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Histone H3 N-terminal mutations allow hyperactivation of the yeast *GAL1* gene *in vivo*

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Recent work has shown that the yeast histone H4 N-terminus, while not essential for viability, is required for repression of the silent mating loci and activation of *GAL1* and *PHO5* promoters. Because histone H3 shares many structural features with histone H4 and is intimately associated with H4 in the assembled nucleosome, we asked whether H3 has similar functions. While the basic N-terminal domain of H3 is found to be non-essential (deletion of residues 4–40 of this 135 amino acid protein allows viability), its removal has only a minor effect on mating. Surprisingly, both deletions (of residues 4–15) and acetylation site substitutions (at residues 9, 14 and 18) within the N-terminus of H3 allow hyperactivation of the *GAL1* promoter as well as a number of other GAL4-regulated genes including *GAL2*, *GAL7* and *GAL10*. To a limited extent glucose repression is also alleviated by H3 N-terminal deletions. Expression of another inducible promoter, *PHO5*, is shown to be relatively unaffected. We conclude that the H3 and H4 N-termini have different functions in both the repression of the silent mating loci and in the regulation of *GAL1*.

Key words: acetylation sites/chromatin/*GAL1*/histone H3/*Saccharomyces cerevisiae*

Introduction

While much light has been shed on the process of transcription initiation as it occurs *in vitro*, on DNA stripped of chromosomal proteins, a great deal is yet to be understood about the mechanisms of gene activation occurring *in vivo* on natural chromosomal templates. To study this problem, we have created mutations in the histones of the yeast *Saccharomyces cerevisiae* and evaluated the effects of these altered proteins on the regulation of transcription. The core histones H2A, H2B, H3 and H4 have limited sequence similarity, yet they share several structural properties. They each contain relatively hydrophobic domains, rich in α-helices (Arents *et al.*, 1991) that are chiefly responsible for both nucleosome assembly and stability. The hydrophilic N-terminal tails are all highly basic and easily accessible to proteases, suggesting that they are found on the surface of the nucleosomal particle. Moreover, the histone N-termini undergo numerous reversible, post-translational modifications including phosphorylation, methylation and acetylation (reviewed by van Holde, 1989). Although the precise function of the individual modifications remains uncertain, a strong correlation exists between increased histone acetylation and the unfolding of chromatin for possible gene activation (Allfrey, 1977; Hebbes *et al.*, 1988; reviewed by Grunstein, 1990). In addition, N-terminal histone H3 phosphorylation is correlated with the activation of *jun* and *fos* (Mahadevan *et al.*, 1991). It has been postulated that modulation in the charge of histone N-terminal domains may alter their potential interactions with nearby DNA (McGhee and Felsenfeld, 1980; Hill and Thomas, 1990) or other proteins (Johnson *et al.*, 1990). In support of this, it is significant to note that nucleosomes alter plasmid DNA topology and that histone H3 and H4 acetylation can affect the extent to which this occurs (Norton *et al.*, 1989, 1990).

Recent evidence argues that nucleosomes are integral components of the machinery that regulates transcription (for recent reviews see Grunstein, 1990; Kornberg and Lorch, 1991; Felsenfeld, 1992). Nucleosomes present at promoter elements have been shown to prevent transcription initiation *in vitro* (Keznetic and Luse, 1986; Lorch *et al.*, 1987; Workman and Roeder, 1987). However, this repression is alleviated when upstream activators are allowed access to the DNA template during *in vitro* nucleosome assembly (Lorch *et al.*, 1987; Workman *et al.*, 1990, 1991; Laybourn and Kadonaga, 1991; Taylor *et al.*, 1991). Considerable evidence has also been generated showing that nucleosomes regulate transcription *in vivo*. For example, nucleosome loss, achieved by repressing histone synthesis in yeast cells while DNA replication is temporarily ongoing, activates transcription initiation even in the absence of upstream activating sequences (UASs) (Han and Grunstein, 1988; Durrin *et al.*, 1992). Modest changes in yeast histone gene copy number can alter promoter choice adjacent to a transposable element (Clark-Adams *et al.*, 1988). Also, a mutant HMG-like protein can suppress the need for a regulator normally required for the expression of the HO endonuclease gene in yeast (Kruger and Herskowitz, 1991). It has further been demonstrated that specific sequences within the N-terminus of histone H4 are required for repression of the yeast silent mating loci (Kayne *et al.*, 1988; Johnson *et al.*, 1990, 1992; Megee *et al.*, 1990; Park and Szostak, 1990). Along with the critical role that histone H4 serves at the yeast mating loci, we have recently shown that the N-terminus of H4, while not essential for viability, is required for the activation of a number of inducible promoters (Durrin *et al.*, 1991). These include the GAL4-regulated gene *GAL1*, whose activation is reduced ~20-fold by H4 N-terminal deletions and *PHO5*, whose activation is reduced 4- to 5-fold. Amino acid substitutions at multiple H4 acetylation sites also cause reduced *GAL1* and *PHO5* induction. As a result of these findings we have postulated that the H4 N-terminus is required for a structural change in the nucleosome which is a prerequisite for transcription initiation (Durrin *et al.*, 1991).

Histone H4 has a number of features in common with histone H3. These two extremely conserved proteins are the first to be loaded onto DNA in the nucleosome assembly

reaction and are the only core histones able to assemble *in vitro* into a complex with structural properties of intact nucleosomes (Dong and van Holde, 1991; Hayes *et al.*, 1991). H3 and H4 are acetylated in a similar manner and the acetylation of both proteins has been correlated with increased transcriptional activity (Chahal *et al.*, 1980; Waterborg and Matthews, 1984; Johnson *et al.*, 1987). In order to determine whether H3 and H4 have similar gene regulatory functions, we have constructed mutant H3 alleles that correspond to the H4 deletions and substitutions used in our previous studies.

We report here that, as for the other core histones, virtually the entire basic N-terminal domain of H3 is dispensable for cell viability. However, unlike the case for H4, even very large deletions within the N-terminus of histone H3 have but a weak effect on repression of the silent mating loci. Moreover, and also in contrast to H4 mutations, deletions and substitutions within the N-terminus of H3 allow hyperactivation, or elevated induction rates, of *GAL1* and certain other GAL4-regulated genes (*GAL2*, *GAL7* and *GAL10*). These data suggest that the N-terminus of H3 serves as a negative regulator of these tightly controlled *GAL* promoters and show that a number of functions of histones H3 and H4 are clearly distinct.

Results

The entire N-terminal domain of histone H3 is dispensable for cellular viability

The haploid yeast cell contains two copies each of the histone H3- and H4-encoding genes *HHT* and *HHF*. They are arranged as unlinked H3–H4 gene pairs which share a common, divergent promoter region and the two copies of each respective gene encode identical proteins. It has previously been shown that while they are differentially transcribed throughout the cell cycle, only one of the two pairs is required for cell viability and that they are functionally interchangeable (Cross and Smith, 1988; Kayne *et al.*, 1988). In order to determine how histone H3 alterations affect cellular functions, a yeast strain, RMY102, was constructed in which both *HHT* and *HHF* loci were deleted and which also contained pRM102, a *CEN* plasmid bearing *HHT2* and *HHF2* under the control of *GAL1* and *GAL10* promoters, respectively. In a second, otherwise isogenic wild type control strain, RMY200, both *HHT2* and *HHF2* were placed under the control of their own wild type promoters in the plasmid pRM200. RMY102 was used as a recipient for all derivatives of pRM200 and the glucose shift test (Kayne *et al.*, 1988) was used to determine whether the variant H3 genes allow cell viability (see Materials and methods). The experimental design for the generation of mutant histone H3 strains is outlined in Figure 1. Assuming a given mutation allows viability, the episomal *GAL*-controlled histone genes should then be lost using their intrinsic mitotic instability, thus completing the construction of the H3 mutant strain.

We first wished to determine how much of the N- and C-terminal, hydrophilic domains of H3, as predicted in a hydropathy plot (Kyte and Doolittle, 1982; data not shown) are required for viability. Site-directed mutagenesis techniques were used to create a set of in-frame deletions within *HHT2* such that progressively truncated H3 N- or C-termini would be encoded. The smallest of the N-terminal

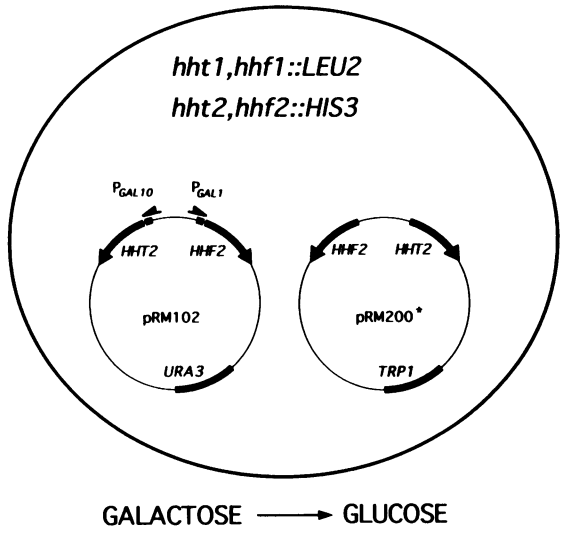

Fig. 1. Mutant histone H3 strain construction and determination of viability. A yeast strain was constructed in which both histone H3–H4 gene pairs (*HHT–HHF1* and *HHT2–HHF2*) were deleted and replaced with selectable markers (*LEU2* and *HIS3*, respectively), *HHT2* and *HHF2* were then introduced on a plasmid in which their expression is controlled by divergent *GAL10* and *GAL1* promoters (pRM102). This strain, which is now dependent on galactose for histone H3 and H4 synthesis, was used for the subsequent introduction of mutant histone H3 genes. These were introduced, under the control of their wild type promoter, on a second plasmid, pRM200*. Shifting the strains from galactose to glucose (glucose shift test; Kayne *et al.*, 1988) allowed repression of wild type H3 synthesis and determination of growth dependence on the mutant H3 protein. If a strain was viable, subsequent loss of pRM102 by intrinsic mitotic instability completed the H3 variant strain construction.

deletions removed amino acids 4–10 (designated H3Δ4–10) and continued in five amino acid blocks up to position 50, which is well within the predicted hydrophobic domain. In every case, the first three amino acids of the protein were left intact to allow normal post-translational processing of the N-terminal residue and stability (Bachmair *et al.*, 1986). Three additional deletions were created which removed five, 10 and 25 residues from the extreme C-terminus of H3. As illustrated in Figure 2, most N-terminal deletions allowed viability, while those that breached the predicted hydrophilic–hydrophobic boundary were lethal. Therefore as with the other core histones (Wallis *et al.*, 1983; Schuster *et al.*, 1986; Kayne *et al.*, 1988; Morgan *et al.*, 1991), the predicted hydrophilic H3 tail is dispensable for viability despite its greater length and extreme conservation. As shown in Table I, the viable N-terminal deletions caused progressively longer doubling times, which has been seen in corresponding H4 deletion strains (Kayne *et al.*, 1988; Morgan *et al.*, 1991). In contrast to the dispensability of the H3 N-terminus, all C-terminal deletions that had been made were lethal.

The N-terminus of histone H3 is not essential for repression of the silent mating loci

It has previously been shown that both deletions and critical amino acid substitutions within the N-terminus of H4 derepress the yeast silent mating loci *HML* and *HMR*, greatly reducing mating efficiency (Kayne *et al.*, 1988; Johnson *et al.*, 1990, 1992; Megee *et al.*, 1990; Park and Szostak, 1990). Deletion of histone H4 amino acids 4–19 or selected substitutions at positions 16–18 reduced the mating

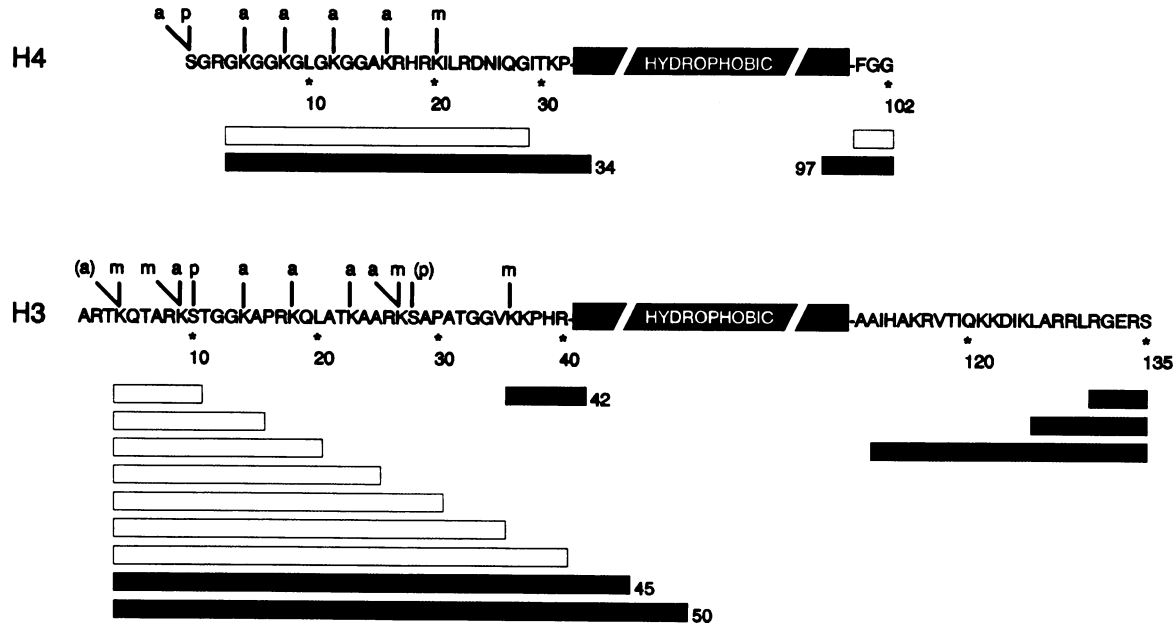

Fig. 2. Predicted structural domains and modification sites of histones H3 and H4. Core histone proteins bear distinct structural domains consisting of a hydrophobic region and hydrophilic N-terminal tail. The structures of histones H3 and H4 are illustrated here schematically. Predicted boundaries between the hydrophobic domains are indicated, as are the sites of post-translational modification (see van Holde, 1989). A fifth H3 acetylation site at position 27 (Marvin *et al.*, 1990), as well as an additional putative phosphorylation site at position 28 (Mahadevan *et al.*, 1991), have been reported and are included. [An additional H3 acetylation site at position 4 has been reported (Thorne *et al.*, 1990), but is seen only after butyrate treatment.] Deletions which allow viability (open bars) or are lethal (solid bars) are indicated. H4 deletions (Kayne *et al.*, 1988) are included for comparison.

efficiency by 5−6 orders of magnitude in *MAT***a** strains. In striking contrast, we found that H3Δ4−30 causes a < 10-fold decrease in mating (Table I). These data are in agreement with an earlier report demonstrating that deletion of H3 residues 1−20 and 1−28 reduced mating 3- to 10-fold (Morgan *et al.*, 1991). H3Δ4−35 and H3Δ4−40 caused slower cell division and a further reduction in mating (up to 100-fold), but we cannot exclude the possibility that this was due to an indirect structural effect on histone H4.

Histone H3 N-terminal deletions elevate the rate of GAL gene induction

GAL promoter activation is sharply reduced in yeast strains carrying histone H4 N-terminal deletions (Durrin *et al.*, 1991). To determine the effect of H3 N-terminal deletions on *GAL1* activation, Northern blot analysis using a ^{32}P-labelled *GAL1* DNA probe was used to measure message levels during induction with increasing galactose concentrations (0−2.0%). In order to obtain an estimate of the message level differences, the radioactivity of individual bands was measured by direct isotope detection and the blot was stripped and then rehybridized with a labelled *PRC1* fragment, since *PRC1* was previously shown to be constitutively transcribed during the yeast cell cycle (Xu *et al.*, 1990); the Northern analysis results are shown in Figure 3a. We found that *GAL1* mRNA levels were higher at lower concentrations of galactose in H3 deletion strains compared with the WT strain (compare 'b', 0.5% galactose and 'c', 1.0% galactose lanes). While most strongly manifested by the larger deletions, most of the effect was seen in the H3Δ4−15 strain. To determine whether other GAL4-regulated genes were similarly affected, the same Northern blot was stripped and re-probed successively with labelled *GAL2*, *GAL7* and *GAL10* DNA sequences (Figure 3b). We found that H3 N-terminal deletions similarly elevated the activity of *GAL2*, *GAL7* and *GAL10*. In contrast, *PRC1* mRNA levels were reproducibly decreased in the H3 N-terminal deletion strains.

Table I. Panel of histone H3 deletion mutant strains: viability, doubling time and mating efficiency

Strain	H3 deletion	Viability	Doubling time (min)	% mating
RMY200	WT	viable	92	1.0
RMY410	Δ4−10	viable	100	0.89
RMY415	Δ4−15	viable	123	0.73
RMY420	Δ4−20	viable	124	0.51
RMY425	Δ4−25	viable	135	0.27
RMY430	Δ4−30	viable	137	0.20
RMY435	Δ4−35	viable	232	0.13
RMY440	Δ4−40	viable	268	0.01
(RMY445)	Δ4−45	inviable	–	–
(RMY450)	Δ4−50	inviable	–	–
(RMY531)	Δ131−135	inviable	–	–
(RMY526)	Δ126−135	inviable	–	–
(RMY511)	Δ111−135	inviable	–	–
(RMY636)	Δ36−42	inviable	–	–

Set of N- and C-terminal deletions created in histone H3. Determination of viability, doubling time and mating efficiency is as described in text and Materials and methods.

Amino acid substitutions at histone H3 acetylation sites also elevate the rate of GAL gene induction

Because histone acetylation has been correlated with increased transcription, we constructed a series of conservative and non-conservative amino acid substitutions at the predicted H3 acetylation sites at positions 9, 14, 18 and 23 (van Holde, 1989). When the first three or all four sites were changed to either arginine (a conservative

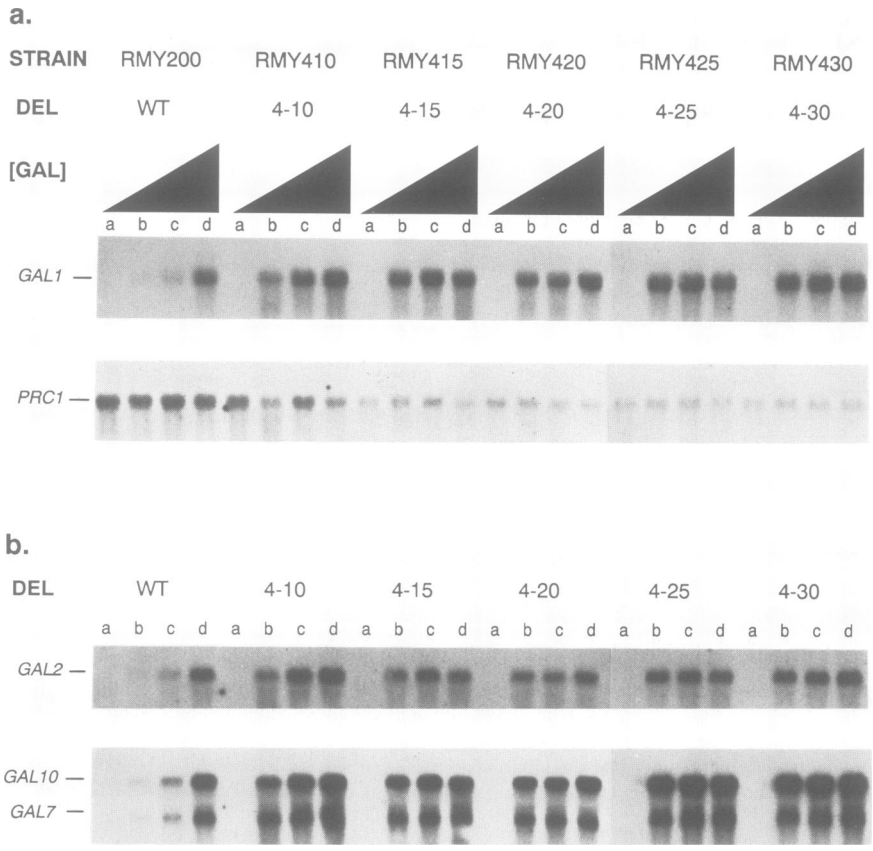

Fig. 3. Northern blot illustrating the hyperactivation of *GAL* genes by H3 N-terminal deletions. Relative rates of *GAL* gene inductions were evaluated by Northern analysis. Strains were initially grown in raffinose in order to relieve glucose repression. *GAL* genes were induced by growing log phase cultures for 6 h in rich media containing the ratios of raffinose and galactose described below. RNA was isolated and 15 μg total RNA was loaded in each lane. (**a**) Northern blots were first probed with a ^{32}P-labelled *GAL1* DNA fragment and subsequently stripped and re-probed with *PRC1* DNA that had previously been shown to be constitutively transcribed during the cell cycle (Xu *et al.*, 1990). After each hybridization, the total radioactivity in each band was determined by direct isotope detection (β-Scanner, Ambis). Because *PRC1* message levels were reduced in H3 mutant strains (see Results and Discussion), we did not normalize the *GAL1* quantification values to the *PRC1* mRNA levels; hence, we relied on even loading of samples. This was confirmed by examining rRNA levels using photographs of ethidum bromide-stained gels and membranes (data not shown). Representative quantifications of relative *GAL1* RNA levels under each growth condition (RMY200 values were set to 1.0); RMY415, lanes: a, values too low to measure accurately; b, 11.4, c, 5.7; d, 1.7. (**b**) The same membranes were stripped and re-probed, successively, with *GAL2*-, *GAL7*- and *GAL10*- specific sequences. Lanes: a, 2.0% raffinose and 0% galactose; b, 1.5% raffinose and 0.5% galactose; c, 1.0% raffinose and 1.0% galactose; d, 0% raffinose and 2.0% galactose. *GAL1*, *GAL7* and *GAL10* probes are described in Kayne *et al.* (1988) and Durrin *et al.* (1991). The *GAL2* probe is a 2.5 kb *Hin*dIII–*Eco*RI fragment from pTLG2 (Tschopp *et al.*, 1986). DEL = deletion; WT = wild type; [GAL] = galactose concentration.

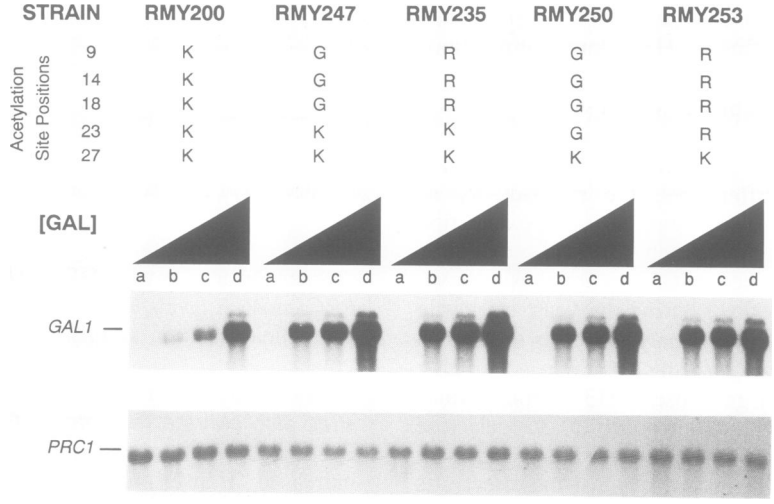

Fig. 4. Northern blot illustrating the hyperactivation of *GAL1* by histone H3 acetylation site substitutions. *GAL1* expression was examined in various strains bearing amino acid substitutions at histone H3 acetylation sites. The wild type strain RMY200 was included. Growth conditions, inductions, Northern analysis and quantifications were as described in Figure 3 and Materials and methods. Representative quantifications of relative *GAL1* RNA levels (RMY200 values are set to 1.0); RMY235, lanes: a, values too low to measure accurately; b, 6.9; c, 4.9; d, 2.4.

Table II. Histone H3 substitution mutant strains: viability, doubling time and mating efficiency

Strain	H3 substitution	Viability	Doubling time (min)	% mating
RMY200	WT($K_{9,14,18,23}$)	viable	92	1.0
RMY247	$G_{9,14,18}$	viable	104	0.45
RMY235	$R_{9,14,18}$	viable	111	0.24
RMY250	$G_{9,14,18,23}$	viable	115	0.40
RMY253	$R_{9,14,18,23}$	viable	135	0.34

change, but an amino acid whose charge cannot be neutralized by acetylation) or glycine (a non-conservative change and an uncharged residue), very little effect on mating efficiency was observed (Table II). However, *GAL1* was hyperactivated in all these strains in a manner similar to that observed in the H3 N-terminal deletions (Figure 4). Although there were differences in expression levels, depending on the substitution made, they all led to a significantly higher rate of *GAL1* induction. This increase was also observed for *GAL2*, *GAL7* and *GAL10* (data not shown). However, we found that there was only a minor effect of the H3 substitution mutations on *PRC1* levels as compared with the effects of the H3 N-terminal deletions (Figure 4).

While the H3 deletion strains were designed to be isogenic, we wished to confirm that the elevated *GAL* transcription levels were due to the plasmid-borne H3 N-terminal mutations and not due to secondary genomic mutations potentially caused by the transformation procedure itself. Therefore we changed the histone gene backgrounds of both the wild type and a representative mutant strain in a plasmid 'shuffle' routine similar to that used in the creation of new mutants (see above and Materials and methods). We found in each case that the wild type and mutant phenotypes were entirely dependent on the episomal wild type or mutant H3 genes (Figure 5).

Induction rate of another regulated promoter, PHO5, is not obviously elevated by a mutant H3 N-terminus

In order to determine whether H3 N-terminal lesions would elevate the activity of another inducible promoter, we also examined the activation of the *PHO5* (acid phosphatase) gene by Northern blot analysis. Initially, *PHO5* was induced by growth in media containing a low concentration of phosphate and expression was monitored over a period of time (data not shown). After 4 h of induction, a point at which we found that RNA levels were still increasing with time, the *PHO5* message levels were determined in control and mutant strains using a labelled *PHO5* DNA probe (Figure 6). We found that alterations within the N-terminus of H3 do not hyperactivate *PHO5*.

Quantifying the effect of H3 N-terminal alterations on transcription by GAL1 and PHO5 promoter–lacZ fusion

To obtain an independent quantification of the effect of H3 N-terminal alterations on promoter activation, we used yeast promoter–β-galactosidase gene (*lacZ*) reporter fusions that have been described previously (Durrin *et al.*, 1991). Prior to *GAL1–lacZ* induction, yeast strains were grown on raffinose-containing liquid media in order to alleviate glucose repression without activating *GAL1*. Induction was carried out by growth in media containing 2.0% galactose (Finley *et al.*, 1990; Flick and Johnston, 1990). The activities of

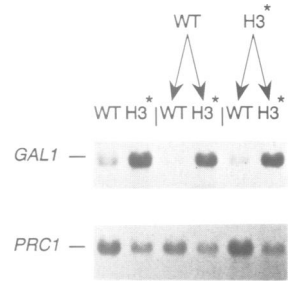

Fig. 5. Hyperactivation is linked to the episomal histone H3 gene. Plasmids bearing the wild type control and mutant histone genes were shuffled into and out of both the wild type controls strain (WT) and a representative of the mutant strains (H3*). RMY200 and RMY235 were transformed with the *GAL* promoter-controlled histone gene plasmid pRM102, after which pRM200 and pRM235, respectively, were allowed to be lost by mitotic instability. The resultant strains were each transformed separately with both of the original plasmids pRM200 and pRM235. After pRM102 was lost, *GAL1* gene induction was analysed as before, except that only one of the inducing conditions was used (1.0% raffinose and 1.0% galactose; same as lane c in Figures 3 and 4). The original RMY200 and RMY235 strains were included for comparison (first two lanes). The blots were stripped and hybridized to *PRC1* DNA.

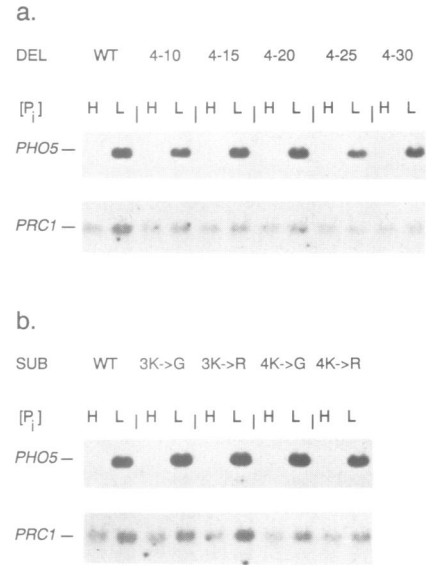

Fig. 6. Activation of the inducible *PHO5* gene is not strongly affected by histone H3 mutations. The acid phosphatase gene *PHO5* was induced in both H3 deletion and substitution mutant strains as described in Materials and methods. Message levels were determined as before by Northern analysis. (**a**) The control strain RMY200 and the H3 N-terminal deletion mutants. (**b**) Control and acetylation site substitution mutant strains; substitutions are indicated. The blots were stripped and rehybridized to *PRC1* DNA. SUB, substitution; [P_i], inorganic phosphate concentration, H (high) or L (low); 3K->G, $K_{9,14,18}$ replaced with G (RMY247); 3K->R, $K_{9,14,18}$ replaced with R (RMY235); 4K->G, $K_{9,14,18,23}$ replaced with G (RMY250); 4K->R, $K_{9,14,18,23}$ replaced with R (RMY253).

both H3 N-terminal deletion strains and a number of the substitution mutants were evaluated and the results are summarized in Table III. We found that deletion of H3 residues 4–20 or 4–30 resulted in an ~3.5-fold increase in induction of the *GAL1* promoter (from ~800 to 2700 units of β-galactosidase activity) and replacement of lysines at positions 9, 14 and 18 with either glycine or arginine results in a similar (3- to 4-fold) increase in activity. The basal levels of activity in these assays were very low, but it is interesting

Table III. Quantitative evaluation of basal and induced levels of transcription: promoter−*lacZ* fusion reporters

Strain	H3 mutation	*GAL1−lacZ*				*PHO5−lacZ*			
		Uninduced		Induced		Uninduced		Induced	
		Raff	REL	Gal	REL	High	REL	Low	REL
RMY200	WT	0.10	1.0	793	1.0	6.2	1.0	71.1	1.0
RMY410	Δ4−10	0.75	7.2	1640	2.1	n.d[a]	−	n.d.	−
RMY415	Δ4−15	1.87	18.1	2786	2.9	n.d.	−	n.d.	−
RMY420	Δ4−20	1.67	16.1	2834	3.6	7.5	1.2	33.4	0.5
RMY430	Δ4−30	1.35	13.0	2682	3.4	10.1	1.6	44.6	0.6
RMY247	$G_{9,14,18}$	0.48	4.6	2288	2.9	7.2	1.2	155.4	2.2
RMY235	$R_{9,14,18}$	0.22	2.1	3477	4.4	4.6	0.7	84.4	1.2
RMY212	R_9	0.14	1.4	1398	1.8	n.d.	−	n.d.	−
RMY213	Q_9	0.18	1.7	1269	1.6	n.d.	−	n.d.	−
RMY214	R_{14}	0.33	3.2	2701	3.4	n.d.	−	n.d.	−
RMY215	Q_{14}	0.30	2.9	1695	2.1	n.d.	−	n.d.	−

Each β-galactosidase assay was performed at least three times (as described in Materials and methods) and the averages are shown. Error was ≤20%. For *GAL1* promoter inductions, very similar results were obtained when raffinose was replaced with glycerol (5%) in the media (data not shown).
[a]Not determined.

to note that there was a very slight elevation of transcriptional activity in the uninduced mutant yeast strains to ~1.5 units in H3Δ4−20 and H3Δ4−30 strains. The fold increase in basal activity in these cases (~10- to 15-fold) must be interpreted with caution due to the extremely low levels (0.1 units) of uninduced activity in the control wild type strain.

In the case of *PHO5*, we observed that induced promoter activities in the control wild type strain as well as H3Δ4−20 and H3Δ4−30 were all within a factor of two. The basal *PHO5−lacZ* activity was very similar in wild type and mutant strains, also varying by only a factor of two. Given the minimal effect of H3 N-terminal deletions and substitutions on *PHO5* mRNA levels as measured by Northern blot analysis (Figure 6) and the lack of an obvious effect of *PHO5−lacZ* activity, we conclude that *PHO5* promoter activity is not affected in a manner similar to the effect on GAL4-regulated genes.

GAL4 message levels are not elevated in H3 mutant strains

Because *GAL1*, *GAL2*, *GAL7* and *GAL10* are strongly affected by H3 N-terminal deletions and amino acid substitutions, and *PHO5* is relatively unperturbed, we wished to ask whether *GAL4* expression was elevated in the mutant strains and hence, responsible for *GAL* hyperactivation. While not responsive to galactose, *GAL4* has recently been found to be glucose repressible (Griggs and Johnston, 1991). Additionally, it was found that even relatively moderate (5-fold) changes in *GAL4* promoter activity account for large (170-fold) differences in *GAL1* transcription. However, at higher *GAL4* levels, a plateau effect was observed such that levels of *GAL4* transcription higher than in wild-type cells did not increase *GAL1* activity appreciably. In spite of this and the fact that we use a non-repressing carbon source prior to induction, we asked whether a strikingly elevated *GAL4* message level might account for the H3-associated hyperactivation of GAL4-regulated genes. Relative message levels were determined in control and mutant strains in both raffinose and galactose. As demonstrated in Figure 7, no obvious elevation in *GAL4* message level was observed in mutant strains. We conclude that a disruption of *GAL4* regulation is unlikely to be the cause of the elevated *GAL* activity observed.

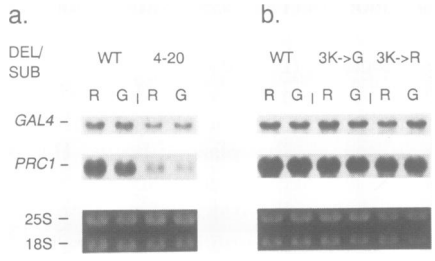

Fig. 7. *GAL4* message levels are not elevated in mutant histone H3 strains. (**a**) *GAL4* message levels were determined by Northern analysis using the control RMY200 and H3 N-terminal deletion strain RMY420 and separately (**b**), RMY200 and the two substitution mutant strains RMY247 (3K->G) and RMY235 (3K->R), in both 2% raffinose (R) and 1% raffinose−1% galactose (G). The *GAL4* DNA probe has been described in Durrin *et al.* (1991). The blots were stripped and rehybridized to *PRC1* DNA.

Glucose repression is partially alleviated in H3 mutant strains

In addition to being activated by galactose, the *GAL* genes are strongly repressed by glucose (for review, see Johnston, 1987). Catabolite repression, which appears to prevent the association of GAL4 with its binding sites and cause repression through upstream repressive sequences (URSs) or operators within the *GAL* promoters (West *et al.*, 1984; Finley *et al.*, 1990), reduces expression to a remarkably low level. As indicated in Table IV, *GAL1* basal activity levels in glucose were modestly elevated in the H3Δ4−20 and H3Δ4−30 strains from 0.1 units to approximately 1.5 units of β-galactosidase activity. Moreover, while the *GAL1* promoter is normally repressed in media containing glucose and galactose, we found that glucose repression was incomplete under these conditions, with β-galactosidase activity increasing to ~5−10 units. In contrast, the removal of the URS or inactivation of the *GAL1* operators can lead to 100−200 units of β-galactosidase activity under repressive or non-repressive/non-inducing conditions, and up to 500 units in media containing both glucose and galactose (West *et al.*, 1984; Finley *et al.*, 1990). None of the mutations in histone H3 caused increases in basal activities in this range. These data show that there is only a partial loss of repression caused by H3 N-terminal lesions.

Table IV. Quantitative evaluation of repressed transcription levels: *GAL1–lacZ* fusion reporters

Strain	H3 mutation	Glu	REL	Glu/Gal	REL
RMY200	WT	0.10	1.0	0.2	1.0
RMY420	Δ4–20	1.87	18.7	11.05	55.3
RMY430	Δ4–30	1.35	13.5	5.63	28.2
RMY247	$G_{9,14,18}$	0.89	8.9	3.62	18.1
RMY235	$R_{9,14,18}$	0.59	5.9	n.d.	–

Discussion

Of the four core histones, H3 and H4 alone can form a nucleosome-like structure. They are both extremely conserved in evolution and share similar domain structures as well as patterns of acetylation that appear to correlate with increased transcription. Given the functions of the H4 N-terminus in both the repression of the silent mating loci and activation of *GAL1*, *GAL7* and *GAL10*, we have asked whether H3 and H4 N-termini have similar transcription-associated functions *in vivo*. By deleting the H3 N-terminus, we have shown that, as with the other core histones, this extremely conserved domain is dispensable for cellular viability. A comparison of our genetic deletion analyses of all four core histones with hydropathy plots and the X-ray crystallographic data of Arents *et al.* (1991) is especially informative. Figure 8 shows the structured and unstructured regions of the core histones as determined at 3.1 Å resolution. For each core histone N-terminus, the length of the largest viable deletion that we have made correlated extremely well with the length of the unstructured region. This correlation was less clear with the very short viable C-terminal deletions.

Despite the similarity and dispensability of the N-termini of H3 and H4, we have shown that these domains have functions that are quite distinct from each other. The H3 N-terminus is neither strongly required for repression of the silent mating locus *HML* nor required for promoter activation of the yeast *GAL* genes. On the contrary, deletions within the N-terminus of H3 result in hyperactivation of the *GAL1* promoter during induction (as well as of *GAL2*, *GAL7* and *GAL10*), a very slight increase in *GAL1* basal level activity and a modest decrease in glucose repression of the *GAL1* promoter. Deletion of residues 4–15 appears to be sufficient for most of the hyperactivation seen in the H3 N-terminal deletion strains. Amino acid substitutions at the sites of H3 acetylation, two of which are found within the first 15 residues, show similar effects on transcription. The activation of *PHO5* was not similarly affected by alteration of the H3 N-terminus. However, it is unlikely that GAL4-regulated genes are the only genes affected by H3 N-terminal deletions. *PRC1* message levels (relative to total RNA) appear to be markedly reduced in H3 N-terminal deletion strains (see Figure 3a) and slightly reduced in strains containing H3 N-terminal substitutions.

The structural features that make H3 and H4 functionally distinct from each other have yet to be determined. However, the N-terminus of H3 is predicted to extend ~10–20 residues further from the nucleosome core and with a different orientation from that of H4 (van Holde, 1989; Arents *et al.*, 1991). Interestingly, the histone–DNA cross-linking studies of Mirzabekov (Mirzabekov *et al.*, 1978; Belyavsky *et al.*, 1980; Shick *et al.*, 1980; Bavykin *et al.*, 1985) have demonstrated that H3 and H4 cause very different cross-linking patterns along the nucleosomal DNA. While portions of H3 appear to be associated with DNA that is both entering and exiting the nucleosome, no such association exists for H4 sequences. It is conceivable that H3 associations with the entry/exit DNA or nearby linker DNA, may be disrupted in H3 mutants such that this DNA is less constrained and perhaps more accessible to the transcription machinery.

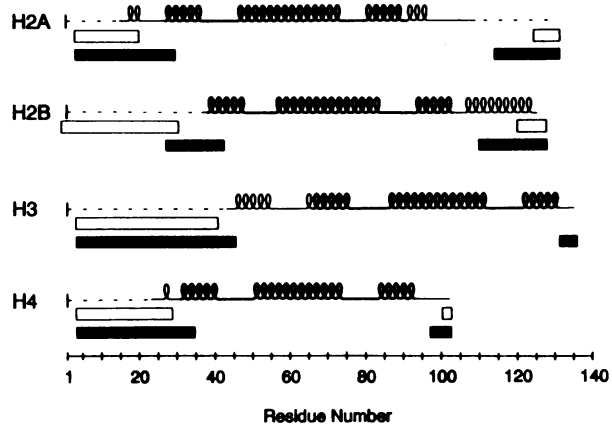

Fig. 8. Comparison of genetic deletion analyses with structural features of the four core histones. The octameric structure of the four chicken erythrocyte core histones has been determined to 3.1 Å resolution and their secondary structures are illustrated schematically, as reported by Arents *et al.* (1991). Continuous lines indicate resolved (structured) regions and dashed lines unstructured regions that do not defract. Bold type helices are involved in the common histone fold. Shown below each histone are the results of our genetic deletion analyses. Deletion of yeast residues 4–20 and 124–131 of H2A, 3–32 and 123–230 of H2B, 4–40 of H3 and 4–28 and 100–102 of H4 allow viability (open bars) while deletion of residues 4–29 and 114–131 of H2A, 30–45 and 113–130 of H2B, 4–45 and 131–135 of H3 and 4–34 and 97–102 of H4 appear lethal (solid bars; see text for references). Note that yeast histones H2A and H2B are three residues (at the C-terminus) and five residues (three at the N-terminus and two at the C-terminus), respectively, longer than the corresponding chicken erythrocyte histones. A close correspondence is observed between viable deletions and unstructured regions at the N-termini of the core histones.

The reasons why H3 N-terminal alterations cause hyperactivation of *GAL1* but not *PHO5* are unclear at this time. We have not excluded the possibility that the H3 lesions increase *GAL1* expression through a decreased level of GAL80 (the inhibitor of GAL4 function). However, it has been observed that *GAL1* gene activity is constitutive in *GAL80* deletion strains grown in non-repressing, non-inducing carbon sources (Torchia *et al.*, 1984; Johnston, 1987; Flick and Johnston, 1990). Since we have observed extremely low uninduced *GAL1* gene activity in H3 N-terminal mutant strains under these conditions, we believe that it is unlikely that the effect of the H3 mutations on *GAL1* occurs through depressed *GAL80* mRNA synthesis. It is possible that the increase we observed in *GAL1* mRNA synthesis is due indirectly to hyperactivation of *GAL2* (the galactose permease) since both genes are activated in a similar manner. We have shown here that it is unlikely that an increased level of *GAL4* mRNA synthesis is responsible. One explanation for the hyperactivation of the *GAL1* promoter may lie in a potentially regulatory nucleosomal arrangement between the *GAL1* UAS and the TATA element. This region contains four adjacent 40 bp sequences

that are ~85% homologous to multimers of the artificial, nucleosome positioning signal, $(A/T)_3NN(G/C)_3NN$, described by Shrader and Crothers (1989), and R.Mann and M.Grunstein (unpublished data). These spans of homology, which are found within a positioned nucleosome (Fedor *et al.*, 1988; Fedor and Kornberg, 1989), also contain the URS (upstream repressive sequence; Flick and Johnston, 1990) and smaller repressive/operator elements (West *et al.*, 1984, 1987; Finley *et al.*, 1990), that have been shown to be required for glucose repression of *GAL1*. Moreover, this region contains a binding site for the repressor protein MIG1 (Nehlin *et al.*, 1991) that corresponds precisely to the previously identified URS-A−operator (O_6) site. These associations may suggest a cooperativity between the histone octamer, a natural nucleosome positioning sequence and the MIG1 factor. Since MIG1 binds *in vivo* within nucleosomal DNA and binds the same sequence only very weakly *in vitro* (Nehlin *et al.*, 1991), it is possible that MIG1 has a greater affinity for its binding element when it is presented as nucleosomal DNA, an interaction that may be weakened by H3 N-terminal lesions. Were this to be the case, *GAL1* and *GAL2* promoters, both of which have sequences that contain sequences homologous to MIG1 binding sites, would be activated by H3 N-terminal lesions. This could then lead to the activation of *GAL* promoters that may lack MIG1 binding sites. In support of this model, we have found (J.Wan and M.Grunstein, unpublished) that glucose repression, through a URS element fused between the glucose insensitive *LEU2* UAS and a *lacZ* reporter gene, is also partially alleviated by the H3 N-terminal deletions described above.

While it is intriguing that both deletions and acetylation site substitution mutations cause similar hyperactivation of certain *GAL* genes, it is not clear that acetylation is a critical variable. One might expect that the placement of either a positively charged amino acid (arginine) or a neutral one (glycine, glutamine) at a site of potential charge modulation (i.e. by acetylation) would cause a dramatically different phenotype. No striking difference was observed, which leads us to believe that the substitutions we have made are causing structural perturbations within the H3 N-terminus which may be epistatic to charge-associated functions.

Materials and methods

Genetic manipulations

Unless otherwise noted, standard yeast genetic techniques were employed throughout this study (Sherman *et al.*, 1986). Most media and chemicals used have been described elsewhere; other media are described in detail below. Yeast transformations were performed by standard lithium acetate protocols. All cloning and plasmid preparations were as described (Sambrook *et al.*, 1989).

Strain constructions and plasmids

RMY 200. Two haploid yeast strains were prepared in which either one or the other of the two *HHT−HHF* clustered loci were deleted and replaced with selectiable markers (Rothstein, 1983). Plasmids pUK192 (in which *HHT1−HHF1* is replaced with *LEU2*) and pUK431 (*HHT2−HHF2* replaced with *HIS3*) were linearized by restriction enzymes, transformed into PKD2-5C and PKD4-2B, respectively, and plated on the appropriate selective medium. Southern analysis was employed to verify that the markers were recombined into the correct loci. (Both gene replacements produced viable strains, confirming that only one copy of H3 is required for viability.) The resultant strains (LJY192 and LJY431-3) were subsequently mated and the diploid (LJY623) was transformed with pRM200, a yeast *CEN4/ARS1/TRP1* plasmid bearing *HHT2* and *HHF2* under their native promoters. This strain was sporulated, its tetrads were dissected and spores were screened for a His$^+$, Leu$^+$, Trp$^+$ phentoype, identifying a strain whose sole source of H3 and H4 is pRM200 (=RMY200).

pUK192 was prepared by digesting pMS19l (Smith and Murray, 1983) with *Sma*I and then Bal31 exonuclease. After *Xba*I linker addition, a 2.2 kb *Xba*I fragment bearing *LEU2* was inserted. Restriction mapping demonstrated that the majority of *HHT1* and *HHF1* coding sequences were removed by the exonuclease digestion (the *Eco*RV site of *HHT1* and the *Xmn*I site of *HHF1* remained). pUK431 was prepared by first inserting an ~1.5 kb, *HHF2*-bearing *Hin*dIII fragment from pMS202 (Smith and Murray, 1983) into the same site of pUC13. This clone, pH4-2, was partially digested with *Hin*dIII, treated with Klenow to fill in cleaved termini and then re-ligated. A resulting construct in which the *Hin*dIII site distal to the *HHF2* coding sequences had been removed, was identified and named pH421DR. An ~2.4 kb, *HHT2*-bearing *Hin*dIII fragment from pMS201 (Smith and Murray, 1983) was then inserted into the now unique *Hin*dIII site of pH42DR, creating pUK430R. pUK430R was cut with *Xba*I, briefly digested with Bal31 and then ligated (this removed the *Eco*RI and *Hin*dIII sites). The resultant plasmid was partially digested with *Xmn*I and an *Xho*I restriction site was created by linker addition. This construct was digested with *Bal*I and *Xho*I (to remove most of *HHT2* and *HHF2*) and the *Xho*I site was filled-in with Klenow. After the addition of an *Xba*I site (using linkers), a 1.8 kb *Xba*I fragment containing *HIS3* was inserted. pRM200 was made by ligating a *Bam*HI−*Sal*I *HHT2* fragment into pLJ999T, which, except for a *TRP1* marker that replaces *URA3*, is equivalent to pUK499 (Kim *et al.*, 1988).

RMY102. RMY200 was transformed with pRM102, a *CEN4/ARS1/URA3* plasmid that bears *HHT2* fused to the *GAL10* promoter and *HHF2* fused to the *GAL1* promoter. After growing the transformants for several days in a non-selective, galactose-containing medium (YEPG), cells were plated and screened for those that had lost pRM200 (Trp$^-$; = RMY102). pRM102 was prepared by inserting a blunted, *HHT2*-bearing *Bam*HI fragment from pUK201 into a blunted *Eco*RI site of pUK420 (Kim *et al.*, 1988) placing *HHT2* under the control of the *GAL10* promoter. pUK201 (U.-J.Kim; M.Grunstein, unpublished construct) features *HHT2* (on a 2.4 kb *Bam*HI fragment) fused to the *GAL1* promoter of pBM258 (Johnston and Davis, 1984).

H3 mutant strains

pRM200 derivatives, bearing altered H3 genes, were prepared and introduced into the strain RMY102. Transformants were selected on SG−ura−trp plates and the glucose shift viability test (Kayne *et al.*, 1988) was used to determine whether individual *HHT2* mutations allowed viability. Site-directed mutagenesis of *HHT2* was accomplished by two different techniques. A 1.1 kb *Bam*HI−*Hin*cII fragment bearing *HHT2* was cloned into pUC13 and subsequently into M13mp19 using *Bam*HI and the *Hin*dIII site of the plasmid polylinker. The Amersham oligonucleotide-directed *in vitro* mutagenesis system was used to create mutations within a single stranded, M13-based template. *Bal*I fragments were excised and inserted into the corresponding sites of pRM200. Alternatively, recombinant PCR was used, which had the advantage of being very rapid. In addition to the 'outside' primers, protocols using either one or two mutagenic oligonucleotides were employed (Sarkar and Sommer, 1990; Higuchi, 1990). A 1.5 kb *Hin*dIII−*Hin*cII fragment from pRM200 was used as a template. The 'outside' oligonucleotide corresponding to the *Hin*cII end of the template was able to be generated by sequencing this previously unsequenced region (sequence derived from pUK320, using the 'reverse' primer). This oligo additionally contained an engineered *Sal*I site. Amplified, mutagenic products were cut with *Bam*HI and *Sal*I and cloned into the corresponding sites of pLJ999T. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA Synthesizer. All engineered mutations were confirmed by dideoxy sequencing using Sequenase (US Biochemical Corp.)

Growth rates and quantitative mating assays

Growth rates were determined essentially as described previously (Kayne *et al.*, 1988) with the following exceptions. Three liquid YEPD cultures of a given strain were started at different dilutions and grown overnight at 30°C with vigorous shaking. The next day, one of the three cultures was usually at a low optical density (OD; absorbance at 600 nm) and growing exponentially. OD measurements were taken every 30 min on a Beckman model DU-65 spectrophotometer and over the approximate range of 0.05−0.80. Quantitative mating assays were performed as previously described (Kayne *et al.*, 1988) except that mating incubations were for 5.5 h.

Transcription induction assays

The activity of each of the promoters examined was evaluated both by Northern analysis of the native transcript and by β-galactosidase assays in strains bearing promoter−*lacZ* fusion reporter plasmids.

Table V. Strains

Strain	Genotype
PKD2-5C	*MATa ade2−101(och) his3Δ201 leu2−3,112, lys2−801(amb) trpΔ901 ura3−52*
PKD4−2B	*MATa ade2−101(och) his3Δ200 lys2−801(amb) trp1Δ901 ura3−52*
LJY192	*MATa ade2−101(och) his3Δ201 lys2−801(amb) trp1Δ901 ura3−52 hht1,hhf1::LEU2*
LJY431−3	*MATa ade2−101(och) lys2−801(amb) trp1Δ901 ura3−52 hht2,hhf2::HIS3*
RMY200	*MATa ade2−101(och) his3Δ200 lys2−801(amb) trp1Δ901 ura3−52 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pRM200 (*CEN4 ARS1 TRP1 HHT2 HHF2*) [Note: we have not determined whether this strain received *LEU2* or *leu2−3,112* at its normal locus]
RMY102	genetic background equivalent to RMY200, but/pRM102 (*CEN4 ARS1 P(GAL10)−HHT2 P(GAL1)−HHF2*)
RMY410	isogenic to RMY200, plus pRM410 (*hht2Δ4−10*)
RMY415	isogenic to RMY200, plus pRM415 (*hht2Δ4−15*)
RMT420	isogenic to RMY200, plus pRM420 (*hht2Δ4−20*)
RMY425	isogenic to RMY200, plus pRM425 (*hht2Δ4−25*)
RMY430	isogenic to RMY200, plus pRM430 (*hht2Δ4−30*)
RMY435	isogenic to RMY200, plus pRM435 (*hht2Δ4−35*)
RMY440	isogenic to RMY200, plus pRM440 (*hht2Δ4−40*)
(RMY445)	isogenic to RMY200, plus pRM445 (*hht2Δ4−45*)
(RMY450)	isogenic to RMY200, plus pRM450 (*hht2Δ4−50*)
RMY212	isogenic to RMY200, plus pRM212 (*hht2* R_9)
RMY213	isogenic to RMY200, plus pRM213 (*hht2* Q_9)
RMY214	isogenic to RMY200, plus pRM214 (*hht2*R_{14})
RMY215	isogenic to RMY200, plus pRM215 (*hht2* Q_{14})
RMY235	isogenic to RMY200, plus pRM235 (*hht2* $R_{9,14,18}$)
RMY247	isogenic to RMY200, plus pRM247 (*hht2* $G_{9,14,18}$)
RMY250	isogenic to RMY200, plus pRM250 (*hht2* $G_{9,14,18,23}$)
RMY253	isogenic to RMY200, plus pRM253 (*hht2* $R_{9,14,18,23}$)
(RMY531)	isogenic to RMY200, plus pRM531 (*hht2Δ131−135*)
(RMY526)	isogenic to RMY200, plus pRM526 (*hht2Δ126−135*)
(RMY511)	isogenic to RMY200, plus pRM511 (*hht2Δ111−135*)
(RMY636)	isogenic to RMY200, plus pRM636 (*hht2Δ36−42*)

Northern analysis

Depending on the promoter of interest, the various mutant histone H3 strains were grown under different culture conditions. Prior to *GAL* induction, strains were first grown in liquid YEPR for ~2 days. Exponentially growing cultures were collected by centrifugation, resuspended in a small volume of YEPR and then inoculated to a final OD of 0.25 into pre-warmed YEP liquid media containing the ratios of raffinose and galactose indicated in figure 3. Cultures were grown at 30°C with shaking for 6 h. *PHO5* inductions were performed essentially as previously described (Han *et al.*, 1988), except cultures were grown to an exponential phase in 'high' phosphate YEPD liquid media, collected by centrifugation, washed once with water and then recollected. After resuspending in a small volume of 'low' phosphate YEPD, cells were immediately added to 'high' and 'low' phosphate YEPD to a final OD of 0.3 and grown for 4 h. Additionally, no phosphate was added back to the 'low' phosphate medium. After all inductions, cultures were quick-cooled by shaking on ice; cells were collected by centrifugation, frozen in dry ice−ethanol and stored at −80°C. Total RNA was isolated by a scaled-down hot phenol extraction technique (Kohrer and Domdey, 1991). Typically, 13 ml of culture was harvested and used for RNA isolation. 15 μg of total RNA was fractionated through a 1% agarose−formaldehyde gel and transferred to solid support by standard techniques (Sambrook *et al.*, 1989). Random primer-labelling (Feinberg and Vogelstein, 1983) of previously described probes (Kayne *et al.*, 1988; Durrin *et al.*, 1991) and Northern analysis were performed as described (Sambrook *et al.*, 1989).

Promoter−lacZ fusions and β-galactosidase assays

The promoter−*lacZ* fusion reporter constructs have been previously described (Durrin *et al.*, 1991). Strains transformed with pRY131 (*GAL1−lacZ*) were grown for 2 days on SR−ura−trp medium. Exponentially growing cultures were collected by centrifugation, resuspended in a small volume of SR−ura−trp, aliquoted into SR−ura−trp and SR/G−ura−trp (2% raffinose: 2% galactose) and grown at 30°C for 6 h. The pre-induction raffinose concentration of 2% was maintained in the inducing medium in order to reduce bias introduced by the fact that those strains that are able to activate *GAL* genes more rapidly presumably will be able to utilize galactose more rapidly and grow. It is also important to note that the high levels of expression that we achieve in both Northern analyses and β-galactosidase assays indicate that raffinose does not repress *GAL* genes.

Transformants with pMH313 (*PHO5−lacZ*) were grown in SD−ura−trp medium to logarithmic phase, collected as above and used to inoculate 'high' phosphate YEPD. After growth for 3−3.5 h, cultures were collected, washed and induced similar to those used in the Northern analysis. After induction, all cultures were placed on ice for ~20 min and β-galactosidase assays were performed on 0.2 ml of each culture as described (Yocum *et al.*, 1984; Han and Grunstein, 1988). Since cell densities were used in the activity calculations, a concern was that histone mutant pleiotropy might alter cell volume or shape. Microscopic examination of both wild type and mutant strains demonstrated that there was no appreciable difference in cell volumes (data not shown).

Strains

Strains used in this study are described in Table V.

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