Note

Identification of a Functional Domain Within the Essential Core of Histone H3 That Is Required for Telomeric and *HM* Silencing in *Saccharomyces cerevisiae*

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ABSTRACT

Fourteen novel single-amino-acid substitution mutations in histone H3 that disrupt telomeric silencing in *Saccharomyces cerevisiae* were identified, 10 of which are clustered within the α1 helix and L1 loop of the essential histone fold. Several of these mutations cause derepression of silent mating locus *HML*, and an additional subset cause partial loss of basal repression at the *GAL1* promoter. Our results identify a new domain within the essential core of histone H3 that is required for heterochromatin-mediated silencing.

CHROMATIN structure provides a key mechanism for the packaging of DNA into the confines of the nucleus, as well as a system for the global regulation of gene expression. Chromatin can exist in a range of structural conformations, from loosely compacted euchromatin to densely packed heterochromatin (Turner 2001). Although the relationship between chromatin structure and gene expression is complex, the general rule is that expressible genes are packed in accessible euchromatin, while genes found in inaccessible heterochromatin are inactive.

Heterochromatin is found at a number of loci in the yeast *Saccharomyces cerevisiae*, including the silent mating loci and subtelomeric regions (Laurenson and Rine 1992; Lustig 1998). Transcriptional silencing at these loci is achieved through interactions between histone proteins and chromatin-associated Sir proteins (Gasser and Cockell 2001). It has been shown that Sir3 and Sir4 interact with chromatin via the N termini of histones H3 and H4 (Hecht *et al.* 1995, 1996; Strahl-Bolsinger *et al.* 1997), creating a condensed and transcriptionally inhibitory chromatin structure (Gottschling 1992; Singh and Klar 1992; Loo and Rine 1994; Weiss and Simpson 1998; Ravindra *et al.* 1999).

Histones are small basic proteins that assemble as an octamer around which ~147 bp of DNA are wrapped to create the nucleosome (Luger *et al.* 1997; White *et al.* 2001). Each histone is composed of a structured essential core and a less structured dispensable N-terminal "tail." The N termini are subject to a variety of post-translational modifications, including acetylation, methylation, and phosphorylation, which play important roles in modulating histone function (Jenuwein and Allis 2001; Berger 2002). Hypoacetylation and methylation of the H3 and H4 N termini are believed to provide a mechanism for restricting histone-Sir interactions to silenced loci (Braunstein *et al.* 1993, 1996; Suka *et al.* 2001; Carmen *et al.* 2002; Krogan *et al.* 2002). Mutations within the histone H3 and H4 N termini disrupt histone-Sir interactions, allowing expression within normally silenced regions (Kayne *et al.* 1988; Johnson *et al.* 1990; Park and Szostak 1990; Aparicio *et al.* 1991; Johnson *et al.* 1992; Thompson *et al.* 1994). Many single-amino-acid substitution mutations in the N terminus of histone H4 that disrupt silencing have been identified; however, no single-amino-acid substitutions in the N terminus of histone H3 that affect telomeric or *HM* silencing have been identified.

Random mutagenesis of histone H3: To better understand the role of histone H3 in chromatin-mediated silencing in yeast, we introduced random substitutions in histone H3 by mutagenic PCR to identify single-amino-acid changes that disrupt telomeric silencing. Candidates were screened for sensitivity to 5-fluoroorotic acid (5-FOA), indicating derepression of the telomere-associated *URA3* reporter gene (which is normally silenced at this locus). Fourteen unique single-amino-acid substitution mutations in histone H3 were identified (Table 1). Four of the substitutions were conserved changes with respect to charge/polarity, while the others represented changes to charge or polarity. Ten of

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the 14 mutations mapped to a discrete region in the central core of H3 corresponding to the α1 helix and L1 loop (Figure 1A). Three mutations mapped to the N terminus, and one mutation localized to the C-terminal end of the α2 helix.

None of the H3 mutant strains exhibited any striking growth defects when grown at 30°, in contrast to the slow-growth phenotype of H3 N-terminal deletion and acetylatable lysine substitution strains (Mann and Grunstein 1992). However, a number of the H3 mutant strains were either slow growing (relative to the wild-type strain) or inviable when grown at 37° (Table 1).

Analysis of telomeric silencing in histone H3 mutant strains: To assess the degree of disruption to telomeric silencing by the H3 mutations, the mutant strains were plated on media with and without 5-FOA to determine the fraction of cells that were resistant to 5-FOA (*i.e.*, cells in which *URA3* is silenced). While the wild-type strain was largely resistant to 5-FOA, H3 mutant strains exhibited a range of sensitivities to 5-FOA, with the fraction of cells surviving on 5-FOA spanning from $\sim$0.001 to $<10^{-6}$ (Table 1). These results indicate that the vast majority of cells within the respective mutant populations have disruptions to telomeric silencing, although some mutations clearly have more pronounced effects than others.

Histone H3 mutations affect silencing at *HML*: Repression at the silent mating loci *HML* and *HMR*, which relies on a mechanism similar to that found at telomeric regions, is essential for haploid mating (Lustig 1998). *HML* and *HMR* possess silent copies of α- and **a**-mating-type regulatory genes, respectively. Derepression of these loci in haploid strains leads to simultaneous expression of both **a**- and α-genes, resulting in a nonmating phenotype (*i.e.*, derepression of *HMLα* causes sterility in **a**-mating-type strains). The degree of *HM* silencing is notably stronger and more stable compared to telomeric regions. As a result, some mutations that disrupt telomeric silencing do not cause a notable loss of repression at the *HM* loci. For example, deletion of the N terminus of histone H3 causes only a weak derepression at *HML* and has no obvious effect at *HMR* (Mann and Grunstein 1992; Thompson *et al.* 1994).

To determine the effect of the histone H3 mutations on silencing at *HML*, histone H3 mutant strains (**a**-mating type) were subjected to a quantitative mating assay to determine the fraction of cells in a population that were able to mate. Six of the histone H3 mutant strains did not demonstrate any statistically significant mating defects (Table 1). Five strains exhibited statistically lower relative mating efficiencies (0.3–0.7), although the differences relative to the wild type were quite small. Three strains had more substantial defects in mating efficiency. Strains expressing the $T_{80}A$ and $K_{79}E$ substitutions exhibited 7- and 50-fold relative reductions in mating efficiency, respectively, while the $E_{73}D$ mutant strain mated more than 4 orders of magnitude less efficiently than the wild-type strain, consistent with strong derepression of *HML*. This mutation had a notably stronger effect on repression at *HML* than did H3 N-terminal deletions (Mann and Grunstein 1992; Thompson *et al.* 1994). Furthermore, the mating deficiencies caused by $E_{73}D$ and $K_{79}E$ were restored to near-wild-type levels by *sir3* suppressor alleles (Table 1), previously identified on the basis of their ability to restore mating in H4 N-terminal point mutation strains (Johnson *et al.* 1990). While the mechanism of suppression by these *sir3* alleles is unknown, the results are suggestive of a direct interaction between Sir3 and both the H4 N terminus and the H3 core domain.

A subset of histone H3 mutations affect basal repression at the *GAL1* promoter: We wished to determine if any of these histone H3 mutations caused generalized and pleiotropic effects on chromatin structure and gene regulation. To address this question, we utilized a reporter construct with the *URA3* coding region under the control of the *GAL1* promoter (Lenfant *et al.* 1996). Expression from the *GAL1* promoter is activated when cells are grown in the presence of galactose, but is strongly repressed in the presence of glucose (Lohr *et al.* 1995). In the presence of raffinose, the *GAL1* promoter is subject to basal level repression mediated by the presence of nucleosomes across the promoter region. Deletions within the H3 and H4 N termini disrupt basal repression of the *GAL1* promoter (Mann and Grunstein 1992; Lenfant *et al.* 1996).

Histone H3 mutant strains possessing the *GAL1-URA3* reporter were examined for 5-FOA sensitivity when grown in the presence of various carbon sources. As expected, all strains exhibited complete sensitivity to 5-FOA when grown in the presence of galactose (data not shown), indicating expression of *GAL1-URA3*. In the presence of raffinose, half of the strains exhibited a significant decrease in 5-FOA resistance (2.5- to 20-fold; Table 1), indicating a modest degree of expression from the *GAL1* promoter. Most of these same mutations also caused low-level expression when the strains were grown in glucose, although the level of 5-FOA resistance was slightly higher in glucose-grown cells than in raffinose-grown cells. Two of these mutations (T_6K and $E_{73}D$) exhibited effects only in raffinose, suggesting that the degree of basal derepression is less pronounced in these two strains. In contrast, the other seven mutant strains displayed no statistically significant differences in 5-FOA sensitivity relative to the wild-type strain. While no strains displayed the extent of 5-FOA sensitivity observed in histone N-terminal deletion strains (Lenfant *et al.* 1996), it is clear that a subset of these mutations allow low-level basal expression at a nonsilenced locus.

DISCUSSION

Through random mutagenesis of histone H3, we have identified a domain within the structured core region

TABLE 1

Histone H3 silencing mutations

Strain name	Amino acid substitution	Growth at 37°[a]	Telomeric silencing (*URA3*-telomere): fraction of 5-FOA^R cells[b]	*HML* silencing: relative mating efficiency[b]	Basal repression (*GAL1-URA3*)	
					Fraction of 5-FOA^R cells in raffinose[c]	Fraction of 5-FOA^R cells in glucose[c]
JTY34U	Wild type	++	0.19 ± 0.08	1.0	0.43 ± 0.12	0.68 ± 0.14
JTY-MS105U	R_2G	+	$4.5 \times 10^{-5} \pm 3.1 \times 10^{-5}$*	0.90 ± 0.40	0.03 ± 0.01*	0.20 ± 0.12*
JTY305U	T_6K	+	$2.1 \times 10^{-5} \pm 1.3 \times 10^{-5}$*	0.97 ± 0.12	0.16 ± 0.03*	0.45 ± 0.27
JTY310U	$A_{29}V$	+	$2.4 \times 10^{-6} \pm 2.8 \times 10^{-6}$*	0.65 ± 0.12*	0.27 ± 0.05	0.46 ± 0.13
JTY301U	$Q_{68}R$	−	$1.2 \times 10^{-3} \pm 2.8 \times 10^{-4}$*	0.90 ± 0.19	0.02 ± 0.01*	0.08 ± 0.03*
JTY309U	$L_{70}S$	−	$1.7 \times 10^{-6} \pm 1.2 \times 10^{-6}$*	0.53 ± 0.29*	0.16 ± 0.01*	0.35 ± 0.07*
JTY311U	$V_{71}A$	+	$1.8 \times 10^{-4} \pm 1.4 \times 10^{-4}$*	1.05 ± 0.23	0.47 ± 0.06	0.70 ± 0.20
JTY317U	$R_{72}G$	++	$1.5 \times 10^{-6} \pm 9.2 \times 10^{-7}$*	0.96 ± 0.25	0.12 ± 0.04*	0.34 ± 0.05*
JTY319U	$E_{73}D$	++	$5.3 \times 10^{-7} \pm 1.3 \times 10^{-7}$*	$5.0 \times 10^{-5} \pm 1.0 \times 10^{-5}$*	0.15 ± 0.04*	0.61 ± 0.24
JTY-MS106U	$Q_{76}R$	++	$6.1 \times 10^{-6} \pm 4.9 \times 10^{-6}$*	1.07 ± 0.31	0.37 ± 0.07	0.66 ± 0.09
JTY308U	$K_{79}E$	++	$3.6 \times 10^{-6} \pm 2.7 \times 10^{-6}$*	0.02 ± 0.01*	0.51 ± 0.15	0.68 ± 0.14
JTY307U	$T_{80}A$	++	$1.9 \times 10^{-6} \pm 9.1 \times 10^{-7}$*	0.14 ± 0.08*	0.56 ± 0.10	0.78 ± 0.12
JTY313U	$T_{80}P$	++	$3.2 \times 10^{-6} \pm 2.9 \times 10^{-6}$*	0.72 ± 0.11*	0.55 ± 0.13	0.78 ± 0.21
JTY304U	$F_{84}L$	+	$1.3 \times 10^{-6} \pm 6.8 \times 10^{-7}$*	0.35 ± 0.06*	0.33 ± 0.10	0.58 ± 0.15
JTY302U	$A_{114}T$	−	$3.5 \times 10^{-5} \pm 2.3 \times 10^{-5}$*	0.30 ± 0.24*	0.11 ± 0.12*	0.09 ± 0.01*
JTY319R1	$E_{73}D$ + *sir3R1*[d]	ND	ND	0.54 ± 0.15*	ND	ND
JTY319R3	$E_{73}D$ + *sir3R3*[d]	ND	ND	0.31 ± 0.12*	ND	ND
JTY308R1	$K_{79}E$ + *sir3R1*[d]	ND	ND	0.98 ± 0.10	ND	ND
JTY308R3	$K_{79}E$ + *sir3R3*[d]	ND	ND	0.76 ± 0.08*	ND	ND

Random mutations were introduced in *HHT2* (encoding histone H3) by PCR mutagenesis and transformed into yeast strain JTY102TU by gapped repair transformation (Muhlrad *et al.* 1992). JTY102TU possesses *HHT2* on a plasmid under the control of the *GAL10* promoter (endogenous *HHT* copies have been deleted) and a copy of the *URA3* gene integrated adjacent to the telomere on the left arm of chromosome VII. About 5000 transformants were replica plated onto media containing 5-fluoroorotic acid (5-FOA; 1 mg/ml) to identify 5-FOA-sensitive colonies. Mutant plasmids were recovered for sequencing and retransformation into the parent strain for phenotype characterization (following loss of the wild-type H3 plasmid). *, statistically significant difference relative to wild type ($P < 0.01$); ND, not determined.

[a] ++, normal growth; +, slow growth; −, inviable.

[b] Quantitative 5-FOA assays (Gottschling *et al.* 1990) and mating assays (Kayne *et al.* 1988) were done as previously described. Average values from a minimum of three assays are indicated ±SD.

[c] Basal repression at the *GAL1* promoter was assayed as previously described using the plasmid pFL800 (Lenfant *et al.* 1996), in H3 mutant strains lacking the *URA3*-telomere integration. The average fraction of 5-FOA-resistant (5-FOA^R) cells from a minimum of four assays is indicated ±SD.

[d] *SIR3* was replaced with *sir3* suppressor alleles *sir3R1* or *sir3R3* (Johnson *et al.* 1990) in strains possessing H3 mutations $E_{73}D$ and $K_{79}E$, which were subsequently tested for mating efficiency as described above. Relative mating efficiencies for all four suppressor strains were statistically different compared to the corresponding H3 mutant strains ($P < 0.01$).

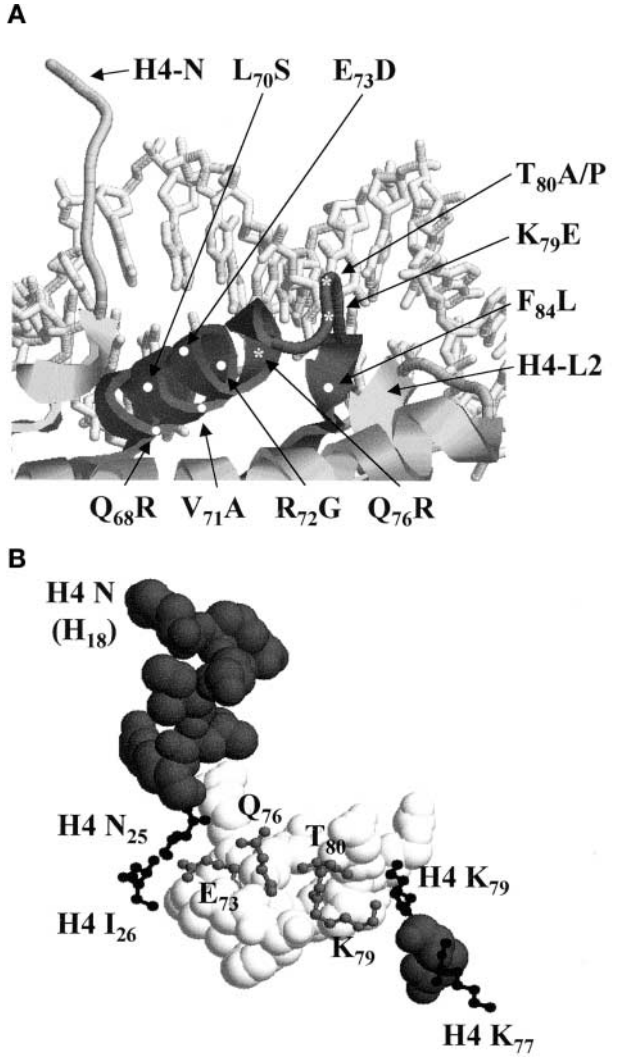

Figure 1.—Schematic representation of the histone H3 α1-L1 silencing domain. (A) Cartoon view of the histone H3 α1 helix (residues 64–78) and adjacent L1 loop (residues 79–85). Histone proteins are shown as ribbons (H3, dark gray; H4, light gray); DNA is shown in stick configuration in background. Positions of H3 silencing mutations as well as the N terminus and L2 loop of H4 are indicated (asterisks represent silencing-specific mutations). The α1 helix is oriented with the C-terminal end (at right) angled away from the nucleosome surface. (B) View of amino acid side chains in the H3 α1-L1 domain (residues 68–84 shown, in light gray) and neighboring H4 N terminus (18–26) and L2 loop (77–79, in dark gray). Key surface-exposed side chains are indicated in ball-and-stick configuration; other amino acids are shown in spacefill format. H3 E_{73} is ~3–4 Å from H4 residues N_{25} and I_{26}; H3 K_{79} is ~3 Å from H4 K_{79}. Images were generated using Protein Explorer, from structural coordinates (White *et al.* 2001) provided by the Protein Data Bank (http://www.rcsb.org/pdb; accession no. 1ID3). Distances and putative interactions were derived with CSU software (Sobolev *et al.* 1999).

that plays an essential role in telomeric and *HM* silencing in yeast. Mutation $E_{73}D$ in particular represents the strongest effect on *HM* silencing for any known H3 mutation. All of the mutated sites identified represent very highly conserved residues in H3 (Wells and Mcbride 1989; Wells and Brown 1991). Of the 14 single-amino-acid mutations identified, 10 substitutions map to the α1 helix and the adjacent L1 loop within the histone H3 fold motif (Figure 1A). We propose that the α1-L1 structure represents a distinct functional domain that plays a key role in silencing heterochromatin.

Many of the mutations identified (R_2G, T_6K, and $A_{29}V$ in the N terminus; $Q_{68}R$, $L_{70}S$, $V_{71}A$, $R_{72}G$, and $E_{73}D$ in the N-terminal end of the α1 helix; $F_{84}L$ in the L1 loop; and $A_{114}T$ in the α2 helix) exhibit a broad range of effects on telomeric silencing, cause minimal effects on silencing at *HML* (with the noteworthy exception of $E_{73}D$), and tend to have effects on basal repression and growth at elevated temperatures. These observations suggest that these particular mutations affect generalized aspects of chromatin structure. These mutations may influence gene expression as a result of their effects on nucleosome structure and integrity via altered histone-DNA or histone-histone interactions. The N-terminal end of the α1 helix (residues 65–69) serves as a DNA docking site, while the L1 loop (including F_{84}) forms a β-strand interaction with the H4 L2 loop (Luger *et al.* 1997). Mutations within these elements may destabilize the nucleosome, as has been previously suggested for other histone mutations (Prelich and Winston 1993; Kruger *et al.* 1995; Santisteban *et al.* 1997), resulting in pleiotropic effects on gene expression. While these structural alterations may directly contribute to disruption of telomeric heterochromatin, it is also possible that the effects on telomeric silencing by these mutations are indirect, perhaps by altering the expression level of Sir proteins or other silencing factors.

In contrast, the mutations spanning amino acids 76–80 within the α1-L1 domain appear to affect silencing in a specific manner. These mutations have very pronounced effects on telomeric and *HML* silencing, but have no obvious effect on basal repression and do not exhibit any apparent growth defects. While we cannot formally rule out indirect effects on silencing by these mutations as suggested above, given these observations, we believe that the C-terminal portion of α1 and the L1 loop play a distinct role in silencing. The side chains of residues 76, 79, and 80 are all exposed on the nucleosome surface (Figure 1B), suggesting that they are involved in silencing protein interactions.

Since the initial submission of this manuscript, the role of the H3 α1-L1 silencing domain described here has been substantiated by experiments demonstrating that K_{79} is methylated by Dot1p in yeast (Ng *et al.* 2002; Van Leeuwen *et al.* 2002), a modification that is conserved in eukaryotes (Feng *et al.* 2002), and is required for silencing in yeast. Although two differing models have been proposed for the role of K_{79} methylation in silencing, they share the common perspective that K_{79} is a potential binding site for Sir proteins in silent chromatin. Our observation that the $K_{79}E$ mating defect is

restored by the *sir3* suppressor mutations is certainly consistent with this view, although we cannot rule out the possibility that suppression occurs via an indirect mechanism that bypasses the requirement of histone silencing domains. The mutations neighboring K_{79} may reflect additional residues involved in Sir protein interactions or may reflect residues required for recognition by the Dot1p methylase. Given the previously established interactions between the H3 and H4 N termini and Sir3 and Sir4, along with the close proximity between the H4 N terminus and the H3 α1-L1 domain (Figure 1B), it is reasonable to speculate that the Sir proteins interact with a noncontiguous histone-binding site encompassing the H3 and H4 N termini and the H3 α1-L1 domain. It is worth noting that K_{79} is part of a cluster of three closely positioned lysine residues on the nucleosome surface (including K_{77} and K_{79} in the L2 loop of histone H4; Figure 1B), suggesting that multiple core domain modifications may play a role in modulating these interactions.

Our results also suggest that the α1-L1 domain possesses an additional silencing function that has yet to be elucidated. This is supported by the $E_{73}D$ mutation, which has a much stronger effect on silencing at *HML* than do any of the other mutations in this domain, indicating that E_{73} must play another role in silencing in addition to any possible role it might play in K_{79} methylation. E_{73} is oriented with its side chain in close proximity to the H4 N terminus, potentially capable of forming hydrogen bonds with residues N_{25} and I_{26} (Figure 1B). The $E_{73}D$ substitution may shorten the side chain enough (~1.5 Å) to disrupt necessary interactions between the H4 N terminus and the H3 α1 helix. This putative interaction may play an additional role in the proposed noncontiguous Sir-binding site described above, or it may function as a recognition site for enzymes that modify histone H4 N-terminal residues. It will be of interest to see if the modification state of the histone H4 N terminus is affected by any of these substitution mutations.

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