXL409 [<i>CEN4 ARS1 TRP1, HHT2, hhf2 gln16</i>] [Thompson et al. 1994]
XLY410α	isogenic to GFY3000 plus pYXL410 [<i>CEN4 ARS1 TRP1, hht2Δ4-30, hhf2 gln16</i>] [Thompson et al. 1994]

of TE (10 mM Tris-HCl, 1 mM EDTA at pH 7.5) and treated with 20 μg of pancreatic ribonuclease A (Worthington, DNase and protease free) at 37°C for 30 min. Each sample was extracted again with phenol-chloroform, and the DNA was precipitated in ethanol. Isolated DNA was electrophoresed on 1.5% agarose gels and stained with ethidium bromide (0.5 μg/ml). Pretreatment buffer consisted of 100 mM EDTA, 0.5% βME, and 1% carbon source; S buffer comprised 1.08 M sorbitol, 20 mM KPO₄ (pH 6.8), 0.5 mM CaCl₂, 0.5% βME, and 1% carbon source; digestion buffer [Stewart et al. 1991] comprised 15 mM Tris 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 300 mM sucrose, 0.2% NP-40, 0.5 mM βME, and 1 mM PMSF; and Stop solution consisted of 500 mM EDTA, and 1% SDS.

Superhelical density analysis

Plasmid DNA was isolated as described by Hoffman and Winston [1987], except that cells and glass beads were shaken vigorously for 4 min in an Eppendorf mixer (5432). DNA (10 μg)

was electrophoresed in 0.8% agarose gels with 10 μg/ml of chloroquine (at this chloroquine concentration, the resolved topoisomers are positively supercoiled) and subjected to Southern blot analysis with ³²P-labeled DNA. The *EcoRI* fragment from plasmid YEp24 that contains 2μ circle sequence was used to probe for endogenous 2μ plasmids.

In vitro chromatin assembly

Strains UKY403 (*GAL-H4*), RMV430 (*H3Δ4-30*), GFY428 (*H4Δ4-28*), and XLY101 (*GAL-H3/GAL-H4; H3Δ4-30/H4Δ4-28*) were grown in galactose-containing medium at 30°C in two parallel 500-ml cultures to an OD₆₀₀ of 3. At this point, one flask of cells was harvested while cells in the duplicate culture were washed once in sterile H₂O and then resuspended in 500 ml glucose containing medium. After 6 hr of culture, the cells were harvested and frozen. Extracts of RMV200 and XLY401a (both viable in glucose; Fig. 2) were prepared from cells grown entirely in glucose-containing medium to an OD₆₀₀ of 3. Whole-

Ling et al.

cell extracts were prepared from frozen cells by the method of Schultz et al. (1991), using dry ice and coffee mills to break the cells. This whole-cell extract contains all the components required for replication-independent nucleosome assembly onto naked, double-stranded plasmid DNA. Chromatin assembly reactions involved incubation of 150 μ g of extract protein with 20 ng of relaxed, internally 32 P-labeled (Razvi et al. 1983) pBlue-Script KS(+) (Stratagene), in the presence of an ATP regeneration system, for 90 min at 30°C (M. Schultz; T. Harkness and M. Schultz; both unpubl.). Following incubation, the labeled DNA was deproteinized, precipitated, resolved on a native 1% agarose gel, and visualized by autoradiography of the dried gel.

Methylation assay

dam methylase accessibility of the site in *GAL1* TATA region was assayed essentially as described by Fisher-Adams and Grunstein (1995), except that the probe is a PCR product using pNN78 as template (*GAL1* DNA; St. John and Davis 1981). The oligonucleotides used for PCR were GAL1P-EcoRI, GGTTAT-CAGCAACAACACAGTCATATCCATTCTCA and GAL1P-Sau3A, CGCTTCGCTGATTAATTACCCAGAAATAAG GCTA.

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