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Yeast histone H4 and H3 N-termini have different effects on the chromatin structure of the *GAL1* promoter

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Deletion of the histone H4 N-terminal residues 4–23 decreases activation of the *GAL1* promoter as much as 20-fold, while deletion of histone H3 N-terminal residues 4–15 hyperactivates *GAL1* ~3-fold. In an attempt to understand the mechanisms by which these two different events take place, we have examined the effects of the H4 and H3 lesions on *GAL1* chromatin structure. The bacterial *dam* methylase, which methylates adenine residues of GATC sequences, was used as an *in vivo* probe for chromatin structure and both indirect end-labeling and ligation mediated PCR (LMPCR) analysis of micrococcal nuclease digestions were used to analyze chromatin in isolated nuclei. We show that while deletions of the H4 and H3 N-termini have similar effects on *dam* methylase access in the *GAL1* coding region, the H4 N-terminal deletion uniquely alters *dam* access at a region near the TATA element. This change is independent of the transcriptional state of *GAL1*. In addition, LMPCR analysis of micrococcal nuclease digests of yeast nuclei demonstrate that H4 N-terminal deletion has unique effects on nuclease accessibility in the nucleosomal region upstream of the TATA element. Our results are consistent with the H4 N-terminus mediating activation of *GAL1* through its effect on the proximal promoter region near the TATA box. These data also suggest that the H3 N-terminus affects *GAL1* hyperactivation through a different promoter element than that affected by H4.

Key words: *dam* methylase/*GAL1*/histone H3/histone H4/micrococcal nuclease

Introduction

There is considerable evidence suggesting that the nucleosomal core particle acts as a repressor of gene expression *in vitro* (Knezetic and Luse, 1986; Lorch *et al.*, 1987; Workman and Roeder, 1987) and *in vivo* (Han and Grunstein, 1988; Durrin *et al.*, 1992). In addition, the basic histone N-terminal domains have been shown to act as complex regulators of transcription with both negative and positive functions. For example, the H4 and H3 N-termini of *Saccharomyces cerevisiae* have repressive functions in the silencing of heterochromatin-like regions at the silent mating loci and regions adjacent to the telomeres (Kayne *et al.*, 1988; Johnson *et al.*, 1990, 1992; Megee *et al.*, 1990; Park and Szostak, 1990; Aparico *et al.*, 1991; Thompson *et al.*, 1994). However, the effects of histone H4 and H3 mutations are not identical. Single amino acid substitutions in the repressive H4 N-terminal domain (residues 16–29) derepress telomeric silencing much more than even large deletions at the H3 N-terminus (within residues 4–20) (Thompson *et al.*, 1994). The different effects of the H4 and H3 N-terminal deletions are even more dramatic at the highly regulated *GAL1* promoter. Deletion of the H4 N-terminal residues 4–23 or mutagenesis of the acetylated lysine residues (at positions 5, 8, 12 and 16) within this domain strongly decreases activation of the *GAL1* promoter (Durrin *et al.*, 1991). In contrast, deletion of the H3 N-terminal residues 4–15 or mutagenesis of the acetylated lysine residues within and adjacent to this region (residues 9, 14, 18 and 23) results in the hyperactivation of *GAL1* (Mann and Grunstein, 1992).

Here, we have attempted to understand the mechanisms by which H4 and H3 N-terminal deletions have opposing effects on *GAL1* transcription by examining their effects on chromatin structure. Roth *et al.* (1992) have shown that histone H4 N-terminal deletions disrupt nucleosomal positioning adjacent to the yeast α2 operator, and it is possible that the histone N-terminal deletions alter *GAL1* promoter chromatin as well. *GAL1* is repressed when cells are grown in glucose. When cells are grown in non-inducing carbon sources such as raffinose, the activator protein, Gal4p, is bound to the UAS_G (upstream activating sequence), but is inhibited by an associated protein, Gal80p. *GAL1* is only active when the cells are grown in galactose and the inhibitory function of Gal80p has been alleviated (Johnston, 1987). Nucleosomes are positioned precisely over the *GAL1* gene. Under repressed conditions, there is a nucleosome positioned adjacent to the UAS_G which protects a region of DNA extending to the TATA element. Downstream from this nucleosome is a second nucleosome which covers the transcriptional initiation start site. Under activating conditions this repressed chromatin structure is disrupted (Lohr, 1984; Selleck and Majors, 1988; Fedor and Kornberg, 1989), a change which is dependent upon the presence of Gal4p, although not necessarily on transcriptional activation (Axelrod *et al.*, 1993).

We have assayed the effects of the histone N-terminal deletions on *GAL1* chromatin structure both *in vivo* and in isolated nuclei. For the former, yeast strains were constructed which contain a series of either H4 or H3 N-terminal deletions and which ectopically express the *Escherichia coli dam* methylase gene. While yeast DNA is not normally methylated, the *dam* methylase modifies adenines contained within 5′-GATC-3′ sequences throughout the genome, a modification which does not appear to

affect yeast cell growth. GATC sequences that are accessible to the methylase can then be distinguished by enzymes that cleave either methylated or unmethylated GATC sequences (Hoekstra and Malone, 1985). In this manner, it has been reported that gene activity may be accompanied by increased access to the methylase, interpreted as the unfolding of chromatin (Gottschling, 1992; Singh and Klar, 1992; Wright *et al.*, 1992). Moreover, *dam* methylase is accessible to GATC sites within the linker between nucleosomes and up to 21 bp into the nucleosome itself while the nucleosome core-contacting DNA and DNA bound by proteins is inaccessible to the *dam* methylase (Kladde and Simpson, 1994). We show here that deletions in the histone H4 N-terminus cause a GATC sequence 15 bp upstream of the TATA element of *GAL1* (the sequence TATAAA) to become much less accessible to *dam* as it is converted from an 'open' to a 'closed' conformation. This closed structure persists regardless of the transcriptional state of *GAL1* and is dependent on the sites of acetylation at the H4 N-terminus. In contrast, this GATC sequence is not obviously altered by H3 N-terminal deletions and sequences within the transcribed portion of *GAL1* are altered in a similar manner by both H4 and H3 N-terminal deletions.

To probe possible changes in nucleosome positioning, we have also used indirect end-labeling of micrococcal nuclease (MNase) digests and high resolution, ligation mediated PCR (LMPCR) of MNase digests of yeast nuclei. These studies investigate whether enzyme access is altered over the *GAL1* regulatory sequence. Both H4 and H3 N-terminal deletions result in different sites of MNase cleavage downstream of the TATA element. These may represent altered nucleosomal positions. However, the H4 N-terminal deletion uniquely results in the protection of a region just upstream of the TATA element, adjacent to the *dam* methylase recognition site. MNase digestion suggests that this protected region contains a nucleosome which may interfere with normal TATA promoter function. These experiments also suggest that the H3 N-terminus mediates its hyperactivation of *GAL1* through a regulatory region other than the one near the TATA element.

Results

Histone H4 and H3 N-terminal deletions have different effects on the accessibility of dam methylase near the GAL1 (TATA) promoter

To assay the effects of histone N-terminal deletions on *GAL1* chromatin structure *in vivo*, yeast strains were constructed carrying a series of N-terminal deletions of either histone H4 (Δ4–19, Δ4–23, Δ4–28) or H3 (Δ4–15, Δ4–20, Δ4–30), and which ectopically express the *E.coli dam* methylase gene. The *dam* methylase can modify 5′-GATC-3′ sites in genomic DNA. These strains were grown in glucose to repress *GAL1* transcription. The GATC sequences examined at the *GAL1* promoter are shown in Figure 1. We first examined the GATC sequence S_1, 15 bp upstream of the promoter (TATA) element. DNA was first cleaved with *Eco*RI and subsequently cut with restriction enzyme isoschizomers: *Dpn*I, which cleaves only dimethylated GATC sequences; *Mbo*I, which cleaves only unmethylated and *Sau*3AI, which cleaves both forms of the GATC sequence. A 285 bp *Eco*RI−*Ban*II probe, probe 1 (Figure 1), was used to limit analysis specifically to site S_1. Densitometry of Southern blots from cells containing wild-type histones shows that S_1 is ~90% cut by *Dpn*I and has therefore been highly methylated *in vivo* (Figure 2A, compare lanes 4 and 5). This argues that the chromatin structure at this site is normally in a very accessible (open) conformation.

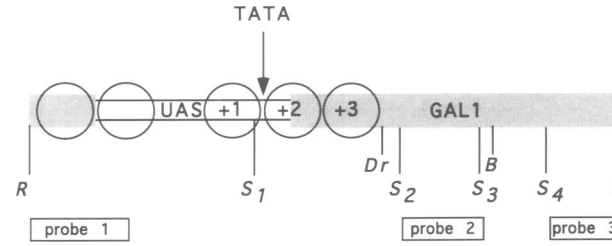

Fig. 1. Schematic of the *GAL1* locus of *S.cerevisiae*. This schematic shows the position of nucleosomes (+1 and +2) relative to the UAS and TATA element of the *GAL1* gene. S_{1-4} represent Sites 1–4, the GATC sequences probed for methylation by the *dam* methylase. Probes labeled are those used for the *dam* methylase Southern analysis and are described in Materials and methods. Please note that probe 2 was used to probe for either S_2 or S_3. To probe for S_2, DNA was first cleaved with *Ban*II, then the methyl-adenine sensitive enzymes. To probe for S_3, DNA was first cleaved with *Dra*I, and for S_1 and S_4, cleavage was first performed with *Eco*RI. These restriction enzyme sites are indicated: R (*Eco*RI), Dr (*Dra*I), B (*Ban*II).

This data contrasts with data of Singh and Klar (1992) which showed that this site is only 3.5% methylated. Since their studies utilized DNA from cells grown to mid-log phase while our work used DNA from cells in late-log to stationary phase, we repeated the *dam* methylase analysis at S_1 on cells harvested in early to mid-log phase. We observed a striking reduction in the amount of methylated S_1 DNA in these log-phase cells (Figure 2A, lane 1 and 2). Only ~10% of the S_1 DNA was methylated in strains bearing wild-type histones or mutant histone H3 as quantified by densitometry of Figure 2A. This is in contrast to ~90% methylation observed for stationary phase cells (Figure 2A, lanes 4 and 5).

One possible explanation for the increased methylation in stationary phase cells may be increased *dam* methylase expression in these cells. To test whether the *dam* methylase may be limiting in the cells harvested in early to mid-logarithmic phase, we isolated total RNA from cultures grown to varying densities from OD_{600} = 0.5 to 6.0. Northern analysis of *dam* methylase message from 20 μg RNA from each of these samples was analyzed and the results are shown in Figure 2B. We used a strain which did not express *dam* methylase as a control (Figure 2B, lanes 1 and 2). Analysis of a strain (GFY3000) which carried the *dam* methylase gene, showed that the *dam* mRNA increased during cell growth (Figure 2B, lanes 3–6) and was most highly expressed in cells of OD_{600} >5 (Figure 2B, lane 6). The increase is ~6-fold as determined by densitometry when normalized to the rRNA levels shown. These data imply that analysis of DNA from cells too early in cell growth may take place under conditions when *dam* methylase is limiting. Thus, to assure maximal methylation of *dam* methylase sites, the data described below utilized DNA isolated from cells in stationary phase.

In Figure 3A we compare the effects of H4 and H3 N-terminal deletions on *dam* access to the S_1 site at the

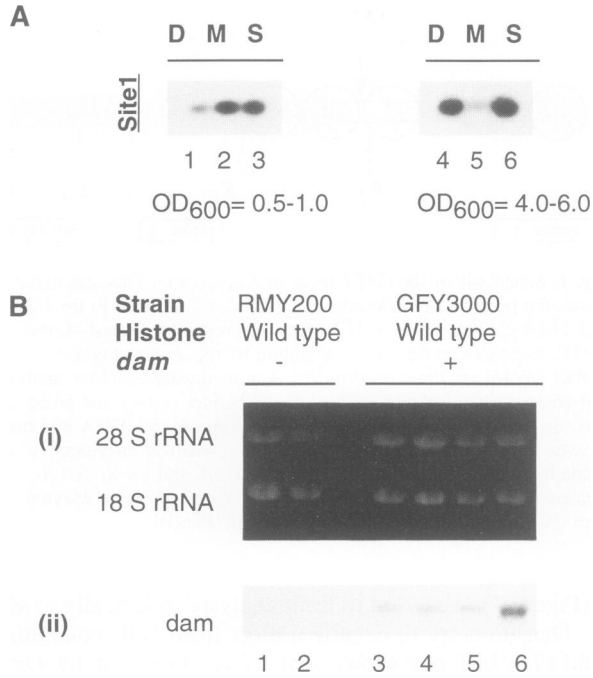

Fig. 2. Analysis of *dam* methylation in logarithmic versus stationary phase cells. (**A**) methylation of Site 1 (S_1). Genomic DNA was isolated from yeast strains constituitively expressing *dam* methylase grown in glucose to OD_{600} = 0.8 (log) or OD_{600} = 5 (late-log stationary). DNA was restricted first with *Eco*RI, then with *Dpn*I (D), which cleaves only methylated DNA, *Mbo*I (M), which cleaves unmethylated DNA or *Sau*3AI (S), which cleaves both forms of DNA. After digest, Southern analysis was performed using probe 1 (Figure 1). (**B**) Total RNA was isolated from a parental strain (RMY200) not expressing dam methylase and from a strain (GFY3000) which does express dam methylase. Cells were harvested when OD_{600} = 0.6 (lane 1), 5.7 (lane 2), 0.5 (lane 3), 1.3 (lane 4), 3.9 (lane 5), 5.7 (lane 6). 20 μg of total RNA was loaded/lane and loads were standardized to rRNA (shown). The RNA was probed for the presence of dam methylase transcript using a probe containing the *dam* coding region (see Materials and methods).

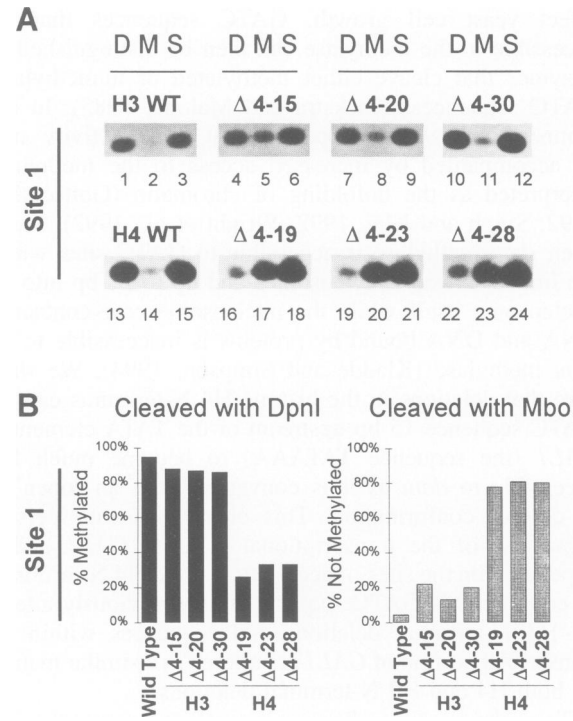

Fig. 3. Analysis of methylase accessibility near the *GAL1* TATA (Site 1). Genomic DNA was isolated from strains bearing both wild-type and mutant histones. The DNA was first cleaved with *Eco*RI, and subsequently with *Dpn*I (D), *Mbo*I (M) or *Sau*3AI (S), as described in Figure 2. (**A**) Methylation patterns at the *GAL1* TATA as assayed by restriction enzyme cleavage are shown for wild-type yeast (WT, lanes 1–3 or 13–15) or yeast bearing histone H3 deletions of amino acids 4–15 (Δ4–15), 4–20 (Δ4–20) or 4–30 (Δ4–30) (lanes 4–12). The methylation patterns in strains carrying histone H4 mutations are shown (lanes 16–24). The mutations shown here are deletions of amino acids 4–19 (Δ4–19), 4–23 (Δ4–23) or 4–28 (Δ4–28). The probe used for Southern analysis is indicated as probe 1 in Figure 1. (**B**) Graphical depiction of densitometry of the Southern blots shown in (A). Percent methylation is calculated as that fraction of DNA cleaved with *Dpn*I as compared with *Sau*3AI. Percent not methylated is quantified as that fraction of DNA cleaved by *Mbo*I as compared with *Sau*3AI.

GAL1 promoter. The extent of cleavage was quantified by densitometry (Figure 3B). In wild-type strains, most (90–95%) of the S_1 site DNA is cleaved by *Dpn*I. In strains bearing H4 N-terminal deletions of amino acid residues 4–19 or greater, the S_1 site is cleaved to a lesser degree by *Dpn*I (~20–30%) and is cut very strongly by *Mbo*I (~80%) (Figure 3A, compare lanes 17, 20 and 23 with lanes 16, 19 and 22). Therefore, the S_1 site is converted to a highly inaccessible or 'closed' structure by deletion of the H4 N-terminus. In contrast, histone H3 N-terminal deletions have very little effect on methylase accessibility of the S_1 site. Rather, the effect of the H3 mutants (87% cleavage by *Dpn*I) as compared with wild-type cells (90–95% cleavage) is clearly similar (Figure 3A, lanes 4, 7 and 10). These data suggest that the histone H4 N-terminus plays a unique role in maintaining an 'open' chromatin conformation just upstream of the *GAL1* TATA region.

Histone H4 and H3 N-terminal deletions have similar effects on dam methylase access in the GAL1 transcribed region

To analyze the effects of histone N-terminal deletions on the chromatin structure of the transcribed portion of *GAL1*, we examined methylation of three GATC sequences (S_2, S_3 and S_4) in this region. Site S_2 lies 409 bp downstream from the initiation of transcription (Figure 1). In a wild-type strain whose *GAL1* gene is repressed in glucose, S_2 is ~66% cleaved by *Dpn*I (quantified in Figure 4B) and is therefore in a largely accessible state (Figure 4A, lanes 1 and 10). When either histone H4 or H3 N-terminal residues are deleted, cleavage at site S_2 is decreased to a similar extent (~30–40% cleavage by *Dpn*I; Figure 4A, lanes 4, 7, 13 and 16). In contrast, *Mbo*I cleavage in the wild-type strain is ~30% (Figure 4A, lanes 2 and 11) as compared with cleavage ranging from 60 to 80% in both H4 and H3 N-terminal deletion strains (Figure 4A, lanes 5, 8, 14 and 17). Thus, both H4 and H3 N-terminal deletions cause the S_2 site to become less accessible to the *dam* methylase. Site S_3, located 708 bp downstream of the initiation of transcription (Figure 1), is cleaved completely by *Dpn*I, implying that S_3 is very highly accessible to the methylase in the wild-type strain (Figure 4A, lanes 19 and 28). Deletions in both the H4 and H3 N-termini cause a decrease in access to *dam* as inferred by decreased *Dpn*I (30–40% cleavage; Figure 4A, lanes 22, 25, 31 and 34) and increased *Mbo*I cleavage (50–70%

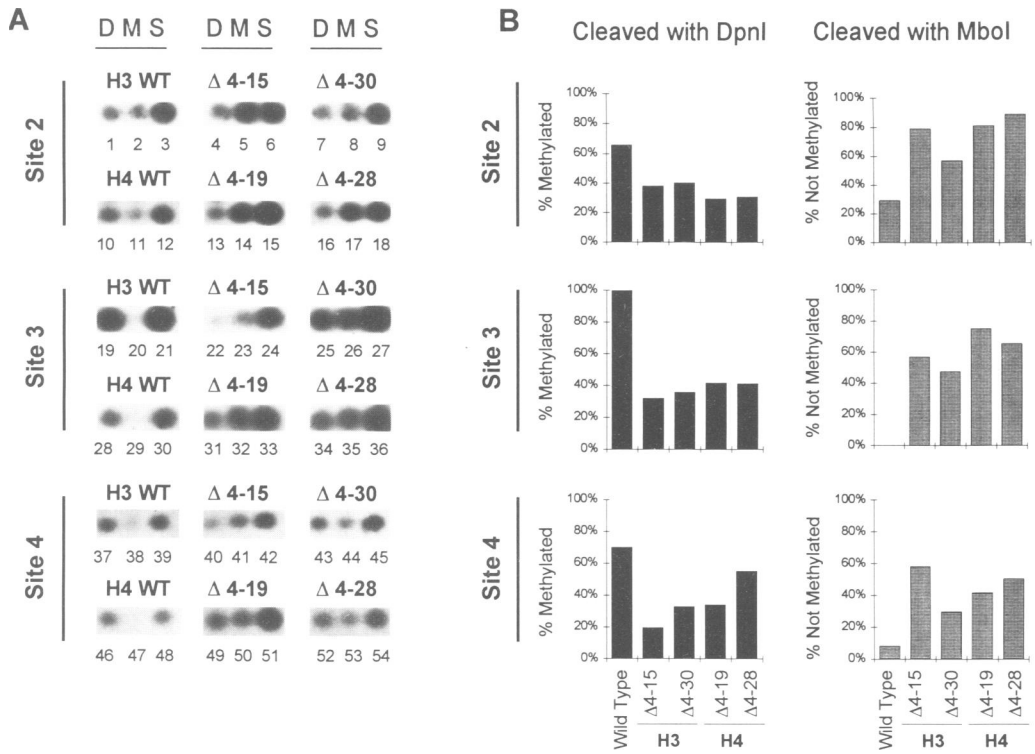

Fig. 4. Analysis of methylase accessibility within the transcribed region of *GAL1* (Sites S_2-S_4). Genomic DNA was isolated from cells grown under repressive conditions and subjected to restriction enzyme digestion and Southern analysis to determine *dam* accessibility. To analyze the first GATC site in the transcribed region (S_2), DNA was first restricted with *Ban*II and subsequently digested by the methyladenine sensitive enzymes described in Figure 2. For the second site probed (S_3), DNA was first restricted with *Dra*I and for the final site examined (S_4) DNA was first restricted with *Eco*RI. WT indicates wild-type histone background; Δ4–15, Δ4–30, are N-terminal deletions of histone H3 and Δ4–19 and Δ4–28 are deletions in histone H4. D (*Dpn*I) represents DNA which is cleaved by *Dpn*I and methylated, M (*Mbo*I) represents unmethylated DNA and S (*Sau*3AI) is indicative of both forms. (**B**) Graphical representation of the data shown in (**A**). For explanation of quantitation see Figure 3.

cleavage; Figure 4A, lanes 23, 26, 32 and 35) at this site. Finally, site S_4, found 962 bp from the start site of transcription, is largely accessible to the *dam* methylase as shown by its cleavage by *Dpn*I (Figure 4A, lanes 37 and 46). However, ~10% of S_4 DNA is cleaved by *Mbo*I in the wild-type strain (Figure 4A, lanes 38 and 47), indicating that, in some cells, this site is inaccessible to *dam*. At this site, both the H4 and the H3 N-terminal deletions caused an increase in *Mbo*I cleavage (up to 60% cleavage, depending on the mutant analyzed), implying a decrease in *dam* accessibility (Figure 4A, lanes 41, 44, 50 and 53). Therefore, while H4 and H3 N-terminal deletions cause quite different effects on chromatin structure at the S_1 site upstream of the *GAL1* TATA region, they result in similar structural changes at sites S_2, S_3 and S_4 in the transcribed region of *GAL1*.

dam methylase accessibility at the GAL1 TATA is decreased by certain mutations that alter the sites of H4 acetylation

Since acetylation of lysine residues (K5, −8, −12, −16) in the H4 N-terminus has been correlated with increased transcription (Allfrey, 1977; Hebbes *et al.*, 1988, 1994; Turner, 1991) and since our deletions remove these lysine residues, we wished to determine whether mutation of these sites alone can reproduce the effects we observe on the access of *dam* to the S_1 site near the *GAL1* TATA. Two mutants were generated: one in which the lysine residues 5, 8, 12 and 16 in the histone H4 N-terminus were changed to arginines [K(5,8,12,16)R], thereby preventing the neutralization of these positively charged residues by lysine specific acetylation, and one in which these lysine residues were changed to glycine [K(5,8,12,16)G] thereby permanently neutralizing these sites. As seen in the Southern blot analysis of Figure 5A (quantified in Figure 5B), changing the lysine residues to arginines results in *dam* methylase accessibility similar to that found in wild-type cells. That is, both the mutant K(5,8,12,16)R and wild-type DNA are cleaved by *Dpn*I (~86% cleavage; Figure 5A, lanes 1 and 10). In contrast, changing the lysines to glycines results in increased cleavage by *Mbo*I (from 20% in wild-type to 70% in K(5,8,12,16)G; Figure 5A, lanes 2 and 8) indicating decreased accessibility to the methylase and mimicking the H4 N-terminal deletion in its effect on *dam* accessibility (Figure 5A, compare lanes 4–6 with 7–9). These experiments suggest that neutralizing the charge of the H4 N-terminus, either by its deletion or mutation to a neutral state, causes the S_1 site at the TATA element to assume a 'closed' conformation resistant to *dam* methylation.

The level of dam methylase accessibility at the GAL1 TATA element in the histone H4 and H3 N-terminal deletion strains is largely independent of the GAL1 transcriptional state

We wished to determine whether the methylase access observed in the histone N-terminal deletion mutants at the S_1 site was affected by activation of *GAL1*. When wild-

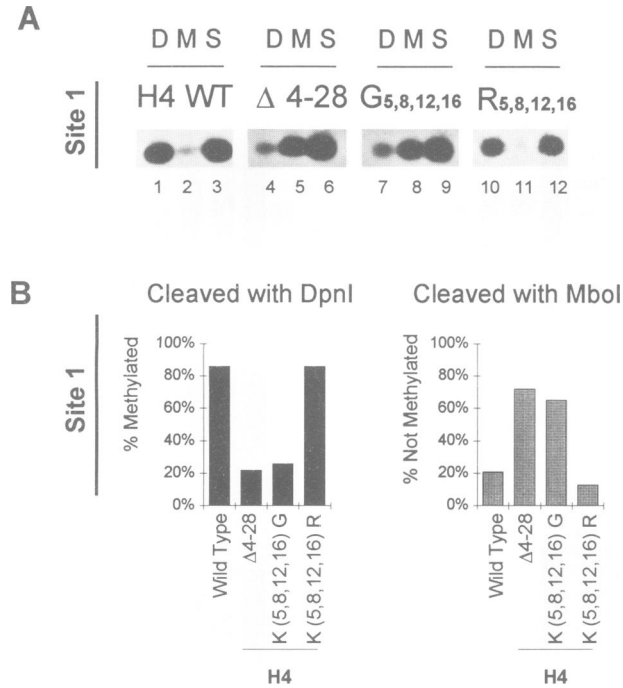

Fig. 5. Mutation of the lysines in the histone H4 N-terminus affects *dam* accessibility at the *GAL1* TATA (S_1 site). Genomic DNA was analyzed for *dam* accessibility at S_1 from cells bearing wild-type histone H4 (WT), N-terminal deletion of amino acids 4–28 (Δ4–28), lysines 5, 8, 12 and 16 changed to glycine [K(5,8,12,16) G], or lysines 5, 8, 12 and 16 changed to arginine [K(5,8,12,16)R]. (**A**) shows Southern analysis; (**B**) shows results of densitometry. Labeling and nomenclature is as described for Figures 2, 3 and 4.

type cells were grown for 24 h under conditions of repression (glucose) or activation (galactose) we observed very similar, high level (~90–95%) methylation of S_1 (Figure 6, compare *Dpn*I and *Mbo*I cleavage in lanes 1 and 2 with that of lanes 7 and 8). Deletion of the H4 N-terminus results in greatly decreased activation of *GAL1* and deletion of the H3 N-terminus causes hyperactivation of *GAL1*. In glucose, strains carrying these deletions have the S_1 site in an 'open' and 'closed' conformation, respectively. Upon growth in galactose, we observe very little change in the accessibility of the S_1 site to *dam* in either the H4 or H3 N-terminal deletion strains (Figure 6, compare lanes 3–6 with 9–12). Therefore, while we cannot exclude minor differences in methylation patterns (there is a slight increase in methylation in the H4 mutant strain in galactose), the effects of the histone N-termini on access to *dam* appear to be independent of transcription.

Both H4 and H3 N-terminal deletions disrupt chromatin structure downstream of the GAL1 TATA region as assayed by indirect end-labeling

The *GAL1* promoter consists of a UAS_G region separated by ~190 bp from the TATA element (Figure 1). The *GAL1* promoter has been shown to contain positioned nucleosomes by indirect end-labeling techniques utilizing cleavage agents (micrococcal nuclease, methidiumpropyl-EDTA) which digest DNA largely between nucleosomes (Lohr, 1984; Fedor and Kornberg, 1989; Axelrod *et al.*, 1993). Such nucleosomal mapping techniques suggest that a nucleosome (+1) lies between the UAS_G and the TATA (the TATA is positioned near the 3′ end of the nucleosome)

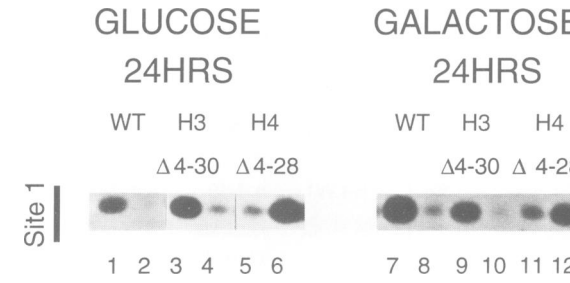

Fig. 6. Analysis of *dam* methylase accessibility near the *GAL1* TATA (S_1 Site) under different transcriptional states. Cells were grown either in 1% glucose:1% raffinose or in 1% galactose:1% raffinose for 24 h and analyzed for methylase accessibility at S_1 as described in Figures 2 and 3. These cells expressed wild-type histones (WT), histone H3 N-terminal deletions of amino acids 4–30 (Δ4–30) or deletion of histone H4 N-terminal residues 4–28 (Δ4–28). Lanes 1, 3, 5, 7, 9 and 11 represent genomic DNA cleaved by *Eco*RI and *Dpn*I, while lanes 2, 4, 6, 8, 10 and 12 represent genomic DNA cleaved by *Eco*RI and *Mbo*I.

and that a second nucleosome (+2) covers the start site of transcription (Fedor and Kornberg, 1989). The UAS_G itself is believed to be nucleosome-free on the basis of its cleavage by methidiumpropyl-EDTA (Fedor and Kornberg, 1989). To determine whether nucleosome positioning is altered by histone N-terminal deletions, we performed indirect end-labeling of micrococcal nuclease digests of yeast nuclei. Chromatin from wild-type, glucose repressed cells, reveals a cleavage pattern of nucleosomes over the *GAL1* promoter region similar to that described previously (Fedor and Kornberg, 1989; Axelrod *et al.*, 1993) (Figure 7).

When either the H4 or H3 N-terminus is deleted, a new cleavage site appears slightly upstream of the +76 cleavage site. In addition to this band, other cleavage sites seen in naked DNA cut with MNase are found in nucleosomal DNAs from H4 or H3 N-terminal deletion strains (Figure 7, lanes 1 and 2 compared with lane 6, asterisks). The H3 N-terminal deletion also results in the loss of a cleavage site (x) found in wild-type chromatin and in the H4 deletion (arrow). The resulting pattern is reminiscent of naked DNA, so we cannot determine whether the disappearance of this band in the H3 mutant is due to protection of this region. We conclude that while the MNase cleavage patterns resulting from H4 and H3 N-terminal deletions are not identical, they are both clearly altered, and that this alteration is most striking in the transcribed region of *GAL1*. Both patterns are likely to be due to altered nucleosomal positioning (see below) and possibly unfolding, thereby exposing new DNA cleavage sites.

LMPCR of micrococcal nuclease digests maps a region of protection caused by the H4 N-terminal deletion to sites near the GAL1 TATA element

Indirect end-labeling did not reveal any strong changes just upstream of the *GAL1* TATA in the H4 N-terminal deletion strains despite the striking change in the S_1 site 15 bp upstream of the TATA. However, indirect end-labeling detects gross changes in micrococcal nuclease sensitivity, while the *dam* methylase assay is specific for GATC sequences only. Therefore, to examine sites adjacent to the S_1 site more precisely, genomic footprinting of

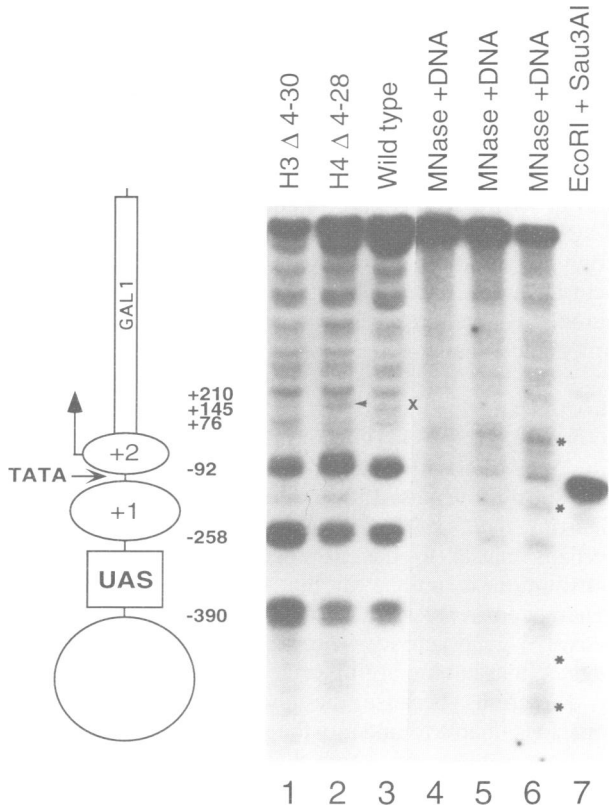

Fig. 7. Indirect end-labeling of MNase digestion of chromatin over the *GAL1* gene. MNase digested chromatin was restricted with *Eco*RI, and subjected to analysis by indirect end-labeling. The *GAL1* gene is depicted with schematic nucleosomes (ovals) for wild-type. Base pair positioning of nucleosomes (+/−) is relative to the start site of transcription. The TATA is located at position −96. This Southern blot was probed with an *Eco*RI−*Ban*II fragment. Lane 3 represents chromatin from wild-type cells, lane 1 is chromatin from histone H3 Δ4–30 and lane 2, chromatin from a strain with histone H4 Δ4–28. Lanes 4–6 represent naked DNA cleaved with either 0.3, 0.6 or 1.25 units of MNase, respectively. Lane 7 shows naked DNA cleaved with *Eco*RI and *Sau*3AI (to serve as a reference for S_1). The chromatin digests shown here were cleaved with 3 units of MNase. The asterisks (*) highlight regions cleaved in naked DNA as well as in histone mutants.

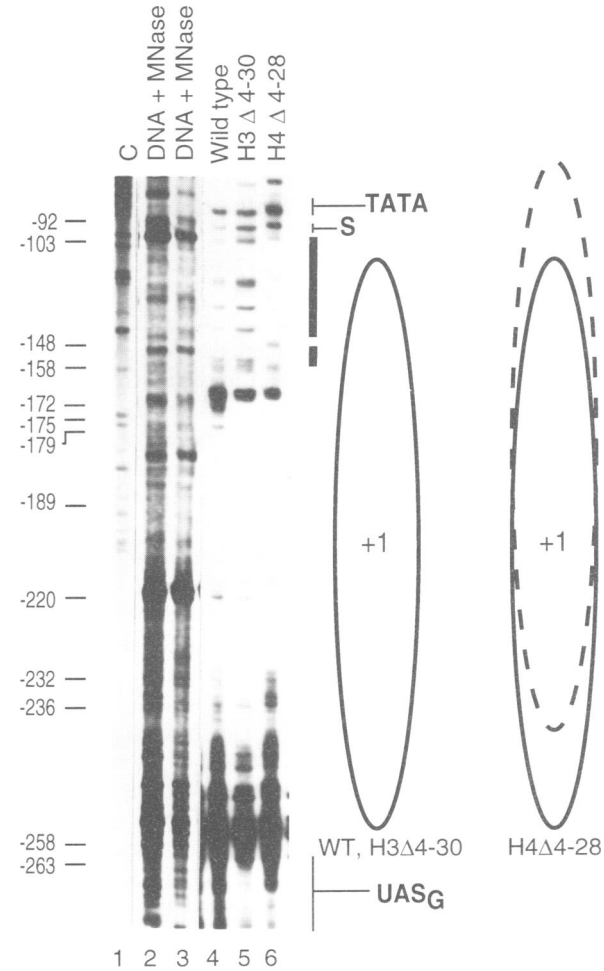

Fig. 8. LMPCR of +1 nucleosome of *GAL1* promoter. Genomic footprint of +1 nucleosome over *GAL1* promoter from chromatin digested with 1.5 units of MNase. Numbering is relative to the start site of transcription. Solid oval represents nucleosome (+1), black bars indicate large regions of protection seen in H4 Δ4–28 strain as compared with wild-type. Dashed oval represents putative nucleosome (+1) shift. The transcriptional start site would be found at the top of the gel, the site S_1 is indicated (S), as is the TATA sequence (−96) and the UAS_G. Lane 1 is the cytosine sequence from *GAL1* and is provided as reference. Lanes 2 and 3 are from naked DNA cut with 0.001 and 0.002 units MNase; lanes 4–6 are from chromatin from glucose repressed wild-type cells , H3 Δ4–30 cells and H4 Δ4–28 cells, respectively.

micrococcal nuclease digests of the region near the +1 nucleosome was conducted using LMPCR as described by Pfeifer *et al.* (1989). DNA was prepared from partial micrococcal nuclease digests of chromatin from wild-type cells, from strains carrying histone H4 Δ4–28, or from strains bearing histone H3 Δ4–30 all grown in glucose (Figure 8). The endpoints of micrococcal nuclease digestion of the +1 nucleosome in the indirect end-labeling experiments are approximately between sites −258 and −92 (166 bp; Figure 7). This length is typical for a yeast nucleosome representing 146 bp nucleosomal DNA and 20 bp linker DNA. It is evident that the apparently homogeneous cleavage (indicative of nucleosomal endpoints) seen at −258 and −92 by micrococcal nuclease indirect end-labeling reflects a large number of cleavage sites detected by LMPCR in the wild-type (Figure 8, lane 4). If we assume that the midpoint of MNase hypersensitivity seen just downstream of the UAS_G in the LMPCR data at −258 (Figure 8, lane 4) represents the upstream boundary of nucleosome +1 then the other boundary of the nucleosome at −92 occurs near the TATA and the S_1 sites.

DNA from the H4 N-terminal deletion strain shows some increased protection within the +1 nucleosome compared with that of wild-type chromatin. A footprinted region of protection occurs between −103 and −158 (Figure 8, lane 6, dark bars) upon H4 N-terminal deletion. It is interesting to note that this region is adjacent to the S_1 site. Since there is also a region of nuclease hypersensitivity between −236 and −232, this protection could be explained if the +1 nucleosome was shifted in its position some 15–25 bp as shown in Figure 8. A similar shift does not appear to take place in the strain carrying the H3 N-terminal deletion although it does demonstrate some increased protection from cleavage in the region between −220 and −172 (Figure 8, lane 5). The cleavage patterns in this strain are remarkably similar

to that of wild-type chromatin in the region between −175 and −92. These data suggest that H4 N-terminal deletion results in the formation of an altered chromatin structure in the region adjacent to the TATA element.

Increased protection near the TATA in the histone H4 N-terminal deletion strains includes a nucleosome

To determine whether the increased protection we observed upstream of the TATA was due to the presence of a nucleosome, we partially digested chromatin with micrococcal nuclease to generate nucleosomal ladders and probed these ladders with DNA fragments from the +1 and +2 nucleosomal regions. If a nucleosome is present, Southern analysis should reveal a fragment of approximately nucleosome size in each case (Straka and Horz, 1991). Chromatin from isolated nuclei was digested with micrococcal nuclease and isolated DNA was electrophoresed and transferred to a nylon membrane. The membrane was probed with a PCR-generated fragment of DNA 102 bp long (A) corresponding to the mapped +1 nucleosome or (B) a 96 bp fragment corresponding to the +2 nucleosome (Materials and methods). As can be seen in Figure 9A, wild-type cells show a nucleosomal ladder emanating from the +1 nucleosome (lane 6). A nucleosomal ladder is also generated from the +1 nucleosome upon H4 or H3 N-terminal deletion, although the less distinct patterns suggest altered nucleosome positioning as the distance increases from the +1 nucleosome. Similar conclusions can be reached upon probing with the +2 nucleosomal DNA. These data indicate that the nucleosomes +1 and +2 of the *GAL1* promoter are present even in the histone N-terminal deletion strains, although they are not positioned as precisely or may be destabilized in a portion of the population.

Discussion

It has previously been shown that H4 and H3 N-terminal deletions result in contrasting effects on *GAL1* transcription. H4 deletions decrease, while H3 deletions increase *GAL1* promoter activity as compared with wild-type strains. Here we report that H4 and H3 N-terminal deletions also have different effects on the chromatin structure of the *GAL1* promoter but similar effects on chromatin in the transcribed region. We show that chromatin at the S_1 site, 15 bp upstream of the *GAL1* TATA regulatory element, is accessible to the *dam* methylase in repressed, wild-type strains. In contrast, we find that the S_1 site becomes inaccessible to *dam* methylase as a result of H4 N-terminal deletion while the H3 N-terminal deletion has no significant effect on methylase access at this site. However, both H4 and H3 N-terminal deletions cause similar changes in *dam* methylase accessibility at sites S_2, S_3 and S_4 in the *GAL1* coding region chromatin. The differences we observe at the promoter between H4 and H3 N-terminal deletion strains are independent of transcription, since they occur under conditions of both repression and activation and also when the TATA element is mutagenized to inactivate it (data not shown). Therefore, the changes in *dam* access at the S_1 site result directly from the histone N-terminal deletions and not from changes in transcription mediated by the histone deletions.

When the lysines at the H4 N-terminus at positions 5, 8, 12 and 16 were replaced with glycines (to neutralize their charge) the effect on the S_1 site was similar to that caused by deletion of the H4 N-terminus. It appears that, as with N-terminal deletion, the neutralized tail loses specific important contacts (either protein or DNA) and allows for the formation of a chromatin structure near the TATA which is inaccessible to *dam* methylase. When arginines replaced lysine residues, preventing *in vivo* neutralization of the H4 N-terminus, the accessible open chromatin structure at S_1 was retained. This suggests, at first glance, that positive charges at the sites of acetylation are necessary for maintenance of the wild-type accessible structure and that this may be a prerequisite for proper gene activation. However, substitution of multiple arginines at sites 5, 8, 12 and 16 causes cell doubling times to increase to 330 min versus 150 min for glycine substitutions (Durrin *et al.*, 1991; data not shown). In addition, and perhaps causal to the slow growth, multiple arginine substitution is unique in that it causes a very strong generalized repressive effect on both constitutive and activated gene activity (Durrin *et al.*, 1991). These data suggest that having arginines at positions 5, 8, 12 and 16 is deleterious because the chromatin structure (while 'open') is unable to undergo the structural change required for transcription and which may be mediated by histone acetylation. On the other hand, completely deleting or permanently neutralizing the H4 N-terminus may prevent normal activity by allowing the assembly of a repressive chromatin structure at site S_1.

Available data argues that the *GAL1* TATA element is present in the linker region between the +1 and +2 nucleosomes and that the S1 site, 15 bp upstream, is at the edge of the nucleosomal DNA. The presence of a repressive chromatin structure adjacent to the *GAL1 TATA* element resulting from the H4 N-terminal deletion is supported by micrococcal nuclease (LMPCR) analysis of *GAL1* chromatin. These data and the nucleosomal ladders (Figure 9) argue that this repressive structure includes a displaced +1 nucleosome, although we cannot exclude the possibility that other factors also contribute to the altered footprint seen. Since *dam* methylation occurs within linker DNA and some 21 bp into the nucleosome itself, a displaced +1 nucleosome (and possible associated factors) shifted by only 15–25 bp (Figure 8) may not only protect the S_1 site from methylation but prevent factors of the basal transcription machinery from properly recognizing their targets. The similarity between the MNase digests (LMPCR) of wild-type and the H3 N-terminal deletion chromatin at this region of the +1 nucleosome argues that access to these factors is not similarly prevented in these strains.

Indirect end-labeling of MNase digests also shows differences in MNase digestion patterns downstream of the TATA element in both H4 and H3 N-terminal deletion strains as compared with wild-type. Both H4 and H3 deletion results in cleavage which argues for changes in nucleosome positioning (multiple frames) at the +2 nucleosome. However, we believe that these differences are unlikely to be the cause of differences in *GAL1* response to H4 and H3 N-terminal deletions since similar sites of cleavage are seen within the region of the +2 nucleosome resulting from both H4 and H3 N-terminal

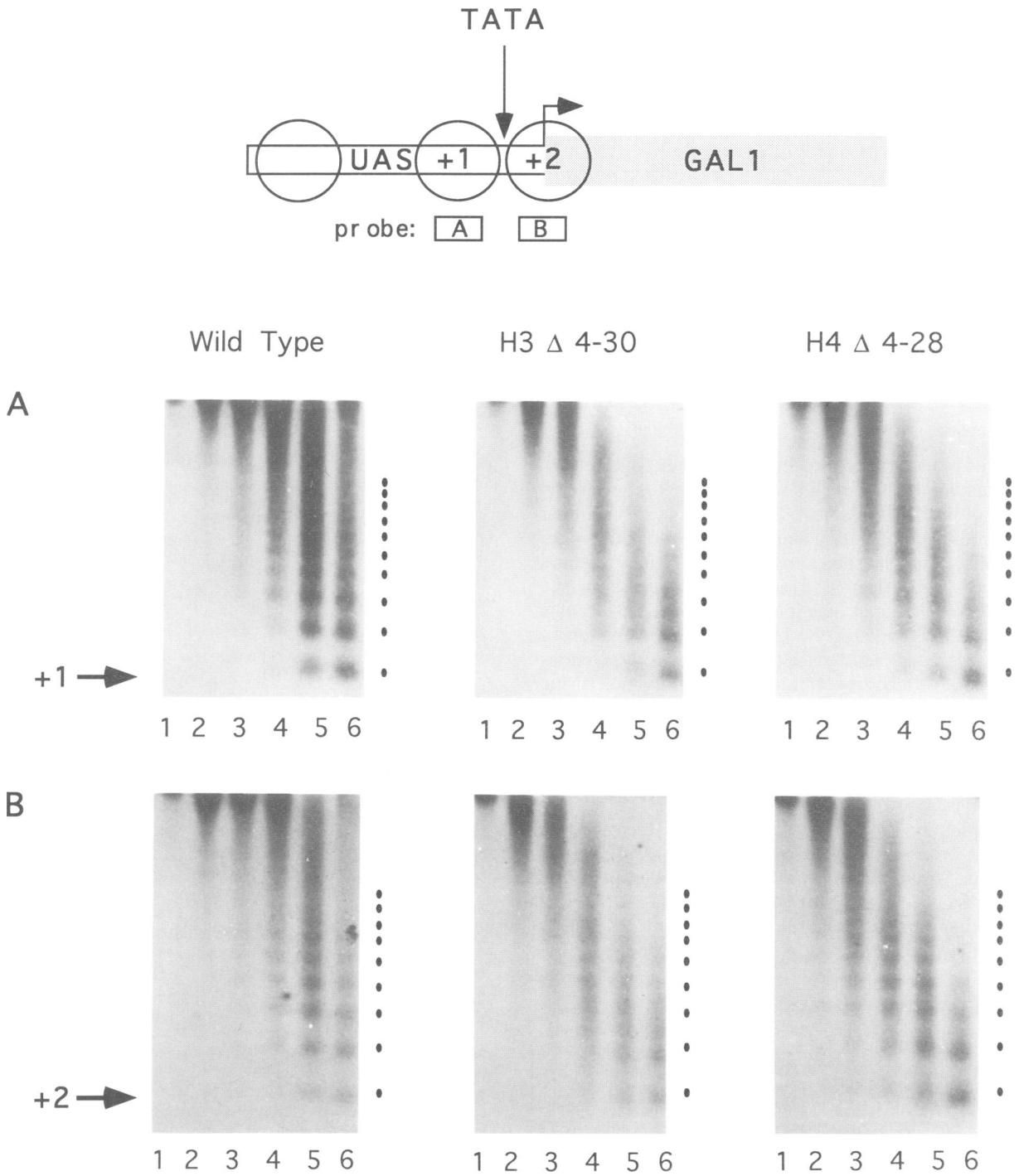

Fig. 9. MNase ladders probed with individual nucleosomal DNA fragments (+1 or +2) to demonstrate the presence of nucleosome sized DNAs at these sites. Southern analysis probing either the +1 nucleosome (**A**) or the +2 nucleosome (**B**) in strains bearing wild-type, H3 Δ4–30 or H4 Δ4–28 mutant histones. Lanes 1–6 have chromatin digested with 0, 3.13, 6.25, 12.5, 25 and 50 units of MNase, respectively. Probes are as indicated in diagram (for details, see Materials and methods).

deletions, yet the mutants have opposing transcriptional phenotypes. In addition, there is a distinct change in the region downstream of the +2 nucleosome specific to the H3 N-terminal deletion strain. While this change is indicative of a chromatin change in the H3 mutant, it is difficult to imagine how such differences quite distant from the promoter element might potentiate *GAL1* hyperactivation. Thus, our experiments suggest that the effect of the H3 N-terminus on *GAL1* hyperactivation occurs through an element other than the TATA and downstream promoter region.

We have previously proposed that the function of the H4 N-terminus in potentiating *GAL1* transcription may be to allow a nucleosomal change in structure to occur near the TATA element. This would allow for the activation of *GAL1* by permitting transcription factors to access the TATA region (Durrin *et al.*, 1991). Consistent with this model, our data presented here suggest that the H4

Table I. Strains

Strain	Genotype
UCC1023	*MAT***a**, *ade2-101, his3Δ201, leu2-2,112, trp1Δ901, ura3-52, lys2-801,:dam$^+$:LYS2* (Wright *et al.*, 1992)
RMY200α	*MAT*α, *ade2-101, his3Δ201, leu2-3,112, trp1Δ901, ura3-52, lys2-801:dam$^+$:LYS2, hht1-hhf1::LEU2, hht2-hhf2::HIS3* and pRM200 (*CEN4 ARS1 TRP1 HHT2 HHF2*)
GFY3000	*MAT*α, *ura3-52, lys2-801, ade2-101, leu2-3,112, his3Δ201, trp1Δ901, hht1-hhf1::LEU2, hht2-hhf2::HIS3* and pRM200 (*CEN4 ARS1 TRP1 HHT2 HHF2*)
GFY3001	Isogenic to GFY3000 with pRM102 [*CEN4 ARS1 URA3 P(GAL10)-HHT2 P(GAL1)-HHF2*]
GFY3415	Isogenic to GFY3000 with pRM415 (*hht2Δ4-15*)
GFY3420	Isogenic to GFY3000 with pRM420 (*hht2Δ4-20*)
GFY3430	Isogenic to GFY3000 with pRM430 (*hht2Δ4-30*)
GFY34414	Isogenic to GFY3000 with pGF28 (*hhf2Δ4-14*)
GFY34419	Isogenic to GFY3000 with pGF17 (*hhf2Δ4-19*)
GFY34423	Isogenic to GFY3000 with pGF27 (*hhf2Δ4-23*)
GFY34428	Isogenic to GFY3000 with pGF29 (*hhf2Δ4-28*)
GFY344KG	Isogenic to GFY3000 with pGF26 (*hhf2 G*9,14,18,23)
GFY344KR	Isogenic to GFY3000 with pGF25 (*hhf2 R*9,14,18,23)

N-terminus functions to prevent the assembly of a repressive structure (which includes a nucleosome) over the vicinity of the TATA element, thereby allowing for proper access of this region during activation. Although improper positioning of nucleosomes over the TATA may account for some of the decreased activation observed in the histone H4 N-terminal deletions, our data argue that this cannot fully account for the H4 phenotype. Mutation of all four acetylated lysine residues to arginines at positions 5, 8, 12 and 16 also results in decreased activation of the *GAL1* promoter despite the apparent 'open' chromatin structure near the TATA. While we cannot exclude indirect effects of this mutation, it is possible that, stimulated by the activator, certain factors, such as the histone acetylase, may interact with the H4 N-terminus to alleviate nucleosomal repression and allow for activation. Thus, the role of the H4 N-terminus may be not only to assure proper positioning of the nucleosomes over the *GAL1* promoter region (particularly near the TATA), but also to interact with another component to allow the required change in nucleosome structure which potentiates gene activity.

Materials and methods

Plasmids

The plasmids used to create the various histone mutant strains are derived from pRM200 (previously described in Mann and Grunstein, 1992). Briefly, pRM200 contains *HHF2* (histone H4) and *HHT2* (histone H3) on a *CEN4 ARS1 TRP1* plasmid. All plasmids bearing *HHT2* mutations have been previously described in Mann and Grunstein, 1992. All *HHF2* mutations were generated by subcloning the *Hin*dIII–*Eco*RI fragments from either pPK (Kayne *et al.*, 1988) or pLD (Durrin *et al.*, 1991) plasmids bearing various *HHF2* mutations into pGF11, a pBR322 based plasmid containing a *Hin*dIII–*Bam*HI fragment which contains the promoter of *HHT2*. From these plasmids the *Eco*RI–*Bam*HI fragment was isolated and recloned into the *Eco*RI–*Bam*HI sites in pRM200.

Yeast strains

The isogenic methylase strains were derived from mating RMY200α [*MAT*α, *ade2-101, his3Δ201, leu2-3,112, lys2-801, trp1Δ901, ura3-52, hht1-hhf1::LEU2, hht2-hhf2::HIS3*/pRM200 (*CEN4 ARS1 TRP1 HHT2 HHF2*)] with UCC1023 (Wright *et al.*, 1992) (*MAT***a**, *ade2-101, his3Δ201, leu2-3,112, trp1Δ901, ura3-52, lys2-801:dam$^+$:LYS2*) and selecting for LYS$^+$ and TRP$^+$ diploids. After sporulation, we screened for HIS$^+$, LEU$^+$, TRP$^+$, ura$^-$, LYS$^+$ spores. The resulting strain, GFY3000 [*MAT*α, *ade2-101, his3Δ201, leu2-3,112, trp1Δ901, ura3-52, lys2-801:dam$^+$:LYS2, hht1-hhf1::LEU2, hht2-hhf2::HIS3*/pRM200 (*CEN4 ARS1 TRP1 HHT2 HHF2*)], was transformed (Gietz *et al.*, 1992) with plasmid pRM102 (*CEN4 ARS1 URA3 GAL10p-HHT2 GAL1p-HHF2*) and pRM200 was subsequently lost as described in Mann and Grunstein, 1992, to create GFY3001.

All combinations of histone mutant strains used in the methylase assay are derivatives of GFY3001. pRM200 derivatives containing altered *hht2* or *hhf2* genes were transformed into GFY3001 by the method of Gietz *et al.* (1992) and subsequently, pRM102 was lost from each transformant by growth on glucose media lacking tryptophan. For a summary of strains, see Table I.

Methylase assays

To assay for *in vivo* chromatin methylation, strains were grown to stationary phase in YEPD (OD_{600} = 4–5), and genomic DNA was isolated. For studies in log phase, cells were grown to OD_{600} = 0.5–1) and genomic DNA was isolated. DNA was restricted by *Eco*RI, *Ban*II or *Dra*I and subsequently analyzed for methylation by cleavage with *Dpn*I (cleaves methylated), *Mbo*I (cleaves unmethylated) and *Sau*3AI (cleaves both forms of DNA) isoschizomers. Fragments were separated by gel electrophoresis on a 1.5% agarose gel, transferred, crosslinked to a nylon membrane by UV irradiation and hybridized to an appropriate random-primer labeled probe (Feinberg and Vogelstein, 1983). The probes utilized are indicated in Figure 1. Probe 1 was a 285 bp *Eco*RI–*Ban*II fragment of pNN78 (St John and Davis, 1981), probe 3 was a 210 bp *Eco*RI–*Eco*RV fragment from the same vector and probe 2 was a 150 bp fragment made from PCR of this region. Image analysis (densitometry) was performed using the NIH Image Software v.1.49.

For the galactose versus glucose studies strains were grown in YEP containing 1% raffinose:1% glucose or 1% raffinose:1% galactose. DNA was isolated at 24 h (as described by Hoffman and Winston, 1989) and the subsequent methylase analysis was performed as described above.

For Northern analysis of dam methylase transcripts, cells were grown in 100 ml of YEPD and ~4.7×10^8 cells were harvested at various time points. The OD_{600} of the culture was noted at these times. Total RNA was isolated as described in Mann and Grunstein (1992). RNA was electrophoresed and transferred to a nylon membrane and probed with a 700 bp *Eco*RI–*Pst*I fragment containing the dam coding region which had been labeled by random priming (see above).

Isolation of yeast chromatin/nuclease assays/indirect end-labeling/LMPCR

Cells were grown in 500 ml of YEPD to an OD_{600} = 1.0, harvested and washed in 2% glucose. Harvesting was followed by pre-treatment in 100 mM EDTA, 1% βME, 2% glucose for 15 min at 30°C. Cells were pelleted at 4 K for 5 min then spheroplasted in 1.08 M sorbitol, 20 mM KPO_4 pH 6.8, 0.5 mM $CaCl_2$, 1% βME, 2% glucose, and 3 mg zymolyase/g cells. Spheroplasts were collected by centrifugation in Beckman Centrifuge at 4 K for 5 min. Spheroplasts were weighed and resuspended in 4 ml digestion buffer per gram of cells. Digestion buffer was as described by Stewart *et al.*, 1991, with the addition of 5 mM $CaCl_2$ and 1 mM PMSF. For each digest, 475 μl of nuclei were added to 25 μl of nuclease of a given concentration (from 1.5 U MNase/digest to 24 units MNase/digest). Micrococcal nuclease (Worthington) digestions were performed at 30°C for 10 min. Digestions were stopped by the addition of 0.5 ml of 20 mM EDTA, 1% SDS. 40 μg of proteinase K was added and incubated overnight at 37°C. DNA was treated with RNaseI, phenol extracted and precipitated.

For indirect end-labeling, DNA from the aforementioned isolation was digested with restriction enzyme (*Eco*RI), electrophoresed on a 1.5% agarose gel and transferred to a nylon membrane. DNA was UV crosslinked to the membrane and probed with a *GAL1 Eco*RI−*Ban*II fragment labeled by random priming.

For LMPCR, DNA from chromatin isolations was ethanol precipitated two more times and 1.5 μg of DNA was subjected to the protocol described in Pfeifer *et al.* (1989). Oligos used were as follows: P1: 5′-GCGTTCCTGAAACGCAGATGTGCCT-3′, P2: 5′-CGCGCCGCAC-TGCTCCGAACAATAA-3′, linker oligos were as described in Pfeifer *et al.* (1989).

For probing MNase ladders, DNA from the above preparations was electrophoresed on 1.5% agarose, transferred to a nylon membrane and hybridized to either a 102 bp +1 *GAL1* fragment (generated by PCR: oligo1: 5′-TGGCCCCACAAACCTTCAAATGAAACG-3′, oligo 2: 5′-CGTTTCGCTGATTAATTACCCCAG-3′) or a 96 bp +2 nucleosome *GAL1* fragment (generated by PCR: oligo 1: 5′-TAACTAATACTTT-CAACATTTTCGG-3′, oligo 2: 5′-AATGAGATTTAGTCATTATA-GTTTT-3′)

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