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-
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/TRPI vector [plasmid B] in a 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. 1] is lethal, but their exchange restores cellular viability. Therefore, the importance of the histone amino termini in nucleosome assembly remains undetermined.
| Strain      | H3 Construct | H4 Construct | Viability | Doubling time (min) |
|-------------|--------------|--------------|-----------|--------------------|
| a) RMY200   | N 1          | C 135        | Viable    | 93                 |
| b) RMY430   | N Δ-4         | C 102        | Viable    | 135                |
| c) GFY428   | N 1          | C 135        | Viable    | 246                |
| d) XLY101   | N 1-28       | C 31-135     | Inviable  | N/A*               |
| e) XLY401a  | N Δ-4         | C 1-30       | Viable    | 264                |
| f) XLY411   | N 1-28       | C 31-135     | Inviable  | N/A*               |
| g) XLY406   | N Δ-1-28     | C 31-135     | 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 H4Δ-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% after 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,
Histone amino termini enable nucleosome assembly

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 poly-nucleosomal 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 2μ 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 2μ 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...
Ling et al.

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 asynchronized 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 G2 at −3 hr. XLY101 cells also contain a minor cell fraction (20%) arrested in G1. (B) Quantification of cellular DNA during glucose arrest demonstrates similar increases in the DNA content of G2-arrested XLY101 and UKY403 cells. G2 DNA content of α-factor-arrested cells was designated as 1N. Cellular DNA content during glucose treatment was compared with the G1 DNA content and plotted against time. G1 and G2 cell populations [XLY101 (a) and XLY101 (b), respectively] were analyzed separately to quantitate cellular DNA amount.

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 medium (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...
Histone amino termini enable nucleosome assembly

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/txl. The left-most column of each gel contains the 123-bp marker ladder.

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.

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 differences 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...
the tails may have discrete, transposable functions in \textit{GAL1} chromatin structure and gene regulation. Chromatin structure was inferred from the access of \textit{Escherichia coli} dam methylase at the \textit{GAL1} TATA element. The H4 amino terminus is required to maintain nucleosomal positioning at the a2 operator (Roth et al. 1992) and at the \textit{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 \textit{GAL1} TATA element (15 bp upstream) there is a GATC site that is adjacent to a nucleosome (Fig. 9A) and that is accessible.

![Figure 7](image)

**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; H34–30/H44–28) 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; H34–30/H44–28: 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...
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 Mbol 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 c]. 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 GALI TATA element.

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

Table: Dam methylase accessibility

| Strain   | Dam methylase accessibility | Strain   | H3 Construct | H4 Construct | Strain   | H3 Construct | H4 Construct | Strain   | H3 Construct | H4 Construct | Strain   | H3 Construct | H4 Construct |
|----------|-----------------------------|----------|--------------|--------------|----------|--------------|--------------|----------|--------------|--------------|----------|--------------|--------------|
|          |                            |          |              |              |          |              |              |          |              |              |          |              |              |
|          | DpnI McC MboI Sau3A         |          |              |              |          |              |              |          |              |              |          |              |              |
| a) GFY3000 | N 1 135 C                 |          |              |              |          |              |              |          |              |              |          |              |              |
|          |                            |          |              |              |          |              |              |          |              |              |          |              |              |
| b) GFY3430 | N 1 102 C                 |          |              |              |          |              |              |          |              |              |          |              |              |
|          |                            |          |              |              |          |              |              |          |              |              |          |              |              |
| c) XLY405α | N 1 102 C                 |          |              |              |          |              |              |          |              |              |          |              |              |
|          |                            |          |              |              |          |              |              |          |              |              |          |              |              |
| d) GFY34428 | N 1 102 C                 |          |              |              |          |              |              |          |              |              |          |              |              |
|          |                            |          |              |              |          |              |              |          |              |              |          |              |              |
| e) XLY404α | N 1 102 C                 |          |              |              |          |              |              |          |              |              |          |              |              |
|          |                            |          |              |              |          |              |              |          |              |              |          |              |              |
| f) XLY401α | N 1 102 C                 |          |              |              |          |              |              |          |              |              |          |              |              |

Figure 9. Transposed H3 and H4 amino-terminal tails cannot function normally to regulate GALI chromatin structure, uninduced transcription, and induced transcription. (A) Schematic representation of the chromatin structure at the GALI promoter. (O) 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 GALI gene. DpnI, Mbol, and Sau3A are isoschizomers. (D) DpnI cuts only methylated (GATC) DNA; (M) Mbol 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 GALI promoter activity was measured using the lacZ reporter gene attached to the GALI 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%. (GALI–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.
we wished to determine whether the exchanged H3/H4 amino termini 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 strain. Therefore, there is an apparent partial alleviation in GAL1 induction that occurs when the H3/H4 amino termini are exchanged and 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 a ∼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 terminus 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 (HMLα and HMRα) 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 MATα strain (a measure of HMLα derepression) approximately twofold (Fig. 10, line b). In contrast, deletion of the H4 amino-terminal residues 4–28 decreases mating by approximately seven orders 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 MATα 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 MATα 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 repressors that are not found at HML (Thompson et al. 1994). This explains the extreme effects of histone mutations on HMR silencing as seen in Fig. 9, lines d and g. However, we observe at HMRα that the presence of the H3 amino terminus on the H4 carboxyl terminus resulted in the complete loss of silencing at HMRα (Fig. 10, cf. lines g and h), although, the transposed H4 tail cannot rescue the silencing defects of a less extreme H4 mutation (H4 K16Q) in its effects on HMRα (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 deletion caused by repressing H4 synthesis. In both cases, G2 arrest occurs although it is more synchronous, more rapid, and affects more cells upon the
Histone amino termini enable nucleosome assembly

| H3 Construct | H4 Construct | MATα Strain | Mating efficiency | MATα Strain | Mating efficiency |
|--------------|-------------|-------------|-------------------|-------------|-------------------|
| a) 1-135 C   | 1-102 C     | RMY200      | 1                 | GFY3000     | 1                 |
| b) 1-30 C    | 31-135 C    | RMY430      | 0.5               | GFY3430     | 0.5               |
| c) 1-28 C    | 31-135 C    | XLY405a     | 0.1               | XLY405α     | 0.1               |
| d) 1-135 C   | 1 gln16 C   | XLY409a     | 8.6 x 10⁻⁷        | XLY409α     | 0.7               |
| e) 1-28 C    | 31-135 C    | XLY410a     | <1.8 x 10⁻⁷       | XLY410α     | 2.0 x 10⁻⁵        |
| f) 1-28 C    | 31-135 C    | XLY407a     | ≤4.0 x 10⁻⁷       | XLY407α     | 6.9 x 10⁻⁵        |
| g) 1-135 C   | Δ4-28 C     | GFY428      | 2.2 x 10⁻⁷        | GFY34428    | 1.5 x 10⁻³        |
| h) 1-30 C    | 29-102 C    | XLY404a     | ≤2.5 x 10⁻⁷       | XLY404α     | 6.3 x 10⁻⁴        |
| i) 1-28 C    | 31-135 C    | XLY401a     | ≤≤2.2 x 10⁻⁷      | XLY401α     | 1.9 x 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 (MATα lys1) as the MATα tester strain and D587-4b (MATα his1) as the MATα tester strain. (gln16) A substitution mutation from lysine to glutamine at histone H4 position 16.
sharply bent region of nucleosomal DNA [Ebralidine 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 MATα 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 pJ438 [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 BamHI–EcoRI fragment in pRM200 [Mann and Grunstein 1992], with a BamHI–EcoRI fragment from pLJ912 [Johnson et al. 1990] containing an H4 mutation [H4 K16Q]. pYXL410 was created by replacing the wild-type H3 BamHI–Sall fragment in pYXL409 with the H3A4–30 BamHI–Sall fragment from pRM430 [Mann and Grunstein 1992]. pGF21 was made by replacing the BamHI–EcoRI fragment of pRM430 containing wild-type H4 with one containing H4A4–28 from pPK613 [Kaye 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 HHT72 (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 [Kaye 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 MATα 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, 1% 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 μl of buffer containing spheroplasts was added to 25 μl of solution containing MNase [Worthington] [0, 0.25, 0.5, 1 U/μl], i.e., from 0 to 25 units per digestion at 30°C for 10 min, 50 μl 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 μl.
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 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 probe for endogenous 2μ circle sequence was used to probe for endogenous 2μ plasmids.

Table 1. Strains used in this study

| Strain    | Genotype                                                                 |
|-----------|--------------------------------------------------------------------------|
| UKY403    | 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, plus pUK421 [TRP1, GAL1-ΔHFF2] [Kim et al. 1988] |
| RMY200    | MATa ade2-101(och) his3-Δ200 lys2-801(amb) trp1-Δ901 ura3-52 hht1, hht1::LEU2, hht2, hht2::HIS3 plus pRM200 [CEN4 ARS1 TRP1, HHT2, HHTF2] [Mann and Grunstein 1992] |
| RMY102    | isogenic to RMY200 plus pRM102 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] [Mann and Grunstein 1992] |
| RMY430    | isogenic to RMY200 plus pRM430 [CEN4 ARS1 TRP1, hht2Δ4-30, HHTF2] [Mann and Grunstein 1992] |
| GFY3000   | MATa ade2-101 his3-Δ201 leu2-3,112 trp1-Δ901 ura3-52 lys2-801:dam+ lys2::HIS3 plus pRM200 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] [Fisher-Adams and Grunstein 1995] |
| GFY3001   | isogenic to GFY3000 plus pRM102 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] [Fisher-Adams and Grunstein 1995] |
| GFY3430   | isogenic to GFY3000 plus pRM430 [CEN4 ARS1 TRP1, hht2Δ4-30, HHTF2] [Fisher-Adams and Grunstein 1995] |
| GFY34428  | isogenic to RMY200 plus pGF29 [CEN4 ARS1 TRP1, HHT2, hht2Δ4-28] [Fisher-Adams and Grunstein 1995] |
| XLY100    | isogenic to RMY200 plus pRM102 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pRM200 [CEN4 ARS1 TRP1, HHT2, HHTF2] |
| XLY101    | isogenic to RMY200 plus pRM102 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pGF21 [CEN4 ARS1 TRP1, hht2Δ4-30, hht2Δ4-28] |
| XLY102    | isogenic to RMY200 plus pRM102 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pRM430 [CEN4 ARS1 TRP1, hht2Δ4-30, HHTF2] |
| XLY103    | isogenic to RMY200 plus pRM102 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pGF29 [CEN4 ARS1 TRP1, HHT2, hht2Δ4-28] |
| XLY104    | isogenic to RMY200 plus pRM102 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pYXL401 [CEN4 ARS1 TRP1, H3N(1-30)H4C(29-102), H4N(1-28)H3C(31-135)] |
| XLY401a   | isogenic to RMY200 plus pYXL401 [CEN4 ARS1 TRP1, H3N(1-30)H4C(29-102), H4N(1-28)H3C(31-135)] |
| XLY404a   | isogenic to RMY200 plus pYXL404 [CEN4 ARS1 TRP1, HHT2, H3N(1-30)H4C(29-102)] |
| XLY405a   | isogenic to RMY200 plus pYXL405 [CEN4 ARS1 TRP1, HHT2, H3N(1-30)H4C(29-102)] |
| XLY406    | isogenic to RMY200 plus pYXL406 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pYXL405 [CEN4 ARS1 TRP1, H4N(1-28)H3C(31-135), hht2Δ4-19] |
| XLY407a   | isogenic to RMY200 plus pYXL407 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pYXL411 [CEN4 ARS1 TRP1, HHT2, hht2Δ4-30, hht2Δ4-28] |
| XLY409a   | isogenic to RMY200 plus pYXL409 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pYXL411 [CEN4 ARS1 TRP1, HHT2, hht2Δ4-30, hht2Δ4-28] |
| XLY410a   | isogenic to RMY200 plus pYXL410 [CEN4 ARS1 URA3, P(GAL1)-HHT2, P(GAL1)-HHTF2] and pYXL411 [CEN4 ARS1 TRP1, hht2Δ4-30, hht2Δ4-28] |
| XLY411    | isogenic to RMY200 plus pYXL411 [CEN4 ARS1 TRP1, hht2Δ4-30, hht2Δ4-28] |
| XLY410p   | isogenic to GFY3000 plus pYXL401 [CEN4 ARS1 TRP1, H3N(1-30)H4C(29-102), H4N(1-28)H3C(31-135)] |
| XLY404p   | isogenic to GFY3000 plus pYXL404 [CEN4 ARS1 TRP1, HHT2, H3N(1-30)H4C(29-102)] |
| XLY405p   | isogenic to GFY3000 plus pYXL405 [CEN4 ARS1 TRP1, HHT2, H4N(1-28)H3C(31-135)] |
| XLY407p   | isogenic to GFY3000 plus pYXL407 [CEN4 ARS1 TRP1, H4N(1-28)H3C(31-135), hht2Δ4-19] |
| XLY409p   | isogenic to GFY3000 plus pYXL409 [CEN4 ARS1 TRP1, HHT2, hht2Δ4-30, hht2Δ4-28] |

Histone amino termini enable nucleosome assembly

In vitro chromatin assembly
Strains UKY403 [GAL-H4], RMY430 [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 RMY200 and XLY401a (both viable in glucose; Fig. 2) were prepared from cells grown entirely in glucose-containing medium to an OD600 of 3. Whole-
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 μg of extract protein with 20 ng of relaxed, internally 32P-labeled (Razvi et al. 1983) pBlueScript 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, GGTTATCAAGCAACAACACAGTCATATCCATTCTCA and GAL1P-Sau3A, CGCCTTGGCTGATTAATTACCCCAGAAATAAGGCTA.

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