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Modifiers of Position Effect Are Shared between Telomeric and Silent Mating-Type Loci in S. cerevisiae

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Summary

Genes placed near telomeres in S. cerevisiae succumb to position-effect variegation. *SIR2*, *SIR3*, *SIR4*, *NAT1*, *ARD1*, and *HHF2* (histone H4) were identified as modifiers of the position effect at telomeres, since transcriptional repression near telomeres was no longer observed when any of the modifier genes were mutated. These genes, in addition to *SIR1*, have previously been shown to repress transcription at the silent mating loci, *HML* and *HMR*. However, there were differences between transcriptional silencing at telomeres and the *HM* loci, as demonstrated by suppressor analysis and the lack of involvement of *SIR1* in telomeric silencing. These findings provide insights into telomeric structure and function that are likely to apply to many eukaryotes. In addition, the distinctions between telomeres and the *HM* loci suggest a hierarchy of chromosomal silencing in S. cerevisiae.

Introduction

Telomeres, the ends of eukaryotic chromosomes, are essential for the stable maintenance and replication of linear chromosomes in eukaryotic cells (Blackburn, 1991; Zakian, 1989). Recently, we have shown that Pol II–transcribed genes succumb to a position effect when placed near the ends of chromosomes in Saccharomyces cerevisiae (Gottschling et al., 1990), reflecting observations made in other eukaryotes that the chromosomal location of a gene can affect its expression (Eissenberg, 1989; Henikoff, 1990; Lima-de-Faria, 1983; Spofford, 1976; Spradling and Karpen, 1990; Wilson et al., 1990). The position effect is manifested as the stable but reversible transcriptional repression of each gene examined. The mechanism by which this repression occurs is unclear, but it is likely due to a structural attribute of S. cerevisiae telomeres. Cytological observations in plants, insects, and mammals indicate that telomeres are heterochromatic; in addition, the telomeres in these organisms and in Trypanosomes occupy unique locations within the nucleus, typically being associated with the nuclear envelope (Chung et al., 1990; Fussell, 1975; Hochstrasser et al., 1986; Lima-de-Faria, 1983; Rawlins and Shaw, 1990; Traverse and Pardue, 1989; White, 1973).

HML and *HMR* are two other loci in S. cerevisiae where a position effect on transcription has been observed (Klar et al., 1981; Nasmyth et al., 1981). The mating-type genes, which are expressed when present at the *MAT* locus, are maintained transcriptionally silent when present at *HML* and *HMR* even though all cis-acting sequences required for full expression at *MAT* are present. Other Pol II– or Pol III–transcribed genes are also repressed when inserted within or near the *HM* loci (Brand et al., 1985; Mahoney and Broach, 1989; Schnell and Rine, 1986).

DNA sequences known as "silencers" flank both *HM* loci and are required for transcriptional repression (Abraham et al., 1984; Brand et al., 1985; Feldman et al., 1984; Mahoney and Broach, 1989). The silencers (denoted E and I) have been genetically dissected into smaller functional elements, which are recognition sites for DNA-binding proteins; these include an *ARS* (autonomous replicating sequence) element, and ABF1- and RAP1-binding sites (Brand et al., 1987; Buchman et al., 1988; Mahoney and Broach, 1989; Mahoney et al., 1991; Shore and Nasmyth, 1987; Shore et al., 1987). The RAP1 protein also binds to the yeast telomeric sequence $(TG_{1\text{-}3})_n$ (Buchman et al., 1988; Longtine et al., 1989). RAP1 is apparently involved in repression of *HM*, since *HMR* is derepressed when *RAP1* temperature-sensitive mutant cells are shifted to the nonpermissive temperature (Kurtz and Shore, 1991).

At least seven additional genetic loci play a role in *HM* silencing. The products of four genes, *SIR1*, *SIR2* (*MAR1*), *SIR3* (*MAR2*, *CMT*), and *SIR4* (silent information regulator), are required for complete silencing at both of the *HM* loci (Haber and George, 1979; Hopper and Hall, 1975; Ivy et al., 1985, 1986; Klar et al., 1979; Rine et al., 1979; see Rine and Herskowitz, 1987, for overview). The molecular mechanism by which the *SIR* genes act to repress transcription is unclear; none of the *SIR* proteins have been demonstrated to bind silencer sequence DNA (Buchman et al., 1988; Shore et al., 1987).

A null allele of either *NAT1* (N-terminal acetyltransferase) or *ARD1* (arrest defective) causes several phenotypes, one of which is derepression of the silent mating-type locus *HML* (Mullen et al., 1989; Whiteway et al., 1987). *NAT1* and *ARD1* appear to encode an N-terminal acetyltransferase; however, it is not known whether the acetyltransferase activity acts directly in silencing at *HML*.

S. cerevisiae harbors two copies of genes encoding histone H4 (*HHF1* and *HHF2*), either of which alone is sufficient for viability (Kim et al., 1988). In strains with deletions of *HHF1* (*hhf1::HIS3*), single point mutations in any of four consecutive amino acids (residues 16–19) near the N-terminus of histone H4 (*HHF2*) relieve transcriptional silencing at *HML*, with no other apparent phenotypic consequence (Johnson et al., 1990; Megee et al., 1990; Park and Szostak, 1990). These results directly implicate chromatin in *HM* silencing. Further evidence for the involvement of chromatin in silencing is suggested by the inaccessibility of *HML* and *HMR* to the HO endonuclease in vivo (Strathern et al., 1982; Kostriken et al., 1983). Additionally, in vitro nuclease sensitivity analysis of *HML* and *HMR* suggests that the *HM* loci exist in a distinct chromatin structure that is refractory to transcription in a *SIR*-dependent manner (Nasmyth, 1982).

The characteristics of position effect and RAP1-binding

sites shared by telomeres and the *HM* loci prompted us to test whether the *SIR*, *HHF2*, *NAT1*, and *ARD1* genes play a role in transcriptional repression at yeast telomeres. The results presented in this paper show that in addition to their roles in silencing at the *HM* loci, the *SIR2*, *SIR3*, *SIR4*, *NAT1*, *ARD1*, and *HHF2* genes are required for the telomeric position effect in S. cerevisiae. Mutations in any of these genes relieve transcriptional repression of either *URA3* or *ADE2* at two different telomeres. In contrast, mutations in *SIR1* did not alter repression at telomeres. These results suggest that telomeres in S. cerevisiae exist in a heterochromatin-like structure, a structure composed of proteins that also function at similar chromosomal domains such as the *HM* loci. Based on the differences in silencing between telomeres, *HML*, and *HMR*, we suggest that a hierarchy of chromosomal silencing exists within the yeast genome.

Results

SIR2, *SIR3*, and *SIR4* Maintain Transcriptional Repression at Telomeres

An isogenic set of *sir*$^-$ strains with the *URA3* gene located at one of four different chromosomal sites was constructed: adjacent to telomere VII-L or V-R, at its normal chromosomal location, or at a second nontelomeric site (*ADH4*, ~20 kb from telomere VII-L). *URA3* expression was measured by two criteria: resistance to 5-fluoroorotic acid (5-FOA^R), and *URA3* mRNA levels as determined by RNA blot hybridization analysis. 5-FOA is converted into a toxic metabolite by the *URA3* gene product, such that cells expressing normal levels of the *URA3* gene product are killed on media containing 5-FOA, whereas *ura3*$^-$ cells are resistant to 5-FOA (5-FOA^R) (Boeke et al., 1987). Cells with *URA3* near a telomere form colonies on 5-FOA medium, yet cells within these 5-FOA^R colonies can grow in the absence of uracil, indicating that genetically identical cells can switch from a clonally inherited repressed state to a transcriptionally active state (Gottschling et al., 1990).

Consistent with these earlier results, when the *URA3* gene was located adjacent to either the VII-L or V-R telomere in a *SIR*$^+$ strain, a significant fraction of cells were resistant to 5-FOA (Table 1; 0.62 for UCC5, 0.15 for UCC35; Figure 1B), and cells from 5-FOA^R colonies retained the ability to form colonies on medium lacking uracil. Similar results were obtained with the *sir1* strain (Table 1 and Figure 1B), indicating that expression of the telomeric *URA3* gene is repressed in a subset of cells in these strains and that the *SIR1* gene product is not required for repression.

In contrast, a telomeric *URA3* gene was not repressed in cells that were *sir2*, *sir3*, or *sir4*. The frequency of 5-FOA^R colonies arising from these strains (~10^{-7}) was equivalent to that seen for all strains with *URA3* at its normal chromosomal locus or at the *ADH4* locus (Table 1; data not shown for *ADH4* locus; Gottschling et al., 1990). Mutations in the *SIR* genes had no effect on the 5-FOA resistance of cells having *URA3* at either of these nontelomeric loci (Table 1; data not shown for *ADH4* locus).

Table 1. The Effects of *sir1*, *sir2*, *sir3*, and *sir4* on the 5-FOA Sensitivity of Strains Having *URA3* at Telomeric and Nontelomeric Loci

Strain	Location of *URA3*	Genotype	5-FOA Resistance
UCC5		WT	0.62 (0.36–0.88)
UCC1		*sir1*	0.30 (0.22–0.42)
UCC2	Telomere VII-L	*sir2*	$<1.8 \times 10^{-7}$
UCC3		*sir3*	$<2.2 \times 10^{-7}$
UCC4		*sir4*	$<2.6 \times 10^{-7}$
UCC35		WT	0.15 (0.14–0.16)
UCC31		*sir1*	0.16 (0.11–0.21)
UCC32	Telomere V-R	*sir2*	$\leq 1.7 \times 10^{-7}$
UCC33		*sir3*	$\leq 1.4 \times 10^{-7}$
UCC34		*sir4*	$\leq 2.1 \times 10^{-7}$
UCC6		WT	$\leq 1.7 \times 10^{-7}$
UCC7		*sir1*	$<2.0 \times 10^{-7}$
UCC8	Normal Locus V	*sir2*	$\leq 2.5 \times 10^{-7}$
UCC9		*sir3*	$<2.0 \times 10^{-7}$
UCC10		*sir4*	$\leq 3.0 \times 10^{-7}$
DBY703	Absent	WT	0.96 (0.91–1.00)

5-FOA resistance was determined from a minimum of four independent trials as described in Experimental Procedures. The range of values for independent trials is given in parentheses. The Roman numerals under the column denoting the location of *URA3* identify which chromosome carries the experimental *URA3* locus, on the left (L) or right (R) arms of the chromosome. WT = wild type: *SIR*$^+$.

RNA blot hybridization analysis shows that sensitivity to 5-FOA as a result of the *sir*$^-$ mutations was a reflection of mRNA levels from the telomeric *URA3* gene (Figure 1A). No *URA3* mRNA was detectable in *SIR*$^+$ or *sir1* strains that had *URA3* at the telomere and were grown under nonselective conditions (Figure 1A, uracil +), even when the autoradiograph was greatly overexposed (data not shown). *URA3* mRNA was only detectable in the *SIR*$^+$ or *sir1* strains when they were grown to select for telomeric *URA3* expression (Figure 1A, uracil −), though this level was significantly lower than when *URA3* was at its normal chromosomal locus.

In sharp contrast, the telomeric *URA3* gene produced high levels of mRNA in *sir2*, *sir3*, and *sir4* strains. These levels were comparable with those from *URA3* at its normal chromosomal locus (Figure 1A). The *sir*$^-$ mutations had no effect on *URA3* expression at its normal chromosomal locus (Figure 1A) or when inserted within the *ADH4* locus (data not shown). These data indicate that the telomeric position effect on *URA3* expression mediated by *SIR2*, *SIR3*, and *SIR4* is at the level of transcription.

To demonstrate that the *SIR* requirement for the telomeric position effect was not gene specific, *sir*$^-$ strains were constructed with the *ADE2* gene located at the VII-L telomere, or at its normal locus. The *ADE2* gene provides a visual color assay for its expression; *ADE2*$^+$ strains form white colonies, while *ade2*$^-$ strains form red colonies (Roman, 1956; Figure 2). Previous work had shown that a *SIR*$^+$ strain containing a single copy of *ADE2* at a telomeric locus exhibited phenotypic variegation of *ADE2*, manifested as red and white sectored colonies (Gottschling et al., 1990). Figure 2 shows that strains with the telomeric *ADE2* that

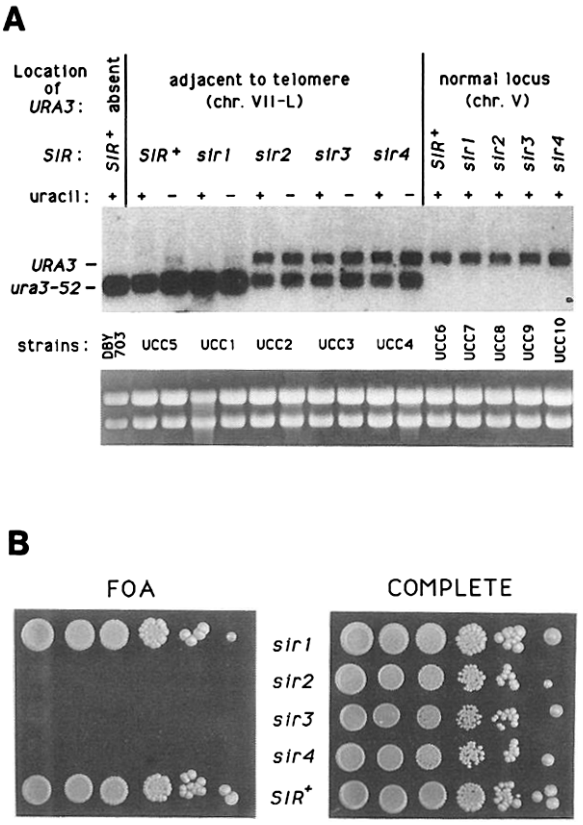

Figure 1. The Effects of *sir* Mutations on *URA3* Expression at Telomeric and Nontelomeric Loci

(A) Total RNA was isolated from mid-log phase cells grown at 30°C in medium containing (100 mg/liter) uracil (+) or lacking uracil (–) and subjected to RNA blot hybridization analysis. The blot was hybridized with a ^{32}P-labeled *URA3* antisense RNA probe, and subjected to autoradiography as described (Gottschling et al., 1990). In strain DBY703 and its isogenic *sir*$^-$ derivatives, the transcript from the *ura3-52* allele at the normal *URA3* chromosomal locus (chromosome V) was truncated, owing to insertion of a Ty element into the *URA3* coding sequence (Rose and Winston, 1984). Thus, when the *URA3* wild-type gene was present together with the *ura3-52* allele in a strain, transcripts from both loci could be analyzed simultaneously. Ethidium bromide staining of rRNA in the gel prior to transfer is shown in the bottom panel. These data were faithfully reproduced a minimum of three times for each strain shown. The very high levels of mRNA from the *ura3-52* locus in DBY703, UCC5, and UCC1 probably result from continuous induction of the mutant gene, owing to a lack of feedback regulation that requires functional *URA3* product (Loison et al., 1980).

(B) Colonies of UCC5 (*SIR*$^+$), UCC1 (*sir1*), UCC2 (*sir2*), UCC3 (*sir3*), and UCC4 (*sir4*) grown 3 days on rich medium at 30°C were suspended in H_2O, and 10-fold serial dilutions were plated onto complete synthetic medium or medium containing 5-FOA as described in Experimental Procedures. One pair of plates is shown.

were *SIR*$^+$ or *sir1* formed red and white variegated colonies, indicating that *ADE2* was repressed in a subset of the cells within these colonies. The *sir2*, *sir3*, and *sir4* strains formed entirely white colonies, demonstrating that the telomeric *ADE2* gene was not repressed (data not shown for *sir2* and *sir3*). These results confirm that the *SIR2*, *SIR3*, and *SIR4* genes are required for maintaining transcriptional repression at telomeres, in addition to silencing the *HM* loci (Rine and Herskowitz, 1987).

Single Point Mutations in Histone H4 Relieve Transcriptional Repression at Telomeres

Single point mutations in any of four consecutive amino acids (residues 16–19) near the N-terminus of histone H4 (*HHF2*) relieve transcriptional silencing at *HML* (Johnson et al., 1990; Megee et al., 1990; Park and Szostak, 1990). *URA3* or *ADE2* was placed at the VII-L telomere in isogenic strains that carried either a single copy of the wild-type histone H4 (*HHF2*) or a mutated copy of *HHF2*. Three such point substitution mutations, all of which derepress *HML*, were tested: a change of Lys-16 to either Gly-16 or Gln-16, and a change of Arg-17 to Gly-17.

Each strain that contained one point mutation in histone H4 exhibited derepression of telomeric *URA3* transcription as shown by their inviability on 5-FOA (Table 2). When *ADE2* was near the telomere in strains with these same histone H4 mutations, colonies were completely white, once again indicating derepression of the telomeric gene (data not shown). Thus, single point mutations at residue 16 or 17 in histone H4 that replace the wild-type basic amino acid with an uncharged residue result in relief of the telomeric position effect.

There is genetic evidence that *SIR3* interacts with histone H4 to silence genes at *HML* (Johnson et al., 1990). Alleles of *sir3* (e.g., *sir3R1*) have been identified that can partially suppress the *HML* silencing defect caused by certain point mutations in histone H4 (e.g., Lys-16 to Gly-16). *URA3* was introduced at the VII-L telomere in an isogenic pair of strains that were either *HHF2-gly16*, *SIR3*$^+$ (UCC2036) or *HHF2-gly16*, *sir3R1* (UCC2035). No suppression by *sir3R1* was observed at the telomere, as judged by complete sensitivity to 5-FOA (Table 2). Equivalent strains with *ADE2* at the telomere produced no red sectored colonies, supporting the conclusion that the *sir3R1* allele could not restore repression at the telomere in an *HHF2-gly16* strain.

NAT1 and *ARD1* Are Required for the Telomeric Position Effect

A null mutation of either *NAT1* or *ARD1* causes derepression of the silent mating-type locus *HML* (Mullen et al., 1989; Whiteway et al., 1987). *URA3* or *ADE2* was introduced at the VII-L telomere into each member of a set of isogenic strains that was either *nat1*, *ard1*, or wild type for both genes. The sensitivity to 5-FOA of *nat1* and *ard1* strains (Table 2) was equivalent to that observed for *sir2*, *sir3*, and *sir4* (Table 1) and the point mutants in histone H4 (Table 2). Thus no position effect was observed for a telomeric *URA3* gene in *nat1* or *ard1* cells. Likewise, the telomeric *ADE2* gene was not repressed in the *nat1* and *ard1* strains, as these strains formed entirely white colonies (data not shown).

Overexpression of *SIR1* Does Not Restore Position Effect at Telomeres

Overexpression of *SIR1* partially suppresses the mating defects of *MAT***a** strains containing *nat1* or *ard1* mutations, or certain *sir3* or *HHF2* alleles by reestablishing silencing at *HMLα* (Stone et al., 1991). We tested whether *SIR1*

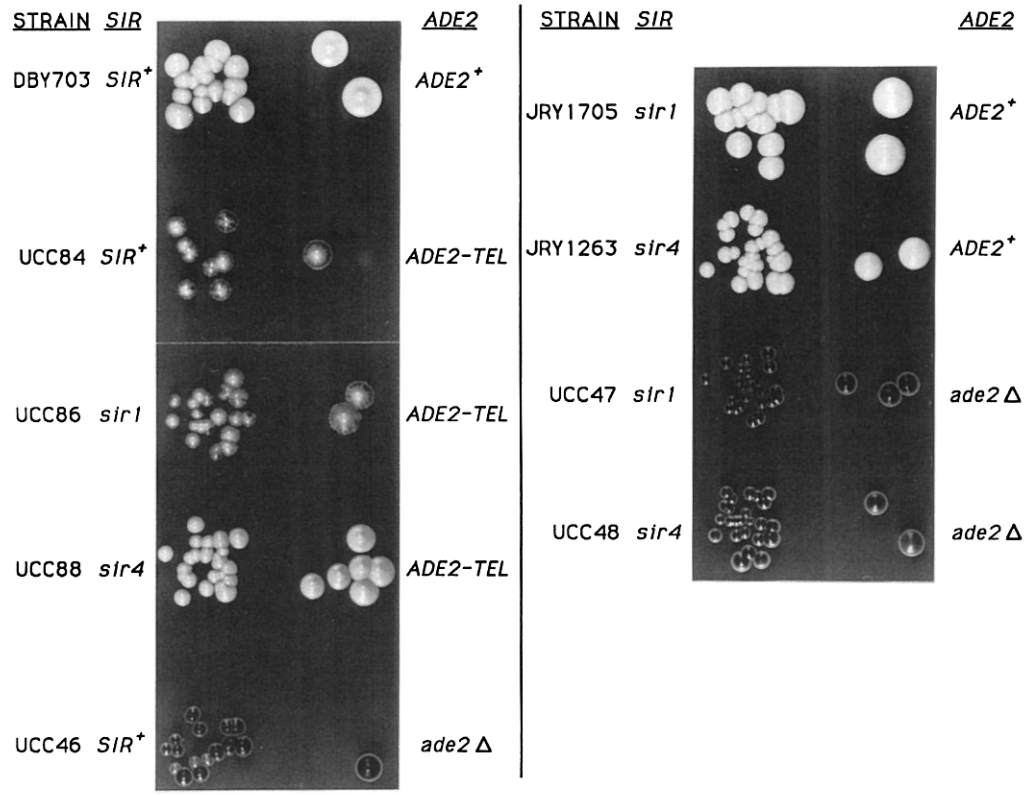

Figure 2. Position-Effect Variegation of a Telomere-Linked *ADE2* Gene Requires *SIR4*, but Not *SIR1*

Colonies grown for 3 days on rich medium at 30°C were suspended in H_2O, and 10-fold serial dilutions were plated on a complete synthetic medium that facilitates the development of red color from *ade2* cells. Multiple independent colonies of each strain were chosen; one independent isolate is shown (two dilutions) for each. The strain names and *SIR* genotypes are shown at the left of the photographs, while the *ADE2* genotype is shown to the right. *ADE2*$^+$, wild-type gene at its normal locus on chromosome XV; *ade2*Δ, deletion of the normal chromosomal copy of the *ADE2* gene; *ADE2-TEL*, *ADE2* gene located adjacent to telomere VII-L in an *ade2*Δ strain.

Table 2. Effects of Mutations in *HHF2*, *NAT1*, and *ARD1*, and *SIR1* Overexpression on the Telomeric Position Effect

Strain	Genotype	5-FOA Resistance
UCC2031	WT	0.63 (0.42–0.83)
UCC2032	*HHF2-gly16*	$<1.4 \times 10^{-6}$
UCC2033	*HHF2-gln16*	$<1.0 \times 10^{-6}$
UCC2034	*HHF2-gly17*	$<1.7 \times 10^{-5}$
UCC2035	*HHF2-gly16 sir3R1*	$<1.4 \times 10^{-6}$
UCC2036	*HHF2-gly16*	$<2.5 \times 10^{-6}$
UCC18	WT	0.21 (0.15–0.29)
UCC16	*nat1*	$<4.3 \times 10^{-7}$
UCC25	*ard1*	$\leq 7.3 \times 10^{-7}$
UCC123	WT/pKL1	0.51 (0.33–0.90)
UCC122	*nat1*/pKL1	$<1.1 \times 10^{-6}$
UCC138	*sir3*/pKL1	$<6.6 \times 10^{-6}$

URA3 is located adjacent to the VII-L telomere in each strain listed in the table. pKL1 is a 2μ-based plasmid that carries the *SIR1* gene (Stone et al., 1991). In strain UCC2035 (*HHF2-gly16*, *sir3R1*), the *sir3R1* allele reestablishes silencing at *HML*, restoring mating efficiency to approximately 10% (data not shown; Johnson et al., 1990). Overexpression of *SIR1* did suppress the loss of *HML* silencing in the *nat1* (UCC122) and *sir3::LEU2* strain (UCC138), as determined by an increase in mating efficiency (data not shown; Stone et al., 1991). Resistance to 5-FOA was determined as described in Experimental Procedures. The range of values is given in parentheses. WT = wild type: *HHF2*, *SIR3*, *NAT1*, *ARD1*.

overexpression could restore silencing of a telomere-linked gene in a *nat1* or *sir3::LEU2* strain. Plasmid pKL1 (Stone et al., 1991), which contains *SIR1* on a 2μ-based vector, was transformed into strains that were *nat1*, *sir3*, or wild type and have *URA3* located at telomere VII-L or at the normal *URA3* locus. As expected, a significant fraction of cells of strain UCC123 (wild type, *URA3*-TEL/pKL1) were resistant to 5-FOA (Table 2). However, the *nat1* and *sir3* strains that have *URA3* at telomere VII-L and harbor pKL1 continue to be sensitive to 5-FOA, as are the strains with *URA3* at its normal chromosomal locus (Table 2; data not shown for *URA3* normal locus). Thus, the overexpression of *SIR1* does not restore silencing at telomeric loci in *nat1* or *sir3* strains. These results are consistent with the results presented above, indicating that *SIR1* plays no role in transcriptional silencing at telomeres.

Discussion

Similarities and Differences in Position Effects at Telomeres and the *HM* Loci

This study shows that the *SIR2*, *SIR3*, *SIR4*, *HHF2*, *NAT1*, and *ARD1* genes are required for the position effect at telomeres in S. cerevisiae. Consequently, it implies that

these gene products constitute a general mechanism for silencing chromosomal domains in S. cerevisiae. In view of the results presented here, it is interesting to note that both *HML* and *HMR* are located quite close to the termini of chromosome III, ~12 kb (Button and Astell, 1986) and ~25 kb (Yoshikawa and Isono, 1990), respectively. When *HML* is present on a circular plasmid or a ring chromosome III derivative, deletion of *HMLE* or *HMLI* results in derepression of *HML* (Feldman et al., 1984; Strathern et al., 1979). However, these mutated *HML* loci are fully silenced when present at the normal telomeric *HML* locus (Mahoney and Broach, 1989), suggesting that the proximity of *HML* to the telomere may facilitate full repression of this locus.

There was no detectable change in the telomere-specific position effect in *sir1* strains or in strains with *SIR1* on a high copy plasmid. Since both of these genotypes have an effect on *HML* and *HMR*, we conclude that *SIR1* function is specific to silencing of the *HM* loci. Single-cell analysis of *sir1* strains indicates that a mixed population of cells exists with ~20% of cells being transcriptionally silent at *HML* and the remainder being transcriptionally active at *HML*; the transcriptional state is clonally inherited, though cells switch between transcriptionally active and repressed states at a low frequency (Pillus and Rine, 1989). We have found that epigenetic switching between transcriptional states occurs at telomeres in SIR^+ (and *sir1*) strains, analogous to that observed at *HML* in *sir1* mutants (Gottschling et al., 1990; Pillus and Rine, 1989; this work). Thus, we propose that *SIR1* provides complete silencing at *HML* and *HMR* by preventing switching from the silent to the active transcriptional state. If this is correct, the *HM* loci should contain elements through which *SIR1* acts, which are absent from chromosomal termini (e.g., the A and B elements [Brand et al., 1987]). In support of this notion, a recent study has identified deletions at *HMLE* that result in epigenetic switching of transcriptional states at *HML* (Mahoney et al., 1991).

A number of differences have been observed between silencing at telomeres, *HML*, and *HMR*, which may yield insights into the functional organization of the silent loci. As indicated above, the epigenetic switching of *HML* expression in *sir1* strains is very similar to the expression of a telomeric gene in a SIR^+ (or *sir1*) strain, indicating that elements through which *SIR1* can act to fully silence *HML* are present at *HML* (and probably *HMR*) but not at telomeres. Also, while a *sir1* mutation has only a slight effect at either *HM* locus, and a mutation in *nat1* alone derepresses *HML* but not *HMR* (Mullen et al., 1989), the *sir1*, *nat1* double mutant is completely derepressed at *HMR*, suggesting that additional mechanisms of silencing exist at *HMR* compared with *HML* (or telomeres) (Stone et al., 1991). Deletion of *NAT1* or *ARD1* results in significant derepression of *HML* but not *HMR* (Whiteway et al., 1987); however, deletion of the *RAP1*- binding site at *HMRE* results in derepression of *HMR* in *nat1* or *ard1* strains (Stone et al., 1991), again indicating that redundant silencing mechanisms exist at *HMR* compared with *HML* and telomeres. Last, *sir3R1* partially restores *HML* silencing in an *HHF2-gly16* strain (mating efficiency is restored from $\sim10^{-5}$ to $\sim10^{-1}$ [Johnson et al., 1990]), but does not restore telomeric silencing (Table 2). This may be explained if suppression of *HHF2-gly16* by *sir3R1* is facilitated by the presence of a redundant silencing mechanism(s), such as that mediated by *SIR1*. Thus we suggest that telomeres exhibit a basal level of transcriptional repression, and that silencing at *HML* and *HMR* is based on the same mechanism(s), but is strengthened and regulated by the presence of additional silencer elements.

How Does the Telomeric Position Effect Occur?

Little is known about the specific mechanism by which the *SIR*, *HHF2*, *NAT1*, and *ARD1* gene products act in transcriptional silencing; however, the available evidence suggests that they modify chromatin structure (Nasmyth, 1982). Single point mutations in histone H4 completely relieve the telomeric position effect and thus provide the best evidence that chromatin structure is intimately involved in telomeric silencing (Table 2). Mutations in any of four contiguous amino acids (residues 16–19) in the N-terminus of histone H4 result in derepression at *HML* (Johnson et al., 1990; Megee et al., 1990; Park and Szostak, 1990); these four positively charged amino acids are conserved throughout eukaryotes, and are sites of post-translational modifications (van Holde, 1989). Significantly, correlative studies note that the modifications (e.g., acetylation and phosphorylation) on histone H4 are associated with the transcriptional status of the chromatin (reviewed in van Holde, 1989). In yeast, suppressors of the histone H4 point mutations, which restore silencing, map as compensatory changes in the *SIR3* gene, thus providing evidence that *SIR3* interacts with chromatin (Johnson et al., 1990). In addition, *SIR2* has been shown to suppress intrachromosomal recombination between rDNA repeats, supporting the idea that *SIR2* may play a general role in chromatin organization (Gottlieb and Esposito, 1989).

NAT1 and *ARD1* apparently encode two subunits of a yeast N-terminal acetyltransferase that acetylates histone H2B along with at least 20 other proteins (Mullen et al., 1989). Thus, while it is tempting to speculate that *NAT1* and *ARD1* play a direct role in silencing by acetylation of H2B, it is premature to assess their precise role because of their pleiotropic effects.

Recently it was reported that *SIR4* shares sequence similarity with the coiled-coil domains of human nuclear lamins A and C (Diffley and Stillman, 1989). These domains facilitate polymerization of lamins into the lamina, which lines the nuclear envelope. Taking into account the cytological observations in interphase nuclei indicating that telomeres are located at the nuclear periphery (see Introduction), it is plausible that the putative polymerization domain of *SIR4* is associated with the nuclear lamina and might therefore mediate binding of telomeres to the nuclear envelope. Since the *SIR4* gene product is believed not to bind DNA directly (Buchman et al., 1988; Shore et al., 1987), an interaction between SIR4 and a telomere-binding protein (e.g., RAP1) may enable an association between telomeres and the nuclear envelope. It is noteworthy that purified mammalian nuclear lamins A and C bind in vitro to synthetic oligonucleotides containing mammalian telomere repeat sequences (Shoeman and Traub, 1990).

Thus, attachment of telomeres, as well as other chromosomal loci or regions, to the nuclear envelope may be a component of nuclear organization and might therefore affect local gene expression (Alberts et al., 1989; Blobel, 1985).

The position effect at S. cerevisiae telomeres may reflect a general feature of eukaryotic telomeres. In Drosophila, stable transposition of the *white* gene to a position near a telomere results in a mottled eye color phenotype (Levis et al., 1985), which is consistent with transcriptional repression of *white* in some cells. Cytological studies in a number of organisms indicate that telomeres are organized into heterochromatin (Lima-de-Faria, 1983; Traverse and Pardue, 1989). While heterochromatin is defined cytologically as a region of the chromosome that remains condensed in interphase, it also displays two distinctive traits: late DNA replication and the ability to repress transcription of euchromatic genes placed nearby (Eissenberg, 1989; Henikoff, 1990; Spofford, 1976; Spradling and Karpen, 1990). S. cerevisiae telomeres possess both of these hallmarks of heterochromatin (Gottschling et al., 1990; McCarroll and Fangman, 1988; this work). The *SIR2*, *SIR3*, *SIR4*, *HHF2*, *NAT1*, and *ARD1* products may be intimately involved with the organization of regions of yeast chromosomes into heterochromatin or heterochromatin-like structures. Because telomeres and histones are highly conserved structurally and functionally among eukaryotes, it seems quite likely that the mechanism of transcriptional repression functioning in S. cerevisiae is also utilized in multicellular eukaryotes.

Experimental Procedures

Plasmid Constructions

Plasmid pADE2 contains the *ADE2* gene on a 3.6 kb chromosomal BamHI fragment from plasmid pL909 (gift from R. Keil). Plasmid pΔADE2 was constructed by replacing the internal 2.2 kb HindIII fragment (contains all but the six C-terminal residues of the *ADE2* open reading frame; Stotz and Linder, 1990) of plasmid pADE2 with the 3.8 kb BamHI–BglII fragment of pNKY51, which contains two direct repeats of the Salmonella *hisG* gene flanking *URA3* (Alani et al., 1987). The HindIII and BamHI ends and the HindIII and BglII ends were blunt ended with T4 DNA polymerase and ligated together, resulting in the destruction of these particular restriction sites. Thus, pΔADE2 contains a 5.2 kb BamHI fragment with about 700 bp of homology to sequences upstream and downstream of the *ADE2* gene flanking the 3.8 kb BamHI–BglII (*hisG-URA3-hisG*) fragment from pNKY51.

A 2.4 kb HindIII fragment from plasmid pJR104 (a gift from J. Rine) that contains the 5′ end of the *SIR3* gene was inserted into pVZ1 to yield plasmid pH3SIR3. Plasmid pH3SIR3 was digested with BglII to excise a 600 bp fragment in the *SIR3* coding sequence, which was replaced with a 1.8 kb BamHI fragment containing the *HIS3* gene. The resulting plasmid was pΔSIR3::HIS3.

Yeast Strains and Methods

Media used for the growth of S. cerevisiae were described previously (Gottschling et al., 1990). S. cerevisiae were transformed by the lithium acetate procedure (Ito et al., 1983) or by electroporation in the presence of sorbitol (Becker and Guarente, 1991).

The *URA3* gene was placed adjacent to the telomere sequence $(TG_{1\text{-}3})_n$ on the left end of chromosome VII (UCC1–UCC5, UCC16, UCC18, UCC25, UCC128, UCC2031–UCC2036), or the right end of chromosome V (UCC31–UCC35); no telomere-associated sequences (i.e., X and Y′ elements [Chan and Tye, 1983a, 1983b]) were present (data not shown). Alternatively, the *ura3-52* or *ura3-1* allele (at the normal *URA3* locus on chromosome V in the parent strains) was converted to *URA3*$^+$ (UCC6–UCC10 and UCC129), or *URA3* was inserted into the *ADH4* locus about 20 kb from the telomere on VII-L (UCC11–UCC15) (data not shown). Strains UCC5, UCC6, UCC12, and UCC35 were derived from DBY703; UCC1, UCC7, UCC11, and UCC31 were derived from JRY1705; UCC2, UCC8, UCC13, and UCC32 were derived from JRY1706; UCC3, UCC9, UCC14, and UCC33 were derived from JRY1264; UCC4, UCC10, UCC15, and UCC34 were derived from JRY1263. Strain UCC18 was derived from W303-1a; UCC16 was derived from AMR1; UCC25 was derived from JRM5. UCC128 and UCC129 were derived from YDS73; strain UCC2031 was derived from LJY153, UCC2032 from LJY405I, UCC2033 from LJY412I, UCC2034 from LJY421I, UCC2035 from LJY305TR1, and UCC2036 from LJY305T. Plasmids and methods for these constructions have been described previously (Gottschling et al., 1990).

Strains UCC46 (*SIR*$^+$), UCC47 (*sir1*), and UCC48 (*sir4*), which were derived from strains DBY703, JRY1705, and JRY 1263, respectively, harbor an *ade2*Δ. The *ade2*Δ was made by transformation of strains DBY703, JRY1705, and JRY1263 with plasmid pΔADE2 digested with BamHI, followed by selection for URA$^+$ transformants. In these transformants the *ADE2* open reading frame has been replaced (all but the six C-terminal residues were deleted) with a DNA fragment containing two direct repeats of the Salmonella *hisG* gene flanking *URA3* (see Plasmid Constructions). Loss of *URA3* by recombination between the two *hisG* repeats within the *ade2* locus was screened for by 5-FOA^R (Alani et al., 1987).

Strains UCC84, UCC86, and UCC88, derived from UCC46, UCC47, and UCC48, respectively, and strains UCC97, UCC98, and UCC99, derived by transformation of strains W303-1a, AMR1, and JRM5, respectively, have a functional *ADE2* gene located adjacent to the chromosome VII-L telomere (*ADE2-TEL*) (construction described previously [Gottschling et al., 1990]); no telomere-associated sequences (i.e., X and Y′ elements [Chan and Tye, 1983a, 1983b]) were present (data not shown). Strains UCC2037–UCC2042, derived from strains LJY153, LJY405I, LJY412I, LJY421I, LJY305T, and LJY305TR1, respectively, were constructed in the same manner to place *ADE2* adjacent to telomere VII-L.

Strain UCC121 was derived from W303-1a by transformation with a 3.6 kb BamHI *ADE2*$^+$ fragment and selection for ADE$^+$ transformants. Strain UCC120 was constructed by introduction of plasmid pJR531 (Kimmerly and Rine, 1987), which had been digested with SphI and EcoRV into UCC97, and selection for HIS$^+$ transformants. Strain UCC131 was constructed by introduction of pΔSIR3::HIS3, which had been digested with EcoRI into UCC84, and selection for HIS$^+$ transformants.

Strains UCC122–UCC125, UCC138, and UCC139 were constructed by transformation of strains UCC16, UCC18, UCC19, UCC21, UCC128, and UCC129, respectively, with plasmid pKL1. Plasmid pKL1 contains the *SIR1* gene on a 2μ-based vector that contains *TRP1* for selection (Stone et al., 1991).

The expected structures of the various chromosomal constructs were confirmed by gel electrophoresis followed by DNA blot hybridization analyses (data not shown). The sir$^-$ phenotypes of strains UCC120 and UCC131 were confirmed by their inability to mate (Sprague, 1991). See Table 3 for the genotypes and sources of the parent strains used in this study.

Quantification of 5-FOA Resistance

Cells from isolated colonies grown on rich medium for 2–3 days at 30°C were inoculated into liquid medium containing (100 μg/liter) uracil. When these cultures reached mid-log phase, serial dilutions were plated onto synthetic complete medium or medium containing 5-FOA (Gottschling et al., 1990). 5-FOA resistance was determined as the average ratio of colonies formed on 5-FOA medium to colonies formed on complete medium, from a minimum of three independent trials, using different colony isolates for each trial. The number of colonies on a plate was determined after 3–4 days of growth at 30°C. Alternatively, colonies of strains grown on rich medium for 2 to 3 days were suspended in H_2O, and 10-fold serial dilutions were plated as described above. For some strains, selection for TRP$^+$ was required to maintain episomal plasmids; these strains were grown on synthetic medium lacking tryptophan for 3 to 4 days and colonies were suspended in H_2O, serially diluted, and plated as above on synthetic medium lacking tryptophan or on 5-FOA medium lacking tryptophan.

Table 3. Genotypes and Sources of Parent Strains

Strain	Genotype	Source
DBY703	*MATα his3 trp1 ura3-52*	J. Rine
JRY1705	DBY703 *sir1::HIS3*	J. Rine
JRY1706	DBY703 *sir2::HIS3*	J. Rine
JRY1264	DBY703 *lys2 sir3::LYS2*	J. Rine
JRY1263	DBY703 *sir4::HIS3*	J. Rine
W303-1a	*MATa ade2 can1-100 his3 leu3 trp1 ura3-1*	R. Rothstein
W303-1b	W303-1a *MATα*	R. Rothstein
AMR1	W303-1a *nat1::LEU2*	Mullen et al. (1989)
JRM5	W303-1a *ard1::LEU2*	R. Sternglanz
YDS73	W303-1b *sir3::LEU2*	Shore et al. (1984)
LJY153	*MATa ade2 his3 leu2 lys2 trp1 ura3 hhf1::HIS3*	L. Johnson and M. Grunstein
LJY405I	LJY153 *HHF2(gly-16) TRP1*	L. Johnson and M. Grunstein
LJY412I	LJY153 *HHF2(gln-16) TRP1*	L. Johnson and M. Grunstein
LJY421I	LJY153 *HHF2(gly-17) TRP1*	L. Johnson and M. Grunstein
LJY305T	*MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3* hhf2::LEU2/pLJ305T (*HHF2-gly16, TRP1*)	Johnson et al. (1990)
LJY305TR1	LJY305T *sir3R1*	Johnson et al. (1990)

Analyses of Nucleic Acids from S. cerevisiae

Preparation and analyses of nucleic acids have been described previously (Gottschling et al., 1990), except that some DNA blot hybridization analyses were carried out using the Genius system from Boehringer Mannheim following the manufacturer's procedures.

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