Cell, Vol. 80, 583–592, February 24, 1995, Copyright © 1995 by Cell Press

Histone H3 and H4 N-Termini Interact with SIR3 and SIR4 Proteins: A Molecular Model for the Formation of Heterochromatin in Yeast

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Summary

The silent mating loci and chromosomal regions adjacent to telomeres of S. cerevisiae have features similar to heterochromatin of more complex eukaryotes. Transcriptional repression at these sites depends on the silent information regulators SIR3 and SIR4 as well as histones H3 and H4. We show here that the SIR3 and SIR4 proteins interact with specific silencing domains of the H3 and H4 N-termini in vitro. Certain mutations in these factors, which affect their silencing functions in vivo, also disrupt their interactions in vitro. Immunofluorescence studies with antibodies against RAP1 and SIR3 demonstrate that the H3 and H4 N-termini are required for the association of SIR3 with telomeric chromatin and the perinuclear positioning of yeast telomeres. Based on these interactions, we propose a model for heterochromatin-mediated transcriptional silencing in yeast, which may serve as a paradigm for other eukaryotic organisms as well.

Introduction

Heterochromatin, often found adjacent to telomeres or centromeres, remains condensed in interphase, is transcriptionally repressed, replicates late in S phase, and in many instances, localizes to the nuclear periphery. Heterochromatic repression may be exploited as a regulatory tool, for example, to assure repression of the silent mating loci in yeast (Laurenson and Rine, 1992) or to compensate for dosage effects of X-linked genes in mammals (Eissenberg, 1989). Probably the most intensely studied aspect of heterochromatin is the epigenetic control of gene expression observed when chromosomal rearrangements transfer a gene from euchromatin to the vicinity of heterochromatin (Eissenberg, 1989). Competition between the formation of a repressive heterochromatic structure and maintenance of the euchromatic state may result in unstable expression, visible as a variegated phenotype of genetically identical cells. Based on the observation that modifier genes, which enhance or suppress position effect variegation (PEV) in Drosophila melanogaster, exhibit dosage effects, it was hypothesized that the formation of heterochromatin relies on the cooperative assembly of multisubunit complexes involving protein–protein and protein–DNA interactions (Tartof and Bremer, 1990). This process is thought to be initiated at specific nucleation sites and to spread along the chromosome until a termination signal is encountered or one of the subunits becomes limiting. However, the molecular nature of heterochromatin is largely unknown.

Although the chromosomes of Saccharomyces cerevisiae are too small to allow cytological detection of heterochromatin, the silent mating loci *HMLα* and *HMRa* (*HM* loci) and chromosomal regions adjacent to telomeres have features highly similar to those of heterochromatin (Thompson et al., 1993). They are likely to have a condensed chromatin structure, replicate late in S phase, are found near the nuclear envelope, and repress gene activity in an epigenetic manner. Telomeric position effects (TPE) originate from the telomere itself and spread continuously for 3–5 kb toward the center of the chromosome (Renauld et al., 1993).

HM silencing, as well as telomeric repression, require the function of *cis*-acting DNA elements, either the E and I silencer elements flanking *HML* and *HMR* (Laurenson and Rine, 1992) or the telomeric $C_{1-3}A$ repeats (Gottschling et al., 1990). Each of these elements appears to be directly or indirectly recognized by *trans*-acting factors needed for the establishment or maintenance of silencing. Among these, RAP1, SIR2, SIR3, SIR4, and histones H3 and H4 belong to a set of proteins that are required for silencing both at the *HM* loci and near telomeres and that may have a structural role (Aparicio et al., 1991; Kurtz and Shore, 1991; Kyrion et al., 1993; Thompson et al., 1993, 1994). Other factors involved in silencing, such as NAT1/ARD1, SIR1, RIF1, ABF1, or the origin recognition complex (ORC), differ in one or both of these regards (Laurenson and Rine, 1992; Rivier and Pillus, 1994). RAP1 recognizes the $C_{1-3}A$ repeats of yeast telomeres and DNA elements at the *HM* silencers (Shore and Nasmyth, 1987; Buchman et al., 1988). RAP1 can also interact with SIR3 and SIR4 (Moretti et al., 1994). While little is known about SIR2 function in silencing, there is evidence for interactions between SIR3 and SIR4 derived from genetic and two-hybrid experiments (Marshall et al., 1987; Moretti et al., 1994). Extra copies of the *SIR3* gene also promote the spreading of TPE, allowing repression of genes at increasing distances from the telomere (Renauld et al., 1993). These data suggest that SIR3 and SIR4 may function as parts of a structural unit.

While silencing appears to be initiated at *cis*-acting DNA elements, the condensed and repressed domains are found in adjacent chromatin, indicating that normal chromatin components contribute to the formation of heterochromatin. A direct link between silencing and chromatin structure has been established by the finding that mutations in the histone H3 and H4 N-termini can lead to derepression of *HMLα*, *HMRa*, and *URA3* at the telomere. Specific histone domains required for silencing are contained within amino acids 4–20 of H3 and amino acids 16–29 of

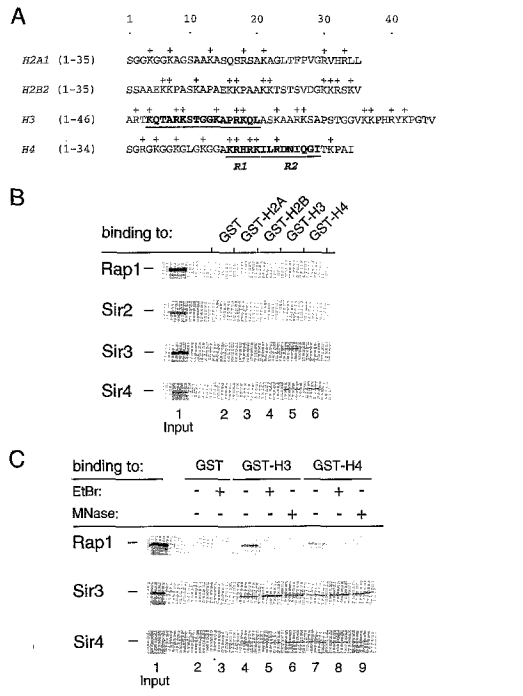

Figure 1. Direct Interactions between SIR3 and SIR4 and the H3 and H4 N-Termini

(A) Amino acid sequence of the histone H2A, H2B, H3, and H4 N-termini as present in the GST fusion proteins. Positively charged K and R residues are marked (plus). The silencing domains in H3 and H4 are underlined, and subdomains R1 and R2 in H4 are denoted.

(B) [^{35}S]methionine-labeled RAP1, SIR2, SIR3, and SIR4 were incubated with GST (lane 2) or GST fusion proteins containing the N-termini of histone H2A (lane 3), H2B (lane 4), H3 (lane 5), or H4 (lane 6) immobilized on glutathione (GSH)–Sepharose beads. After extensive washing, interacting proteins were eluted from the beads and analyzed along with a fraction of the input material (lane 1) by SDS–PAGE and autoradiography.

(C) Binding of RAP1, SIR3, and SIR4 to GST (lanes 2 and 3), GST–H3 (lanes 4, 5, and 6), or GST–H4 (lanes 7, 8, and 9) was analyzed in the presence or absence of EtBr or MNase as indicated. To demonstrate DNA-dependent RAP1 interactions, the NaCl concentration was lowered to 100 mM and no glutamate was present in binding and wash buffers. Lane 1, a fraction of the input material (see Experimental Procedures).

H4 (Kayne et al., 1988; Johnson et al., 1990, 1992; Megee et al., 1990; Park and Szostak, 1990; Aparicio et al., 1991; Mann and Grunstein, 1992; Thompson et al., 1994). Mutations in the H4 silencing domain can be suppressed by single amino acid substitutions in SIR3, suggesting direct or indirect interactions between these proteins (Johnson et al., 1990, 1992). Interestingly, N-terminal deletions in histones H2A and H2B are not known to affect TPE or *HM* repression (Kayne et al., 1988; Thompson et al., 1994), arguing that the involvement of histones H3 and H4 in these processes is specific.

RAP1, SIR3, and SIR4 are also involved in the subnuclear positioning of telomeres. RAP1 is a component of the telosome particle (Wright et al., 1992), and immunofluorescence studies with antibodies against RAP1 and SIR3 were used to detect telomeres clustered together in multiple foci at the periphery of yeast nuclei (Klein et al., 1992; Palladino et al., 1993). This was confirmed by in situ hybridization with telomere-specific DNA probes (H. Scherthan, T. L., and S. M. G., unpublished data). Telomeres were no longer found clustered and located near the nuclear envelope in either *sir3*$^-$ or *sir4*$^-$ strains (Palladino et al., 1993). These data suggest a structural role for RAP1, SIR3, and SIR4 at the telomeres.

The finding that mutations of the histone H3 and H4 N-termini can abolish heterochromatin-like gene repression in yeast implies that histones participate in the formation of a repressive chromatin structure. To understand the molecular nature of the silencing complex, we have investigated possible interactions between histones and other silencing factors. Here, we provide evidence that SIR3 and SIR4 directly interact with the genetically identified silencing domains of the histone H3 and H4 N-termini. We propose that the interactions between histone N-termini and SIR proteins are fundamental for the establishment of heterochromatin-like structures at the nuclear periphery in yeast.

Results

Direct Interaction between SIR3 and SIR4 and the Histone H3 and H4 N-Termini In Vitro

Mutations within the N-terminal silencing domains of histones H3 and H4 (Figure 1A) cause derepression of genes adjacent to telomeres and at the *HM* loci, possibly by preventing interactions of H3 and H4 with other factors involved in transcriptional silencing. To test this hypothesis, the histone N-termini, fused to glutathione S-transferase (GST), were immobilized on glutathione (GSH)–Sepharose beads and used as potential ligands for [^{35}S]methionine-labeled RAP1, SIR2, SIR3, or SIR4, which were transcribed and translated in vitro. Proteins interacting with the histone N-termini were eluted from the GSH beads, resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and visualized by autoradiography. Both SIR3 and SIR4 were found to interact similarly with the GST–H3 and GST–H4 fusions (Figure 1B, lanes 5 and 6). No interaction between the SIR proteins and GST–H2A or GST–H2B (Figure 1B, lanes 3 and 4) was detected. In contrast with SIR3 and SIR4, RAP1 and SIR2 did not bind to, or interacted only very weakly, with the GST–histone fusions (Figure 1B, lanes 3–6).

Although the histone N-termini are highly charged and are known to interact with DNA, it seems unlikely that we observe nonspecific, charge-dependent or indirect, DNA-mediated interactions. First, all four histone N-termini contain a large number of positively charged residues (Figure 1A), yet SIR3 and SIR4 do not interact with the H2A and H2B N-termini. Second, the binding reactions in Figure 1B included ethidium bromide (EtBr) to prevent protein–DNA interactions (Lai and Herr, 1992). Nonetheless, the DNA independence of the SIR protein–histone interactions was confirmed in binding experiments with or without EtBr or micrococcal nuclease (MNase) (Figure 1C). For comparison, RAP1 was included in this set of experiments. Omission of EtBr and lowering the ionic strength allows RAP1 to interact with the H3 and H4 N-termini (Figure 1C, lanes 4 and 7). However, this interaction was sensitive to

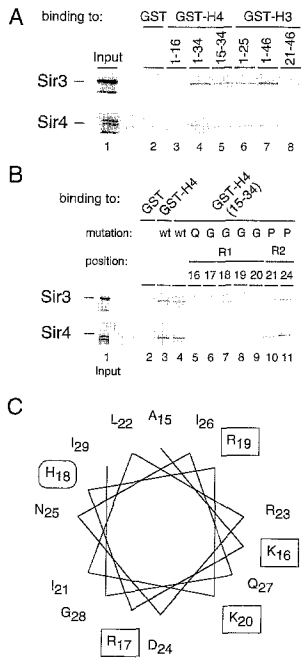

Figure 2. The Interactions between the H3 and H4 N-Termini and SIR3 and SIR4 Are Mediated by the Genetically Identified Histone Silencing Domains

(A) Deletion mutants of the H3 or H4 fusion proteins were bound to GSH beads and incubated with labeled SIR3 or SIR4. Proteins bound by the GST–histone fusions were analyzed together with a fraction of the input material as described in Figure 1. Amino acids present in the respective fusions are indicated. In H4, amino acids 16–29, and in H3, amino acids 4–20, correspond to the silencing domains. To generate the H4 15–34 construct, amino acids 4–14 were eliminated from the full-length H4 N-terminus fusing amino acids 1–3 to amino acids 15–34. This construct is also referred to as GST–H4 Δ4–14 in Figure 3.

(B) GST–H4 fusions with single amino acid substitutions in silencing subdomains R1 or R2 were interacted with SIR3 or SIR4 as described in Figure 1.

(C) Helical wheel depiction of the putative α helix (Johnson et al., 1992) of the H4 silencing domain. R1 residues 16, 17, 19, and 20, required for SIR3 and SIR4 binding, are boxed, as is H18.

EtBr and MNase (Figure 1C, lanes 5, 6, 8, and 9) consistent with an indirect, DNA-mediated association. In contrast, neither EtBr nor MNase treatment disrupted the SIR3– or SIR4–histone interactions (Figure 1C, lanes 5, 6, 8, and 9). Rather, removal of DNA interactions appeared to facilitate the SIR protein–histone binding. Therefore, both SIR3 and SIR4 interact directly and specifically with the H3 and H4 N-termini in vitro.

Genetically Identified Silencing Domains at the H3 and H4 N-Termini Mediate the Interaction with the SIR Proteins

By mutational analyses, amino acids 4–20 in H3 and 16–29 in H4 were shown to be required for silencing in vivo. The silencing domain in H4 can be further subdivided into a highly basic subdomain R1 (residues 16–20), whose function can be disrupted by various single site substitutions, and a relatively uncharged subdomain R2 (residues 21–29), affected mostly by proline exchanges (Johnson et al., 1992). To compare the sequence requirements for the silencing function of histones in vivo and SIR protein binding in vitro, we used deletion mutants of the GST–histone fusions (Figure 2A). In agreement with the genetic data, constructs retaining the silencing domains (GST–H4, 15–34; GST–H3, 1–25) (Figure 2A, lanes 5 and 6) were found to interact with SIR3 and SIR4 with little or no difference in binding relative to the full-length N-termini (GST–H4, 1–34; GST–H3, 1–46) (Figure 2A, lanes 4 and 7). In contrast, despite their basic nature (Figure 1A), sequences outside the silencing domains (GST–H4, 1–16; GST–H3, 21–46) did not interact, or interacted very weakly, with the SIR proteins (Figure 2A, lanes 3 and 8). Thus, the interactions between the histones and the SIR proteins are mediated by H3 and H4 N-terminal regions containing the genetically identified silencing domains.

Binding of the SIR proteins to the H4 N-terminus was further characterized using single amino acid substitutions in silencing subdomains R1 and R2. All of the mutations used disrupt *HM* silencing or telomeric repression, including the glycine replacement of lysine at position 20 (A. H., J. Thompson, and M. G., unpublished data), which therefore is now included in R1. Replacing basic residues at positions 16, 17, 19, and 20 in R1 with neutral amino acids (glutamine or glycine) abolished binding of both SIR3 and SIR4 (Figure 2B, lanes 5, 6, 8, and 9). Changing position 18 from histidine to glycine (H18G) had a smaller effect (Figure 2B, lane 7). Substituting prolines for isoleucine at position 21 or aspartic acid at position 24 in subdomain R2 also had less or no effect on the interaction with the SIR proteins (Figure 2B, lanes 10 and 11), implying that the importance of H18 and subdomain R2 for silencing may be based on mechanisms other than an interaction with SIR3 or SIR4.

A rationale for understanding the effects of the mutations in R1 on SIR protein binding is provided by a helical wheel projection of the H4 silencing domain (Figure 2C). Residues 16, 17, 19, and 20 are all found on one side of the hypothetical amphipathic α helix (Johnson et al., 1992), whereas H18 is found on the opposite face. Thus, residues 16, 17, 19, and 20 may present a surface for the interaction with SIR3 and SIR4, while H18 may contact nucleosomal DNA, to which it can be cross-linked in vivo (Ebralidse et al., 1988).

Intramolecular Suppression of the K16Q Mutation by Residues 4–14 of the H4 N-Terminus

In vivo, replacing K16 of the H4 N-terminus abolishes silencing. In vitro, however, we found that substituting K16 with glutamine (K16Q), when present as the sole mutation in the H4 N-terminus, had no effect on the interaction with SIR3 (Figure 3A, lane 4). Binding of SIR3 was destroyed only when, in addition to K16Q, amino acids 4–14 were deleted (Figure 3A, lane 8), or when K5, K8, and K12 or K5 and K12 were also mutated to Q (Figure 3A, lanes 6 and 9). When K12 and K16 alone were replaced with Q, we observed only a partial loss of SIR3 binding (Figure 3A, lane 10). Δ4–14 or G substitutions at K5, K8, and K12 by themselves had no visible effect on SIR3 binding (Figure 3A, lanes 5 and 7). These results suggest that one or more of the lysines at K5, K8, and K12, while not normally re-

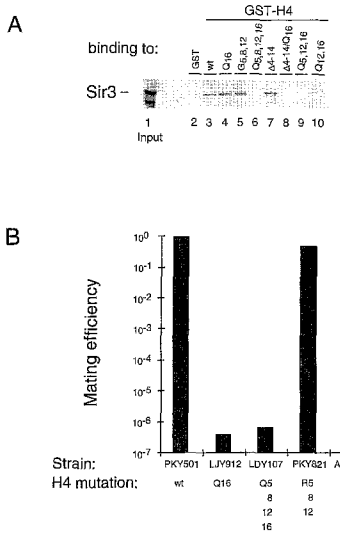

Figure 3. Intramolecular Suppression of the K16Q Mutation in H4 by Amino Acids within Region 4–14

(A) Binding of SIR3 to wild-type or mutant GST–H4 fusion proteins was analyzed as before (Figure 1). Glutamine (Q) substitutions at positions K5, K12, and K16 (lanes 9 and 10) of the H4 N-terminus were used to mimic the combinatorial effect of mono- or diacetylation of K12 or K5 and K12 together with the K16Q mutation.

(B) Mating efficiencies of yeast strains with wild-type or mutant histone H4. Shown are the ratios of diploid cells formed per total number of cells in the reaction. The average values of at least three independent experiments are shown.

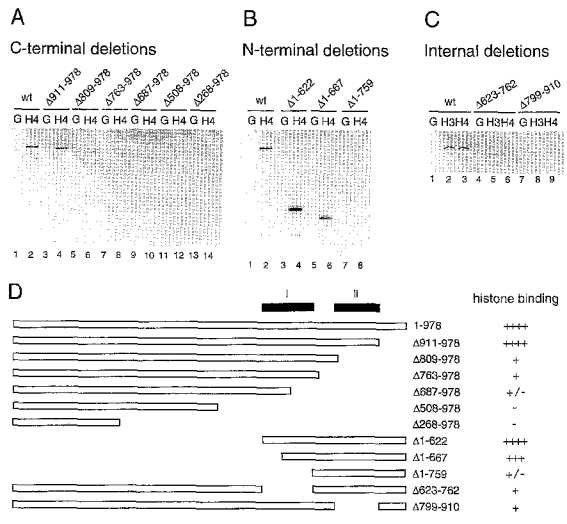

Figure 4. Histone Interaction Domains Are Found at the C-Terminus of SIR3

(A–C) Binding of [^{35}S]methionine-labeled C-terminal, N-terminal, or internal deletion mutants of SIR3 (schematically shown in [D]) to GST (G), GST–H4 (H4), or GST–H3 (H3) fusion proteins was analyzed as described (Figure 1). The amount of input material of the different deletion mutants was adjusted to give similar intensities of the bands corresponding to the full-length translation products (data not shown). Owing to fewer methionines in the deletion mutants, this results in an up to 4-fold increase in the amount of protein used in the binding assay. Therefore, loss of histone binding of a particular deletion mutant is even more significant.

(D) Schematic presentation of the SIR3 deletion mutants (open bars) and their histone binding properties. Regions involved in histone binding are indicated (closed bars).

quired for binding (Figures 2A; Figure 3A, lanes 5 and 7), can complement the defect imposed by the K16Q mutation. This suppression appears not to take place in vivo, possibly because the redundancy in the H4 N-terminus is masked by a histone modification not present in the bacterially produced GST–H4 fusion proteins. This could be either heterochromatin-specific acetylation of K12 (Turner et al., 1992; J. Broach, unpublished data) or diacetylation of H4 during deposition on replicating chromatin (Chicoine et al., 1987). Since DNA replication and ORC components are required for the establishment of silencing (Miller and Nasmyth, 1984; Rivier and Pillus, 1994), our results could indicate an involvement of diacetylated H4 in the histone–SIR protein interaction and may provide insight into the process of heterochromatin assembly and maturation.

The data above suggest that K5, K8, and K12 in their charged state would suppress the mating defect caused by the K16Q mutation in vivo. In fact, a yeast strain with arginines at positions 5, 8, and 12 (preserving the positive charge of unacetylated lysines) combined with K16Q (AHY104, R5, R8, R12, Q16) mated at a 50- to 100-fold higher frequency than LJY912 (H4, Q16) (Figure 3B). As expected, this suppression occurs with R, but not with Q substitutions (Figure 3B), which mimic the hyperacetylated, neutralized state of H4. The relatively low level of suppression may not be surprising, as multiple R substitutions themselves can interfere with silencing (Park and Szostak, 1990; Thompson et al., 1994). Thus, our binding experiments and the suppression analyses both demonstrate that charged residues at positions 5, 8, and 12 of histone H4 can compensate, at least in part, for the effect of the K16Q mutation.

Domains in SIR3 Required for Histone Interaction In Vitro Are Also Required for Its Function In Vivo

To delineate the domains of SIR3 involved in the histone interaction, we made deletion mutants of SIR3 and analyzed their histone binding (Figure 4). Deletion of amino acids 911–978 from the C-terminus of SIR3 had little, if any, effect on binding to GST–H4 (Figure 4A, lane 4), while removal of amino acids up to residue 808 caused a strong decrease in the interaction (Figure 4A, lane 6). The residual histone binding was not changed upon further deletion up to amino acid 762 (Figure 4A, lane 8). It was then diminished in the mutant lacking amino acids 687–978 (Figure 4A, lane 10) and lost when all sequences C-terminal of amino acid 507 were removed (Figure 4A, lanes 12 and 14). Therefore, histone-interacting sequences lie between amino acids 808–910 and in a region N-terminal of amino acid 762.

Consistent with this, an N-terminal deletion mutant of SIR3 containing only amino acids 623–978 interacted strongly with GST–H4 (Figure 4B, lane 4). Histone binding was slightly reduced by further removal of amino acids 623–667 (Figure 4B, lane 6). The C-terminal portion of SIR3 consisting of amino acids 760–978 interacted only

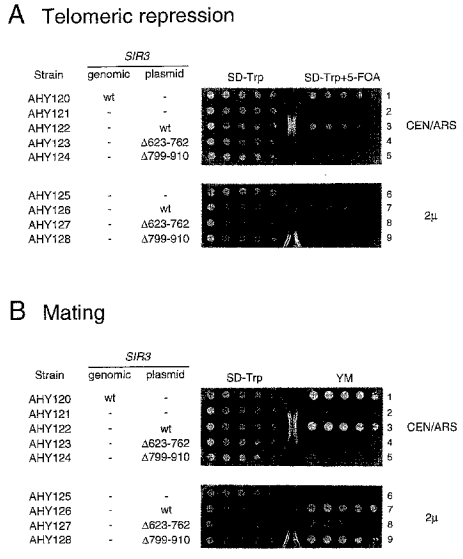

Figure 5. Histone Interaction Domains Are Required for SIR3 Silencing Functions

Yeast strains AHY121–AHY128 are deleted for *SIR3* (AHY 121, AHY125; lines 2 and 6), or harbor wild-type *SIR3* (AHY122, AHY126; lines 3 and 7) or mutant *SIR3* genes lacking one of the histone interaction domains (AHY123, AHY124, AHY127, AHY128; lines 4, 5, 8 and 9), on either *CEN/ARS*- or *2μ*-based yeast shuttle vectors. AHY120 is a control strain with wild-type genomic *SIR3*. All strains have *URA3* integrated at the telomere of the left arm of chromosome VII (Aparicio et al., 1991; Thompson et al., 1994).

(A) To analyze telomeric repression, serial 10 × dilutions of each strain, pregrown in the absence of tryptophane (Trp) to maintain the plasmids, were placed onto media with or without 5-FOA and Trp (SD-Trp, SD-Trp plus 5-FOA).

(B) To test for repression of *HMLα* (restoring mating competency), equal numbers of AHY120–AHY128 (pregrown in the absence of Trp) and an MATα tester strain were combined, after which serial 10-fold dilutions of each mating reaction were placed onto synthetic minimal media (YM) selective for diploid cells. Corresponding dilutions from mock mating reactions performed with the same number of AHY cells but in the absence of the MATα tester strain were spotted on SD-Trp to control for cell viability and the presence of the shuttle plasmids. Absence of functional SIR3 leads to a loss of mating and causes sensitivity to 5-FOA due to derepression of the *HM* loci and telomeric *URA3*.

very weakly with GST–H4 (Figure 4B, lane 8), despite the presence of amino acids 808–910 (see above). Therefore, the interaction of wild-type SIR3 with the H4 N-terminus appears to require two domains, one from amino acids 623–762, the other from amino acids 808–910, neither of which binds strongly to GST–H4 when present alone.

SIR3 from which either amino acids 623–762 or amino acids 799–910 had been removed, each showed a partial decrease in H4 binding (Figure 4C; compare lanes 3, 6, and 9). The same two internal deletions also weakened the interaction of SIR3 with H3, to an extent similar to the H4 interaction (Figure 4C; compare lanes 2, 5, and 8), suggesting that the same domains in SIR3 are required for binding of the H3 and the H4 N-termini. Whether precisely the same SIR3 residues within these two domains are involved in both the H3 and the H4 interactions remains to be determined.

To test the importance of the histone interaction regions for SIR3 function in vivo, we asked whether mutant *SIR3* genes, lacking amino acids 623–762 and amino acids 799–910, could restore mating and telomeric repression of a *sir3*⁻ strain. Wild-type and mutant *SIR3* genes on *CEN/ARS*- or *2μ*-based yeast shuttle vectors were introduced into a yeast strain carrying *URA3* at the telomere of the left arm of chromosome VII (Aparicio et al., 1991; Thompson et al., 1994). The disruption of the endogenous *SIR3* gene in the parental strain causes the derepression of *HMLα* and telomeric *URA3*, giving rise to a nonmating phenotype and sensitivity to 5-fluoro-orotic acid (5-FOA) since the *URA3* gene product converts 5-FOA into a cytotoxic substance. Wild-type *SIR3*, present on either a low copy number, *CEN/ARS* plasmid, or a multicopy, *2μ* vector, restored both telomeric repression and mating (Figures 5A and 5B; compare lines 2 and 6 with lines 3 and 7) to levels comparable to those of a control strain with intact endogenous *SIR3* (Figures 5A and 5B, lines 1). In contrast, neither of the *SIR3* deletion mutants was able to complement the *SIR3* disruption when carried on *CEN/ARS* plasmids (Figures 5A and 5B, lines 4 and 5). Even the increased dosage of the mutant *SIR3* genes on *2μ* plasmids did not reinstate telomeric repression (Figure 5A, lines 8 and 9), and the mating defects of the SIR3 mutants were only partially overcome, restoring mating (and therefore repression of *HMLα*) to a level of approximately 100-fold and 10-fold, respectively, below wild type (Figure 5B, lines 8 and 9). Therefore, domains required for histone interaction in vitro are also required for SIR3 function in vivo.

Histone H3 and H4 N-Termini Are Required for the Subnuclear Localization of SIR3 and the Perinuclear Positioning of Telomeres

Immunofluorescence of yeast spheroplasts with antibodies against RAP1 and SIR3 reveals 8–12 intensely staining foci per nucleus, which correspond to clusters of telomeres. Superimposition of the immunostaining pattern and nuclear DNA staining suggests that the telomeric foci are localized to the nuclear periphery in most cells (Klein et al., 1992; Palladino et al., 1993) (Figures 6A–6E, wild-type strains). Clustering and perinuclear localization of telomeres depends on both SIR3 and SIR4 (Palladino et al., 1993). To provide further evidence for the in vivo occurrence of the SIR protein–histone interaction, we used immunofluorescence studies to compare the subnuclear localization of RAP1 and SIR3 in isogenic strains with either wild-type or mutant N-termini of histones H3 and H4.

In the H3 (Δ4–30) or the H4 (Δ4–28) N-terminal deletion strains, the RAP1 staining pattern is less discrete and delocalized relative to wild-type cells (Figures 6C and 6D). The alterations in RAP1 positioning are more evident in the H4 mutant than in the H3 mutant (Figures 6C and 6D). Even more pronounced than the apparent delocalization of telomeres is the change in subnuclear localization of SIR3. In the absence of the H3 or H4 N-termini, we observe diffuse SIR3 staining throughout the nucleus, in contrast with the punctate and perinuclear signal in the wild-type strains (Figures 6C and 6D). Although some foci can still be observed, this suggests that the majority of SIR3 is no longer associated with telomeric chromatin. Deletion of

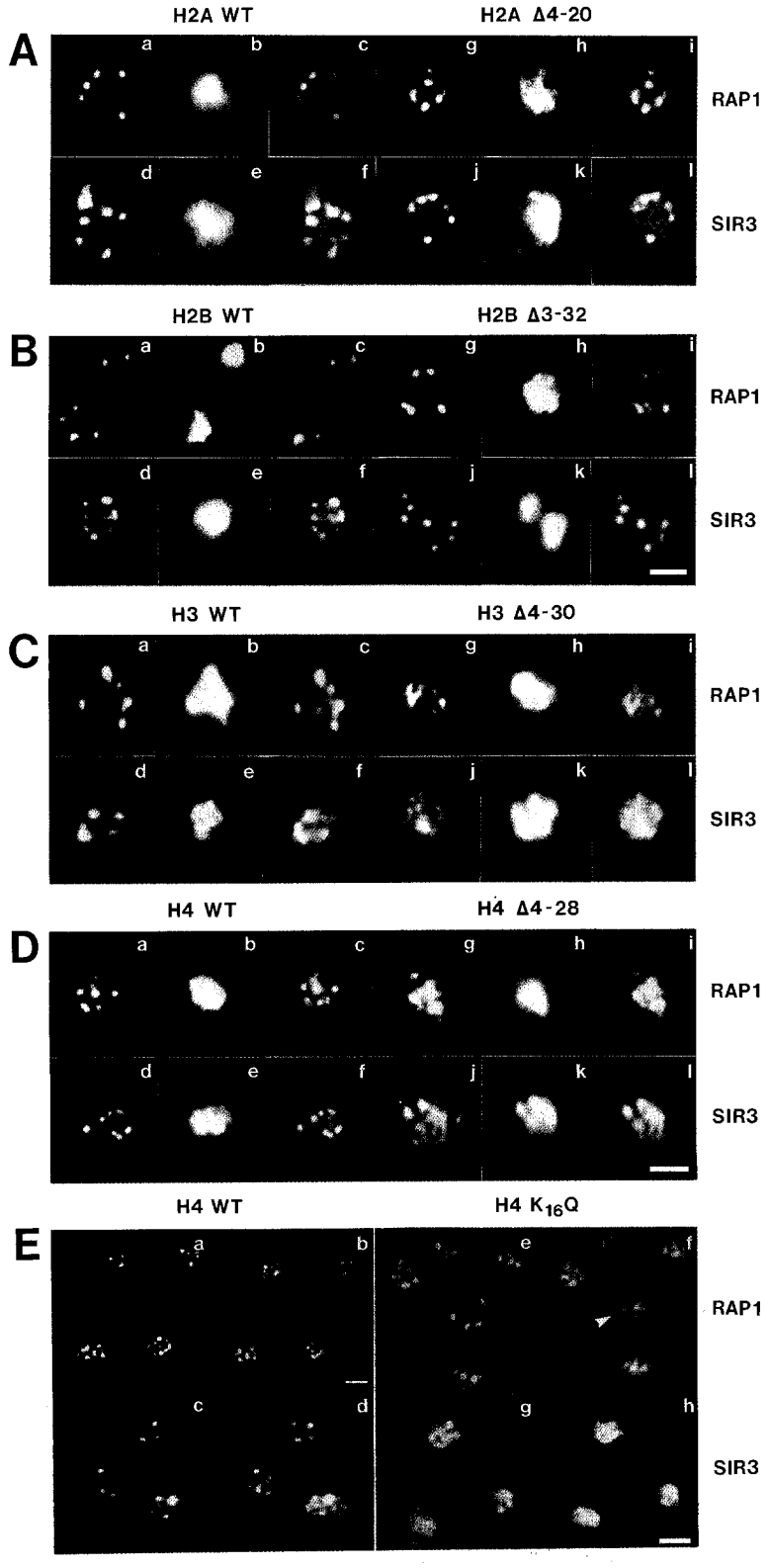

Figure 6. Histone H3 and H4 N-Termini Are Required to Maintain the Discrete Perinuclear Staining Patterns of RAP1 and SIR3

(A) Confocal immunofluorescence microscopy using affinity-purified antibodies against RAP1 (a–c and g–i) or SIR3 (d–f and j–l) and FITC-conjugated secondary antibodies was performed on the diploid strains STY5 (H2A wild type) and STY6 (H2A Δ4–20) carrying wild-type or mutant histone genes as indicated. Immunostaining (a, d, g, and j) and genomic DNA staining patterns (b, e, h, and k) of individual nuclei are shown in black and white. (c), (f), (i), and (l) show the merger of the two (RAP1 and SIR3 signals in green; DNA signals in red). Where the two patterns overlap, the image is yellow. Both anti-RAP1 and anti-SIR3 give a low level general staining of the nucleus in addition to the bright foci shown. Confocal filtering (identical in all images shown) diminishes the low level background staining in the presence of the brighter foci of immunofluorescence.

(B) as in (A), using the haploid strains JTY505 (H2B wild type) and JTY506 (H2B Δ3–32). The bar indicates 1.3 μm. The diameter of nuclei varies depending on the focal plane visualized.

(C) As in (A), using the diploid strains STY8 (H3 wild type) and STY7 (H3 Δ4–30) The punctate staining patterns of RAP1 and SIR3 are less evident in the mutant strain.

(D) As in (A), but with the diploid strains STY9 (H4 wild type) and STY10 (H4 Δ4–28). The bar indicates 1.3 μm.

(E) Diploid strains STY3 (H4 wild type) and STY18 (H4 K16Q) were reacted with anti-RAP1 (a and e) or with anti-SIR3 antibodies (c and g) as in (A). Superimposition of genomic DNA staining patterns (in red) and immunostaining patterns (in green) is shown in panels (b), (d), (f), and (h). Yellow color marks areas in which the DNA and FITC signals coincide. The arrow in (f) indicates one nucleus in which the RAP1 foci are still largely peripheral, although the majority of nuclei show a more random staining pattern. Scale bars indicate 1.5 μm.

the H2A (Δ4–20) or H2B (Δ3–32) N-termini, which were not bound by SIR3 in vitro and which are not involved in silencing did not disturb the subnuclear localization of SIR3 and RAP1 (Figures 6A and 6B).

K16 of the H4 N-terminus plays a critical role in silencing in vivo and in the interaction between the H4 silencing domain and SIR3 in vitro. To assess the importance of this residue for the interaction with SIR3 in vivo, we examined the SIR3 and RAP1 staining patterns in a strain carrying the H4 K16Q allele. In this strain, the anti-SIR3 stain-

ing is diffuse and delocalized relative to the isogenic wild-type strain (Figure 6E, compare [c] and [d] with [g] and [h]). Moreover, the RAP1 foci are less condensed and more randomly distributed in nuclei from the H4 mutant cells, even though the RAP1 staining pattern is still largely punctate, and in few cases perinuclear (Figure 6E, arrow in [f]). This residual aggregation of the telomeres may result from SIR3 interacting with RAP1 (Moretti et al., 1994) rather than with histones (see Discussion). Nonetheless, like the N-terminal deletions of H3 and H4, the single site substitution in H4 prevents the association of SIR3 with telomeric chromatin and consequently affects aggregation and perinuclear localization of the telomeres. Thus, the H3 and H4 N-termini are not only involved in telomeric repression, but are also required for the spatial organization of chromosomes in the nucleus.

Discussion

Histone Function in Silencing

The involvement of histones H3 and H4 in repression of heterochromatin-like regions in yeast seems paradoxical. Histones are found throughout the yeast genome, yet mutations in the silencing domain of H4, which completely derepress *HMLα* or telomeric *URA3*, have no obvious effect on the regulation of a number of euchromatic genes (Kayne et al., 1988; Durrin et al., 1991). One possible explanation for the specific effects of histone mutations on silencing is that distinct domains within the H3 and H4 N-termini directly interact with other silencing factors and that these interactions are determined by the chromosomal context in which they occur. We have shown here that the H3 and H4 N-termini directly interact with the SIR3 and SIR4 proteins in vitro. The specificity and the physiological relevance of these interactions are demonstrated by the following observations. The interactions between the histones and the SIR proteins require the genetically identified N-terminal silencing domains of H3 and H4. Other N-terminal basic regions in these proteins or in the similarly charged H2A and H2B histones were not bound, arguing against nonspecific, electrostatic interactions between SIR3 and SIR4 and the H3 and H4 N-termini. The SIR proteins were found to interact with the basic subdomain R1 of the H4 N-terminus, which may form an amphipathic α helix. Certain single amino acid exchanges in R1, which abolish silencing in vivo, disrupt the interaction with the SIR proteins in vitro. Conversely, deletions in SIR3, which weaken the histone binding in vitro, prevent telomeric and silent mating type repression in vivo. Also, deletions of the H3 and H4 N-termini and even a single site exchange in the H4 silencing domain cause a delocalization of SIR3 and the disruption of the perinuclear positioning of yeast telomeres. Therefore, the results of our in vitro binding studies correlate with the results of functional analyses and suggest that the histone H3 and H4 N-termini serve as chromosomal anchoring sites for SIR3 and SIR4.

Direct or indirect interactions between histones and SIR proteins had already been suggested by the identification of the N-terminal SIR3R1 and SIR3R3 mutations that can suppress the mating defect caused by single amino acid substitutions in H4 (Johnson et al., 1990, 1992). However, while efficient suppression requires the presence of the H4 N-terminus, several observations argue that the mechanism of suppression is not due to a physical interaction between histone H4 and the altered residues in SIR3R1 and SIR3R3. The SIR3 suppressors are not allele specific, suppressing single amino acid mutations even in the H4 silencing subdomain R2. In vitro, SIR3R1 and SIR3R3 did not restore binding to the K16Q mutation (tested in the context of only the silencing domain), neither did they show improved binding over wild-type SIR3 (A. H. and M. G., unpublished data). Consistent with this, both the SIR3R1 and the SIR3R3 mutations are located outside of the histone interaction domains, which map to the C-terminus of SIR3. Therefore, the SIR3R1 and SIR3R3 suppressors may act indirectly by potentiating interactions with other silencing factors.

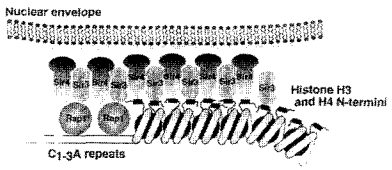

Figure 7. A Model for the Formation of Heterochromatin and Transcriptional Repression at Yeast Telomeres

See Discussion for details.

A Model for the Formation of Telomeric Heterochromatin

Our experiments show that SIR3 and SIR4 can bind to the H3 and H4 N-termini. Yet, the function of the SIR proteins in vivo is limited to the *HM* loci and telomeric chromatin, suggesting that a specific chromosomal context is required in order for the interactions between histones and SIR proteins to occur. We propose that the activity of specific *cis*-acting DNA elements generates this context as outlined in the following model for the formation of telomeric heterochromatin (Figure 7). An important component of the model is RAP1, which binds to the telomeric $C_{1-3}A$ repeats and is able to interact with SIR3 and SIR4. RAP1 may thus recruit SIR proteins to the telomeres and initiate the assembly of a multimeric protein complex (Moretti et al., 1994). In this manner, the sequence-specific DNA binding of RAP1 would determine the region converted into heterochromatin. However, silencing of genes does not occur within the $C_{1-3}A$ repeats, but in neighboring chromatin. Therefore, we suggest that, at the transition point between the telomeres and adjacent chromatin, SIR3 and SIR4 begin to polymerize into a heterochromatic complex that can spread along the chromosome through an interaction of SIR3 and SIR4 with the histone H3 and H4 N-termini. The formation of such a complex, based on multiple weak interactions between H3 and H4 and the SIR proteins and between the SIR proteins themselves (Chien et al., 1991; Moretti et al., 1994), would explain the continuity of the silenced domain extending from the telomere and is supported by the finding that increasing SIR3 levels promotes spreading of telomeric repression

Table 1. Yeast Strains

Strain	Genotype	Reference
UKY403	*MATa, ade2-101, his3-Δ200, leu2-3,-112, lys2-801, trp1-Δ901, ura3-52, thr⁻, tyr⁻, arg4-1, hhf1::HIS3, hhf2::LEU2, pUK421 (CEN3, ARS1, TRP1, GAL1-HHF2)*	Kim et al., 1988
PKY501	as UKY403, but with pPK301 (*CEN3, ARS1, URA3, HHF2*)	Durrin et al., 1991
PKY821	as UKY403, but with pPK621 (*CEN3, ARS1, URA3, HHF2-R5, -R8, -R12*)	Johnson et al., 1990
LDY107	as UKY403, but with pLD107 (*CEN3, ARS1, URA3, HHF2-Q5, -Q8, -Q12, -Q16*)	Durrin et al., 1991
LJY912	as UKY403, but with pLJ912 (*CEN3, ARS1, URA3, HHF2-Q16*)	Johnson et al., 1990
AHY104	as UKY403, but with p291.3 (*CEN6, ARS4, URA3, HHF2-R5, -R8, -R12, -Q16*)	This study
JTY153U	*MATa, ade2-101, his3-Δ200, leu2-3,-112, lys2-801, trp1-Δ901, ura3-52, hhf1::HIS3, adh4::URA3-Tel*	Thompson et al., 1994
JTY155U	as JTY153U except *sir3::LEU2*	Thompson et al., 1994
JTY505	*MATa, ade2-101, ura3-52, trp1-Δ901, met13, htb1-1, htb2-1,* pJT138 (*CEN6, ARS4, TRP1, HTB2*)	Thompson et al., 1994
JTY506	as JTY506 but with pJT139 (*CEN6, ARS4, TRP1, HTB2-Δ3–32*)	Thompson et al., 1994
AHY120	as JTY153U but with pRS424 (2μ, TRP1)	This study
AHY121	as JTY155U but with pRS414 (CEN6, ARS4, TRP1)	—
AHY122	as JTY155U but with p309.1 (*CEN6, ARS4, TRP1, SIR3*)	—
AHY123	as JTY155U but with p368.1 (*CEN6, ARS4, TRP1, SIR3 Δ623–762*)	—
AHY124	as JTY155U but with p348.1 (*CEN6, ARS4, TRP1, SIR3 Δ799–910*)	—
AHY125	as JTY155U but with pRS424 (2μ, TRP1)	—
AHY126	as JTY155U but with pHR67-23 (*2μ, TRP1, SIR3*)	—
AHY127	as JTY155U but with p370.3 (*2μ, TRP1, SIR3 Δ623–762*)	—
AHY128	as JTY155U but with p349.1 (*2μ, TRP1, SIR3 Δ799–910*)	—
STY5	*Mata/Matα, ura3-52/ura3-52, his3/his3, hhta1-1/hhta1-1, hhta2-1/hhta2-1,* pJC102 (*CEN3, ARS1, URA3, HHTA1*), pJT142 (*CEN6, ARS4, HIS3, HHTA1*)	—
STY6	as STY5 but with pTS2 (*CEN3, ARS1, URA3, HHTA1-Δ4–20*) and pJT143 (*CEN6, ARS4, HIS3, HHTA1-Δ4–20*)	—
STY8	*Mata/MATα, ura3-52/ura3-52, ade2-101/ade2-101, his3-Δ200/his3-Δ200, leu2-3, -112/leu2-3, -112, lys2-801/lys2-801, trp1-Δ901/trp1-Δ901, hht1, hhf1::LEU2, hht2, hhf2::HIS3,* pRM200U (*CEN4, ARS1, URA3, HHT2, HHF2*), pRM200 (*CEN4, ARS1, TRP1, HHT2, HHF2*)	—
STY7	as STY8 but with pRM430U (*CEN4, ARS1, URA3, HHT2-Δ4–30, HHF2*) and pGF29 (*CEN4, ARS1, TRP1, HHT2-Δ4–30, HHF2*)	—
STY3	*Mata/MATα, ura3-52/ura3-52, ade2-101/ade2-101, arg4-1/arg4-1, his3-Δ200/his3-Δ200, leu2-3, -112/ leu2-3, -112, lys2-801/lys2-801, trp1-Δ901/trp1-Δ901, thr/thr, tyr/tyr, hhf1::HIS3/hhf1::HIS3, hhf2:: LEU2/hhf2::LEU2,* pPK301 (*CEN3, ARS1, URA3, HHF2*)	—
STY9	as STY3 with additional presence of pLJ999T (*CEN4, ARS1, TRP1, HHF2*)	—
STY10	as STY9 but with pPK613 (*CEN3, ARS1, URA3, HHF2-Δ4–28*) and pSB1 (*CEN4, ARS1, TRP1, HHF2-Δ4–28*)	—
STY18	as STY3 but with pLJ912T (*CEN3, ARS1, URA3, HHF2-Q16*)	—

(Renauld et al., 1993). Coating of nucleosomes with SIR proteins could then lead to silencing by restricting the access of transcription factors to their DNA recognition elements.

The C-terminus of SIR4 has similarity to nuclear lamin proteins (Diffley and Stillmann, 1989). Disruption of either the *SIR3* or the *SIR4* gene prevents the aggregation and perinuclear positioning of telomeres. SIR3 and SIR4 may therefore provide the means by which telomeric heterochromatin is tethered to the nuclear periphery. According to this model, association of SIR proteins with chromosomes is achieved in part through binding to RAP1 and in part through an interaction with histones H3 and H4. The RAP1–SIR protein interaction is presumably not affected by the histone mutations, since RAP1 does not bind to the histone N-termini and histones appear not to be part of the telosome (Wright et al., 1992). The fraction of SIR proteins bound to RAP1 could therefore account for the residual associations of telomeres with each other and the nuclear envelope as revealed by RAP1 staining (Figure 6). That N-terminal mutations of the H3 and H4, nonetheless, have a strong delocalizing effect on SIR3 emphasizes the importance of the proposed interactions between H3, H4, SIR3, and SIR4 and suggests that these are largely responsible for the formation of a repressed heterochromatic structure and its localization to the nuclear periphery. Although additional repressor mechanisms appear to operate at the telomere associated *HM* loci (Chien et al., 1993), *HM* and telomeric silencing use many similar factors (Aparicio et al., 1991), suggesting that the principles of the model are also applicable to the *HM* loci. Thus, the *HM* silencer elements may function in a manner analogous to the telomeric RAP1-binding sites and nucleate the formation and spreading of a heterochromatic structure, also based on interactions between histones and SIR proteins.

The *HM* loci and chromosomal regions adjacent to yeast telomeres share a number of features with heterochromatin from more complex eukaryotes. It has been suggested that heterochromatin is a multimeric assembly of histone and nonhistone chromatin components. Here, we have shown that histones H3 and H4 can directly interact with other components of yeast heterochromatin. Given that histones are among the most conserved proteins known and that reducing the copy number of histone genes suppresses PEV in D. melanogaster (Moore et al., 1983), our model for the formation of heterochromatin in yeast may serve as a paradigm for other eukaryotes as well.

Experimental Procedures

Yeast Strains

Strains are detailed in Table 1. AHY120 is derived from JTY153U and harbors pRS424 (Christianson et al., 1992). To generate AHY121–

AHY128, JTY155U was transformed (Gietz et al., 1992) with pRS414 (Sikorski and Hieter, 1989), pRS424 (Christianson et al., 1992), p309.1 (*CEN/ARS*, wild-type *SIR3*), pHR67-23 (2μ, wild-type *SIR3*), p348.1 (*CEN/ARS*, *SIR3* Δ799–910), p349.1 (2μ, *SIR3* Δ799–910), p368.1 (*CEN/ARS*, *SIR3* Δ623–762), or p370.3 (2μ, *SIR3* Δ623–762). STY3, STY5–STY10, and STY18 were obtained by crossing of suitable parental strains and plasmid shuffling as described (Mann and Grunstein, 1992). The parental strains were PKY501 and PKY903 (Kayne et al., 1988; Johnson et al., 1990) for STY3, STY9, STY10, and STY18; TSY223, TSY226, JTY507, and JTY508 (T. Schuster and M. G., unpublished data; Schuster et al., 1986) for STY5 and STY6; RMY200U, RMY200α, RMY430U, and RMY430α (Mann and Grunstein, 1992) for STY7 and STY8.

Plasmids

GST–histone fusion genes with the N-termini of histones H2A (amino acids 1–35, copy I), H2B (amino acids 1–35, copy II), H3 (amino acids 1–46, copy I), or H4 (amino acids 1–34, copy II) were generated by inserting PCR-derived DNA fragments covering the desired histone residues and having BamHI and EcoRI sites attached to their 5′ and 3′ ends into pGEX2T (Smith and Johnson, 1988). Mutant versions of the H3 and H4 GST fusion genes were also obtained by PCR using suitable primers and templates (Johnson et al., 1990, 1992; Durrin et al., 1991).

A histone H4 gene (R5, R8, R12 with K16Q) was obtained by recombinant PCR (Higuchi, 1990) using appropriate primers and templates (Durrin et al., 1991; Johnson et al., 1990, 1992). The final PCR product was cloned into pRS316 (Sikorski and Hieter, 1989) to give rise to p291.3. Sequences were verified by dideoxy sequencing using the Sequenase version 2.0 system (USB).

Coding regions of *RAP1*, *SIR2*, *SIR3*, and *SIR4* were inserted into pBAT (Annweiler et al., 1991). Fragments used were an RsaI–XbaI fragment with *RAP1* positions 748–3670, a PCR-generated EcoRI fragment with *SIR2* positions 1158–2846, a Fnu4HI–HpaI fragment with SIR3 positions 543–4096, and a HindIII–ClaI fragment with *SIR4* positions 559–4773. Deletions in the *SIR3* coding region were introduced by digesting the wild-type plasmid with restriction enzymes at the desired locations within the gene and downstream or upstream of the coding region and religating the DNA fragments. Stop codons are derived from plasmid sequences within 25 amino acids or less downstream of the *SIR3* sequences. Start codons were provided by complementary adapter oligonucleotides 5′-AGCTTGCCACCATGGCT-3′ and 5′-AGCCATGGTGGCA-3′. Noncompatible sticky ends were converted to blunt ends with Klenow enzyme or T4 DNA polymerase prior to ligation.

To obtain the low copy number yeast vector for SIR3 (p309.1), a HpaI–SalI fragment with the *SIR3* gene was inserted into pRS414. The corresponding high copy number plasmid used was pHR67-23 (Renauld et al., 1993). Low copy number and high copy number plasmids encoding internal deletions Δ623–762 (p368.1 and p370.3) and Δ799–910 (p348.1 and p349.1) in the *SIR3* gene were made by exchanging appropriate restriction fragments between in vitro expression vectors and p309.1 or pHR67-23.

Protein Binding Assays

GST and GST–histone fusions were expressed in Escherichia coli BL 21(DE3) as described (Smith and Johnson, 1988). RAP1, SIR2, SIR3, and SIR4 were made in vitro in the presence of [^{35}S]methionine using the T3 TNT-coupled reticulocyte lysate system (Promega). For binding studies (Hagemeier et al., 1993), 25 μg of GST fusion protein bound to 10 μl of GSH beads (Pharmacia) was incubated for 15 min at room temperature in 195 μl of TGD_{150} (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM glutamate, 1 mM DTT) with 50 μg/ml EtBr and 1 μg/μl crude BL21 extract, unless otherwise stated. Radiolabeled RAP1, SIR2, SIR3 (5 μl), or SIR4 (7.5 μl) were added and binding proceeded for 60 min. MNase digests were performed 45 min after binding with 5 U of MNase, 2 mM $CaCl_2$ and terminated after 30 min with EGTA (10 mM). Following binding, the GSH beads were washed once with 200 μl of TGD_{150} and four times with 200 μl of TGD_{100} (equals TGD_{150} with 100 mM NaCl). Proteins were eluted in SDS–PAGE sample buffer and 40% of the eluate, and 20% of the input material was resolved by SDS–PAGE and visualized by autoradiography.

Quantitation of Mating Efficiency and 5-FOA Sensitivity

Quantitative matings were done as described (Kayne et al., 1988). To test mating of AHY120–AHY128, 3×10^6 logarithmically growing cells of each strain were mixed with 3×10^6 cells of the α tester strain D587-4b. Cells were spread on a sterile filter paper disk (Millipore HAWP 025 00, 0.45 μm pore size) and placed on the surface of a rich media agar plate. After 5.5 hr at 30°C, cells were rinsed off the filter, pelleted, and resuspended in 100 μl of sterile water, and four serial 10× dilutions of the cell suspension were made. From each of the dilutions, 10 μl were spotted onto an agar plate selective for diploids (YM plates). Cells from mock matings done in the absence of the D587-4b cells were incubated, collected, and diluted as above and spotted on selective media lacking Trp (SD-Trp). For 5-FOA sensitivity, 3×10^6 cells were collected, resuspended, and diluted as before, and 10 μl aliquots were placed on SD-Trp plates (plus or minus 5-FOA).

Immunofluorescence

Strains with histone genes on *CEN/ARS* plasmids were precultured in selective media and then grown for 10–16 hr in YPD. Cells were converted into spheroplasts, fixed with formaldehyde (Palladino et al., 1993), and reacted with affinity-purified anti-RAP1 (Klein et al., 1992) or anti-SIR3 antibodies, raised against a full-length SIR3–β-galactosidase fusion protein. Secondary antibodies conjugated to fluorescein isothiocyanate (FITC) were preadsorbed against fixed spheroplasts. The DNA fluorescence signal was detected by EtBr staining (2 μg/ml) in the mounting solution (1× PBS, 80% glycerol, 24 μg/ml 1,4 diazabicyclo-2,2,2, octane [DABCO; Sigma]) or by exploiting the background signal from fluorescent secondary antibodies. Slides were mounted and viewed on a Zeiss Axiovert 100 microscope with the Zeiss Laser Scanning Microscope 410 system, or the Bio-Rad 600 Laser Scanning Microscope. A 100× Plan-Neofluar objective (1.3 oil) was used. Image processing was standardized for all images (similar filtration and threshold levels) (Palladino et al., 1993).

Acknowledgments

Correspondence should be addressed to M. G. We are grateful to D. Gottschling, D. Shore, L. Pillus, and J. Thompson for the gift of plasmids, A. Formenton, M. Gotta, and M. Roberge for antibodies, G. Fisher-Adams for valuable discussions, and H. Kim for excellent technical help. This work was supported by grants from the National Institutes of Health (GM42421 to M. G.), the Swiss National Science Foundation and the Human Frontiers Research Program (to S. M. G.), and fellowships from the Deutsche Forschungsgemeinschaft to A. H. and S. S.-B.

Received October 13, 1994; revised December 2, 1994.

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