# Telomere Structure Regulates the Heritability of Repressed Subtelomeric Chromatin in *Saccharomyces cerevisiae*

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ABSTRACT

Telomeres, the protein-DNA structures present at the termini of linear chromosomes, are capable of conferring a reversible repression of Pol II- and Pol III-transcribed genes positioned in adjacent subtelomeric regions. This phenomenon, termed telomeric silencing, is likely to be the consequence of a more global telomere position effect at the level of chromatin structure. To understand the role of telomere structure in this position effect, we have developed an assay to distinguish between the heritability of transcriptionally repressed and derepressed states in yeast. We have previously demonstrated that an elongated telomeric tract leads to hyperrepression of telomere-adjacent genes. We show here that the predominant effect of elongated telomeres is to increase the inheritance of the repressed state *in cis*. Interestingly, the presence of elongated telomeres overcomes the partial requirement of yCAF-1 in silencing. We propose that the formation of a specific telomeric structure is necessary for the heritability of repressed subtelomeric chromatin.

THE mechanism by which cells "remember" their transcriptional state from one generation to the next involves epigenetic effects in development, variegation, and heterochromatin formation. Telomeric silencing specifically refers to the epigenetic and metastable transcriptionally repressed state that is conferred onto genes inserted adjacent to telomeric chromatin in both yeast and other organisms (Gottschling *et al.* 1990; Lustig 1998). This effect is similar to position-effect variegation (PEV) and heterochromatin formation observed in Drosophila and in higher eukaryotes (Henikoff 1996).

The budding yeast *Saccharomyces cerevisiae* has served as an excellent model for investigating both the effect of telomeres on the structure of subtelomeric sequences and the formation of heterochromatic domains. Telomeric silencing is clearly associated with specific changes in subtelomeric chromatin structure (Gottschling 1992; Wright *et al.* 1992; Renauld *et al.* 1993; Hecht *et al.* 1995, 1996; Strahl-Bolsinger *et al.* 1997). First, specific subclasses of acetylated subtelomeric histones (Braunstein *et al.* 1993) are present in subtelomeric regions. Second, subtelomeric chromatin is compacted into nuclease-resistant domains (Gottschling 1992; Wright *et al.* 1992; Renauld *et al.* 1993). Finally, novel "fold-back" structures appear to form between the telomeric and subtelomeric domains (Hecht *et al.* 1996; Grunstein 1997; Strahl-Bolsinger *et al.* 1997). These heterochromatin-like regions are found at both natural and modified termini, although the regulation of natural telomeric silencing has a higher level of complexity (Vega-Palas *et al.* 1997; Fourel *et al.* 1999; Pryde and Louis 1999).

Five fundamental processes have been proposed to operate both in silencing of the cryptic *HM* mating-type loci and in telomeric silencing (Pillus and Rine 1989; Loo and Rine 1995; Lustig 1998). Within the context of a telomere, these include (1) recruitment of silencing factors to specific DNA binding sites (termed silencers; Cockell *et al.* 1998), (2) a molecular "communication" between the telomeric and subtelomeric sequences that initiates subtelomeric silencing (Park *et al.* 1998), (3) the maintenance of the repressed state during the cell cycle, and (4) the heritability of the repressed state. A fifth parameter is the distance that repressed states are promulgated unidirectionally from the telomeric initiation sites into distal subtelomeric regions (termed propagation in this study; Renauld *et al.* 1993).

Telomeric silencing is related to silencing of *HML* and *HMR*, which share proteins essential for silencing. The yeast duplex telomere-binding protein *r*epressor/*a*ctivator *p*rotein 1 (Rap1p) recruits the *s*ilent *i*nformation *r*egulators Sir3p and Sir4p to the telomere via the Rap1p C-terminal domain (Moretti *et al.* 1994; Cockell *et al.* 1995; Liu and Lustig 1996). Indeed, the protein encoded by the *rap1-17* mutation, Rap1-17p, lacks the C-terminal 165 amino acids that are obligatory for silencing and has defects in telomere size control (Kyrion *et al.* 1992, 1993). In wild-type cells, the repressed state spreads unidirectionally through nucleoso-

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mal chromatin. Silencing is likely to be maintained in part through associations among the Sir2, Sir3, and Sir4 proteins, and between the N termini of histones H3 and H4 and both Sir3p and Sir4p (reviewed in Cockell *et al.* 1998; Lustig 1998).

Yeast chromatin assembly factor-1 (yCAF-1) has also been implicated in silencing (Enomoto *et al.* 1997; Kaufman *et al.* 1997; Monson *et al.* 1997). yCAF-1 is composed of three subunits, Cac1p, Cac2p, and Cac3p (Kaufman *et al.* 1997), each of which is necessary for full telomeric silencing and for heritability of the repressed state. yCAF-1 also plays a role in a multiplicity of additional processes, including ribosomal DNA silencing (Smith *et al.* 1999), repair of DNA damage (Game and Kaufman 1999), and transposition (Qian *et al.* 1998; Huang *et al.* 1999). Both hCAF-1 and yCAF-1 have been characterized biochemically. Human CAF-1 acts specifically during DNA replication (Kaufman *et al.* 1995), possibly depositing a subset of acetylated histones H3 and H4 or forming a structure that is more accessible to specific acetylation. yCAF-1 has a similar subunit structure and shares homology with hCAF-1 (Kaufman *et al.* 1997). Biochemically, yCAF-1 enzyme activity is also capable of histone deposition activity and is likely to be involved in chromatin formation. Interestingly, recent data indicate that proliferating cell nuclear antigen (PCNA), the β-clamp component of the DNA polymerase complex, associates with CAF-1 *in vitro* (Krude 1999; Shibahara and Stillman 1999). Conceivably, PCNA targets yCAF-1 to specific chromosomal sites that require a unique function of yCAF-1.

Far less is known about heritability. Heritability refers to the ability of either repressed or derepressed transcriptional states to give rise to an identical transcriptional state in subsequent progeny. Several studies have begun to investigate this phenomenon. At the silent *HM* loci, several topological and single-cell pedigree analyses have demonstrated that silencers act in both the formation and heritability, but not maintenance, of the repressed state (Holmes and Broach 1996; Bi and Broach 1997; Ansari and Gartenberg 1999). These studies also strongly suggest that repressed chromatin is erased after DNA replication in the absence of silencers.

The *HM* silencer and putative internal silencers compete with the telomere for limiting components (Lustig *et al.* 1996; Maillet *et al.* 1996; Gotta *et al.* 1997; Smith *et al.* 1999). Earlier studies in our laboratory indicated that longer telomeres introduced into wild-type cells display higher levels of silencing (Kyrion *et al.* 1993). Interestingly, such longer telomeres impair silencing at *HMR*, most likely through sequestration of Sir4p at the telomere (Buck and Shore 1995).

Indeed, this competition is probably a manifestation of a larger role for an exchange of Sir factors to other sites in the genome. In particular, aging cells exhibit relocalization of Sir2p, Sir3p, and Sir4p to the nucleolus (Austriaco and Guarente 1997; Kennedy *et al.* 1997; Sinclair *et al.* 1997), possibly as a prelude to apoptotic-like fragmentation of the nucleolus (Sinclair *et al.* 1997). In addition, the production of yeast double-strand breaks relocalizes both the yKu heterodimer and Sir3p to strand breaks (Martin *et al.* 1999; Mills *et al.* 1999). It is unclear at present whether Sir3p acts at double-strand breaks in heterochromatin formation or in a protective role.

We have previously demonstrated that when elongated telomeres are introduced into wild-type strains, repression levels exceed wild-type values (Kyrion *et al.* 1993; Li and Lustig 1996). In this investigation, we examine the effect of telomere length on "cellular memory" of repressed and derepressed states. To this end, we developed a novel assay to discriminate between the stability of these transcriptional states. We find that the increased length of a telomeric tract confers higher levels of heritability of repressed subtelomeric chromatin in a process unaffected by loss of yCAF-1. In addition, increased heritability of the repressed state acts *in cis* and hence is likely to be mediated by an intramolecular process. Our data also suggest the presence of redundant pathways, governed by both telomere structure and yCAF-1, that may be necessary for molecular memory.

## MATERIALS AND METHODS

**Plasmids:** Tethering plasmids pBTM and pBTM-SIR3 were constructed as described (Lustig *et al.* 1996). Bacterial transformations were carried out by standard techniques.

**Yeast strains and methods:** The genotypes of the strains used in this study, the percentage of total telomeres that are elongated, and the presence (or absence) of wild-type or elongated VIIL:: *URA3/ADE2*-marked telomeres are shown in Table 1. CZY1/*RAP1* and CZY4/*RAP1* were each transformed with pBTM (encoding LexA) or pBTM-SIR3 (encoding LexA-Sir3) as described (Lustig *et al.* 1996). MBH9 was derived from CLY1/*rap1-17* (Liu and Lustig 1996) and contained a 3.6-kb telomeric tract with an artificially introduced *Hae*III site 750 bp from the subtelomeric/telomeric junction (M. Bucholc and A. J. Lustig, unpublished data). Transformations were carried out by the LiOAc method. Standard fluctuation assays were carried out as previously described, using 7–10 colonies per assay (Liu *et al.* 1994). Medians of propagation assays were determined by measuring the red $FOA^r$ colonies/total red colonies in each member of the distribution.

**Assessment of marked and global telomere length:** All strains containing elongated telomeres were derived from an initial cross of wild-type and *rap1-17* strains. After sporulation, wild-type spore colonies containing the elongated telomeres were used for subsequent studies. The size of the VIIL-marked telomeres was determined by Southern analysis. The global elongated telomere size ranges from ~400 bp to 4 kb in length (Kyrion *et al.* 1992). The approximate percentage of elongated telomeres was based on statistical probability and random assortment (*i.e.*, first cross, 50% elongated; second cross, 25% telomeres, etc.).

**Determination of interactions among homologous telomeres:** To distinguish individually among the silencing of two homologs, diploids were generated containing one homolog with either a wild-type or elongated marked VIIL:: *ura3*/*ADE2*-marked telomere. The other homolog contained an elongated

**TABLE 1**

**Strains used in this study**

| Strain | Genotype | VIIL length[a] | %ET[b] | Reference |
|---|---|---|---|---|
| W303a | *MAT***a** *his3 ade2-1 trp1 leu2-3,112 ura3-1* | — | 0 | Kurtz and Shore (1991) |
| W303α | *MATα HIS3 ade2-1 trp1 leu2-3,112 ura3-1* | — | 0 | Kurtz and Shore (1991) |
| AJL 418-3a | *MAT***a** *HIS3 ade2-1 trp1 leu2-3,112 ura3-1* | E | 50 | Li and Lustig (1996) |
| AJL 412-2c | *MAT***a** *HIS3 ade2-1 trp1 leu2-3,112 ura3-1 VIIL::URA3/ADE2* | E | 50 | Kyrion *et al.* (1993) |
| AJL 412-4d | *MATα HIS3 ade2-1 trp1 leu2-3,112 ura3-1 VIIL::URA3/ADE2* | E | 50 | Li and Lustig (1996) |
| PKY 021 | *MATα leu2-3,112, HIS3 ade2-1 trp1 ura3-1 cac1::LEU2* | — | 0 | Kaufman *et al.* (1997) |
| YBO 152 | *MAT***a** *leu2,112 his3 ade2-1 trp1 ura3-1 cac2::TRP1* | — | 0 | P. Kaufman, personal communication |
| BL22-2b | *MATα his3 ade2-1 trp1 leu2-3,112 ura3-1 VIIL::URA3/ADE2* | E | 50 | Li and Lustig (1996) |
| BL27-11a | *MAT***a** *his3 ade2-1 trp1 leu2-3,112 ura3-1 VIIL::ura3/ADE2* | E | 50 | Li and Lustig (1996) |
| AJL 275-2a::UA[c] | *MATα his3 ade2-1 trp1 leu2-3,112 ura3-1 VIIL::URA3/ADE2* | WT | 0 | Li and Lustig (1996) |
| AJL384-3b,386-3c | *MATα HIS3 ade2-1 trp1 leu2-3,112 ura3-1 RAP1* | — | 50 | Kyrion *et al.* (1993) |
| CZY1/RAP1 | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 VIIL::URA3 pRS313/RAP1* | E | 100 | Li and Lustig (1996) |
| CZY4/RAP1 | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 VIIL::URA3/ADE2 LexAS3 pRS313/RAP1* | WT | 0 | Lustig *et al.* (1996) |
| AJL 437-1d | *MAT***a** *HIS3 ade2-1 trp1 leu2-3,112 ura3Δ1::TRP1::ura3Δ1 VIIL::ura3/ADE2* | WT | 11.5 | Li and Lustig (1996) |
| MBH9 | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 VIIL::URA3/ADE2* (750-bp *Hae*III site) *pRS313/rap1-17* | E | 100 | A. J. Lustig, unpublished results |
| AJL 418 | W303a × 275-2aVIIL::UA | WT | 0 | This study |
| AJL 419 | AJL 412-2c × AJL 412-4d | E/E | 50 | This study |
| AJL 421 | AJL 418-3a × AJL 412-2c | WT/E | 25 | This study |
| AJL 425 | AJL 418-3a × W303α | WT | 0 | This study |
| AJL 426 | AJL 418-3a × AJL 384-3b | WT | 25 | This study |
| AJL 427 | AJL 418-3a × AJL 386-3c | WT | 25 | This study |
| AJL 459 | BL22-2b × BL27-11a | E/E | 50 | This study |
| YP8 | BL22-2b × AJL 437-1d | E/WT | 25 | This study |
| BL4-2 | W303α × AJL412-2c | RD | 25 | This study |
| BL4-3 | W303α × AJL412-2c | E | 25 | This study |
| YP1 | PKY021 × AJL275-2a::UA | WT | 0 | This study |
| YP2 | YBO152 × AJL 275-2a::UA | WT | 0 | This study |
| YP3 | PKY021 × MBH9 | E | 50 | This study |
| YP4 | YBO152 × MBH9 | E | 50 | This study |

[a] The telomere is wild type (WT) or elongated (E) in size. —, The absence of a marked telomere; RD, a rapid deletion of marked telomere to near wild-type size.
[b] The overall percentage of elongated telomeres.
[c] AJL 275-2a::UA is an abbreviation of the strain previously termed AJL 275-VIIL-ADE2 (Li and Lustig 1996).

VIIL:: *URA3*/*ADE2*-marked telomere. Cells were first grown on FOA-limiting adenine media and screened for FOA$^r$ white colonies. Given the unidirectionality of silencing, FOA$^r$ cells would be indicative of repression at the telomeric *ADE2* gene. On the other homolog, the *ADE2* gene must be derepressed to give rise to FOA$^r$ white colonies. These telomeres, therefore, must define the derepressed/repressed state. We then conducted fluctuation analysis on FOA-containing media for ≈20 generations to determine the degree of switching from derepressed/repressed to repressed/repressed states. The degree of switching is defined in this study as the number of repressed cells/total cells and is a semiquantitative indication of the rate of switching. This is required because the number of switched cells can be influenced by an increase in both the rate of switching and the stability of the switched state. This assay measures switching only at the VIIL:: *URA3*/*ADE2*-marked telomere.

To determine the degree of switching from derepressed to repressed states in diploids containing one elongated VIIL:: *URA3*/*ADE2*-marked telomere, fluctuation analysis was performed after the identification of white colonies. Cells were grown for ≈20 generations on limiting adenine media. The low level of switching from the derepressed state to the repressed state (Figure 2) precludes multiple switches. Therefore, in this case, the degree of switching is equal to the frequency of red-centered colonies within the population. In the case of AJL 419 and AJL 421, the percentage of FOA$^r$ cells was determined by a standard fluctuation analysis (Figure 4).

## RESULTS

**An assay for the heritability of transcriptional states at telomeres:** In this study we examine three parameters to investigate the process of "cellular memory." At a theoretical level, heritability refers to the ability of a given transcriptional state to be continuously transmitted to subsequent progeny. Operationally, we define the heritability of the repressed state as the degree of sectoring of cells initially repressed for transcription at the telomeric *ADE2* gene to cells derepressed for *ADE2* expression. An analogous operational definition holds for the heritability of derepressed cells. The degree of switching can be estimated by semiquantitative methods based on the frequency of conversion from one transcriptional state to the other. Finally, propagation refers to the extent to which silencing can spread from the telomere to distal chromatin regions. This is defined here as the percentage of *ADE2*-repressed cells that are repressed at the distal *URA3* gene (Figure 1). We have developed a model system to distinguish between the heritability of repressed and derepressed transcriptional states after selection for each state for a specified number of generations (Figure 1). The heritability assays use a yeast strain with a marked VIIL:: *URA3*/*ADE2* telomere, with *URA3* in the centromere-distal position (Figure 1, top). In the strain background used in these studies, the *ADE2* gene is repressed only poorly (Li and Lustig 1996), providing the greatest sensitivity for conditions that measure repression (Figure 1).

To measure the stability of the repressed state, newly arisen red (repressed) sectors, derived from the periphery of white colonies, were grown on limiting adenine media containing 5-fluoro-orotic acid (5-FOA) for a specified number of generations (Figure 1A). 5-FOA allows the growth of Ura3$^-$, but not Ura3$^+$, cells. Because the *URA3* gene is telomere-distal to the *ADE2* gene and silencing spreads unidirectionally, all Ura$^-$ cells must be Ade$^-$ (Renauld *et al.* 1993; Hecht *et al.* 1996). As a consequence, all 5-FOA$^r$ cells produce red colonies (data not shown). After ≈20 generations, cells are dispersed onto nonselective plates and the sectoring patterns are evaluated.

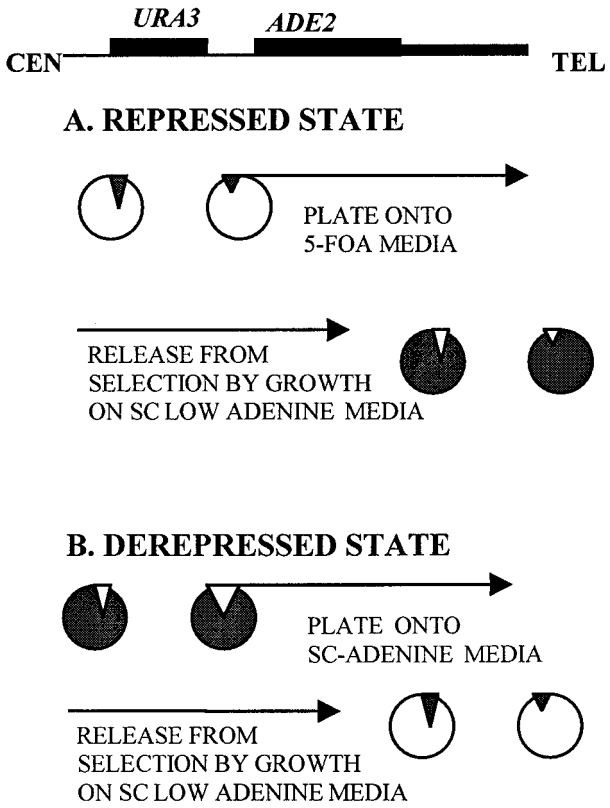


Figure 1.—Determining the heritability of the repressed and derepressed state at telomeres. (Top) A diagram of the VIIL:: *URA3*/*ADE2*-marked telomere used in these studies. The black boxes (from left to right) are the *URA3* and *ADE2* genes, respectively. The telomeric tract is depicted as a thick black line on the right. (Bottom) A pictorial representation of the assay (described in the text) to determine the heritability of repressed (A) and derepressed (B) states. Black and white indicate repressed (black) and derepressed (white) sectors or colonies, respectively.

In the converse experiment, we picked newly arisen derepressed Ade$^+$ (white) sectors from the extreme periphery of repressed Ade$^-$ (red) colonies and cultured the cells on adenine omission media [conditions that induce the *ADE2* gene (Gedvilaite and Sanauskas 1994)] for ≈20 generations (Figure 1B). We dispersed cells onto nonselective plates and evaluated the colony sectoring patterns. Southern analysis was used to confirm the lengths of telomeres in each experiment.

**Elongated telomeres increase the heritability of the**

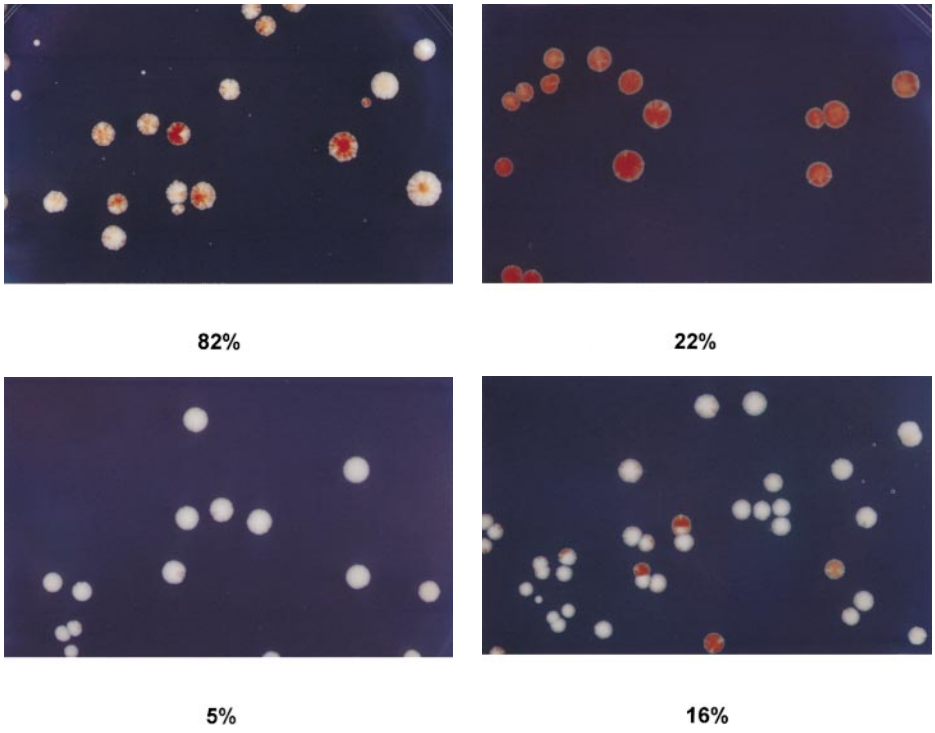


Figure 2.—The relationship of telomere length to the heritability of repressed and derepressed states. (Top left) Red sectors from the periphery of white colonies were picked from AJL 275-2a:: UA, carrying wild-type length telomeres. Cells were treated as described in Figure 1A, and the sectoring pattern (shown here) was analyzed. (Top right) Red sectors from the periphery of white colonies were picked from BL22-2b, carrying an elongated telomere. Cells were treated as in Figure 1A, and the plates were analyzed. (Bottom left) White sectors from the periphery of red colonies were picked from AJL275-2a:: UA, carrying wild-type length telomeres. Cells were treated as in Figure 1B and the plates were subsequently analyzed. (Bottom right) White sectors from the periphery of red colonies were picked from BL22-2b, carrying an elongated VIIL:: *URA3*/*ADE2*-marked telomere. Cells were treated as in Figure 1B and the plates were analyzed. In all experiments, elongated telomere size was confirmed by Southern analysis. The approximate switch rates from repressed to derepressed states are listed on the top, while the switch rates between derepressed and repressed states are shown on the bottom.

**repressed state:** We have previously demonstrated that elongated telomeres (produced in *rap1-17* mutant cells) confer a hyperrepressed state onto adjacent Pol II-transcribed genes (*e.g.*, VIIL:: *URA3*/*ADE2*) after reintroduction into a wild-type *RAP1* background (Kyrion *et al.* 1992; Li and Lustig 1996; Figure 1). We wished to explore whether tract-length-dependent hyperrepression, in otherwise wild-type cells, is associated with changes in the heritability of the repressed or derepressed states. We therefore conducted the stability assays using the *RAP1* cells containing a VIIL:: *URA3*/*ADE2*-marked telomere with either 300 bp wild type or elongated telomeric tract sizes of >700 bp (Figure 2).

Strikingly, the presence of an elongated VIIL:: *URA3*/*ADE2*-marked telomere in wild-type cells has a dramatic effect on the stability of the repressed state. After growth on 5-FOA-containing media, cells with the marked elongated telomere remain in the repressed state after an additional round of solid subculturing (*i.e.*, ≈20 generations; Figure 2, top right). Indeed, the majority of cells (63%) at the periphery of repressed colonies remained in that state after an additional 20 generations of growth. In contrast, isogenic cells containing a marked telomere of wild-type size gave rise to a high level of sectoring, indicating increased reversion to the derepressed state (Figure 2, top left).

To assay the heritability of the derepressed transcription state at the telomeric *ADE2* gene, cells were selected for *ADE2* by growth on adenine omission media and plated onto low adenine media. Cells containing the elongated VIIL:: *URA3*/*ADE2*-marked telomeres maintain the derepressed state after selection through 20 generations of colony growth. However, a slightly greater number of derepressed sectors appeared to be present at the periphery than in cells with wild-type telomeres (Figure 2, bottom).

The qualitative differences can be estimated semiquantitatively if two assumptions are fulfilled. First, the telomere length must be present at elongated sizes throughout colony growth. Rapid deletion events can shorten telomeres to wild-type length in <20 generations (Li and Lustig 1996). Such a process would distort any estimates that we may derive. Second, switches in state must form primarily at the periphery of colonies so that the semiquantitative methods reflect the observed switching. Both parameters are fulfilled based on Southern analysis and visual inspection of the colonies.

We first estimated the degree of switching from repressed to derepressed state. After growth on 5-FOA media (Figure 1), cells from 10 independent colonies were grown on nonselective media and assayed for the percentage of derepressed colonies in strains containing short (AJL275-2a::UA) or elongated (BL22-2b) telomeres. We found that strains carrying the elongated telomere contained an approximately fourfold decrease in repressed cells over cells containing wild-type telomeres, with switching values of ≈22 *vs.* ≈82%, respectively [Figure 2, top left (AJL275-2a::UA) and top right (BL22-2b)].

To assay the degree of switching from derepressed to

repressed states, we followed an analogous approach with cells grown on adenine omission media (Figure 2, bottom). We found that strains carrying the elongated telomere contained a threefold increase in repressed cells over cells containing wild-type telomeres, with switching values of ≈16 *vs.* ≈5%, respectively (Figure 2, bottom left and bottom right). Hence, the degree of switching from derepressed to repressed states is also increased in strains by the elongated telomeres. However, the apparent increase in switching to the derepressed state (Figure 2) may also be a reflection of increased heritability of the repressed state once formed.

**Elongated telomeres increase the propagation of the repressed state:** We were interested in examining the relationship between heritability and propagation. The distance that the repressed state is spread from telomeric sequences through subtelomeric chromatin (*i.e.*, propagation) is also clearly increased by telomere length. In cells containing wild-type-length telomeres, 2.5% of red ($Ade^-$) colonies were $Ura^-$. In contrast, 39% of red colonies that contain elongated telomeres formed 5-$FOA^r$ colonies (see Figure 5). Hence, telomere elongation appears to enhance the heritability and propagation of the repressed state, and consequently to decrease switching to the derepressed state relative to wild-type-length telomeres. These data suggest a mechanistic relationship between heritability and propagation.

**The relationship between heritability and telomere length:** Two models can explain the results described above. First, a specific relationship may exist between telomere length and heritability. Alternatively, any state that results in hyperrepression may lead to an increase in heritability of the telomeric *ADE2* and *URA3* genes. To test the latter possibility, we performed the heritability assays under a second distinct hyperrepressed state formed by tethering of LexA-Sir3p at the telomeric/subtelomeric junction in wild-type *RAP1* strains (Figure 3, top). Under these conditions, despite the wild-type telomere length, extremely high levels of Sir-dependent repression are attained at the telomeric *ADE2* gene (Lustig *et al.* 1996).

Assays for the heritability of the derepressed state were performed using CZY4/RAP1, which includes three LexA sites just distal to the telomeric tract (Figure 3, top), transformed by pBTM-SIR3 (encoding LexA-Sir3). Fluctuation analysis following expression of LexA-Sir3p yielded values of 98% repressed colonies, while expression of LexA displayed values of 11%. Remarkably, sectoring patterns of this strain display an unusual red (pink)/white ring-like colony phenotype (Figure 3, bottom). No effects on wild-type levels were observed in the absence of a LexA binding site or after transformation with LexA alone (data not shown). This result contrasts with the profound increase in the stability of the repressed state found in elongated telomere-mediated hyperrepression. These data from tethered strains containing wild-type-length telomeres in wild-type *RAP1* strains suggest the presence of rapid switches between repressed (red), partially repressed (pink), and derepressed (white) states during growth of the colony. These data also suggest that there may be multiple pathways to the hyperrepressed state.

We note that tethered LexA-Sir3p also displayed a marked decrease in propagation ($\approx 1 \times 10^{-4}$ $FOA^r$) compared to propagation induced by elongated telomeres (0.39 $FOA^r$, Figure 5). These data suggest a 3800-fold drop in propagation between tethering-induced and telomere-elongation-induced hyperrepression. These data once again link heritability to propagation.

**Elongation-mediated hyperrepression is telomere-autonomous:** The enhancement of silencing due to elongated telomeres might act solely intramolecularly (*in cis*) or through increased interactions with other telomeres or telomeric factors (*in trans*). To test this, we compared the effect of differing amounts of elongated telomeres on repression of a wild-type-length VIIL::*URA3*/*ADE2* telomere in diploid cells (see Figure 4). Silencing was measured in this case by the expression of the *URA3* gene distal to the *ADE2* gene. Wild-type cells containing 25% elongated telomeres were compared to strains carrying only wild-type-length VIIL::*URA3*/*ADE2*-marked telomeres (Figure 4A, lines 1 and 2). Repression was assayed by the frequency of 5-$FOA^r$ ($Ura^-$) colonies.

Our results indicated that the presence of the subpopulation of elongated telomeres did not increase the level of telomeric silencing at VIIL::*URA3*/*ADE2*. Another interpretation of these data is that 25% of elongated telomeres may not be sufficient to confer hyperrepression. To test this, we conducted the converse experiment in which 25% elongated telomeres including an elongated VIIL::*URA3*/*ADE2* (BL4-3; Figure 4A, line 3) were present. Under these conditions, hyperrepression in diploid strains was as high as observed in the haploid strains containing 50% elongated telomeres (≈34%; Figure 5), suggesting that the percentage of elongated telomeres between 25 and 50% does not alter silencing.

Furthermore, cells that differed from BL4-3 in only a deletion of the elongated VIIL::*URA3*/*ADE2*-marked telomere to wild-type size, eliminated hyperrepression (BL 4-2; Figure 4A, line 4). Because the only difference between the two strains is the presence of the elongated VIIL::*URA3*/*ADE2*-marked telomere, these data suggest that the degree of telomeric silencing is not the consequence of general telomere elongation nor the interaction among the telomeres of nonhomologous chromosomes. These data support the notion that an elongated telomere affects hyperrepression *in cis*.

Consistent with this hypothesis, a haploid strain containing an elongated VIIL::*ura3*/*ADE2* telomere cannot confer hyperrepression on the short *URA3*-marked telo-

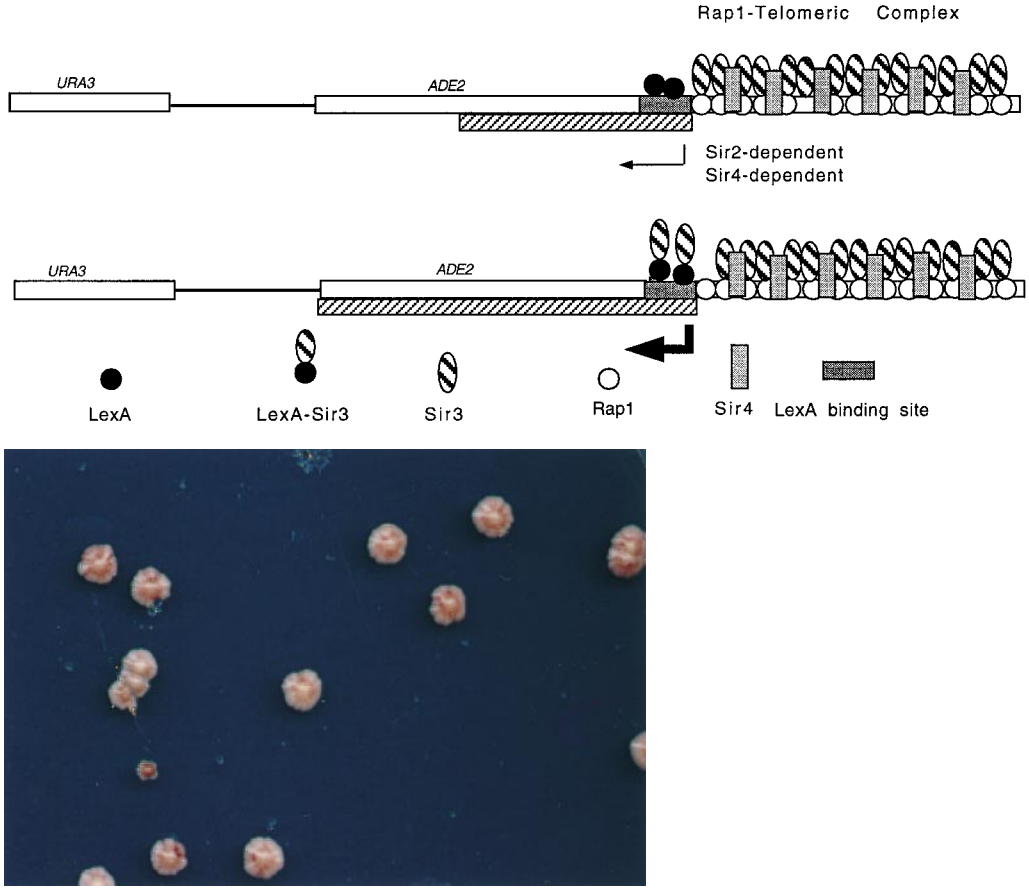


Figure 3.—Hyperrepression does not ensure enhanced heritability. (Top) Depiction of tethered silencing system. Tethering of the LexA protein does not affect silencing, while tethering by LexA-Sir3p leads to hyperrepression of silencing *in cis*. The degree of *ADE2* silencing is indicated by the thickness of the arrow. The extent of propagation is shown by the hatched bar. Other symbols are shown on the figure. Some of the proteins normally involved in wild-type telomeric silencing are also shown. (Bottom) White sectors from red or pink colonies of the *RAP1* strain CZY4/RAP1, containing wild-type length telomeres, were transformed with pBTM-SIR3 (encoding LexA-Sir3) and treated as described in Figure 1B. The sectoring patterns were analyzed as described in the text.

mere on the right side of chromosome V (VR:: *URA3*; Li 1998).

Can the two telomeres of homologs interact to facilitate silencing? To test this, we analyzed the degree of switching of a derepressed homolog containing a marked elongated telomere as a function of the structure of the homologous telomere in diploid cells (see materials and methods).

For YP8 (containing one elongated VIIL:: *URA3*/*ADE2* telomere and a second wild-type-length VIIL:: *ura3*/*ADE2*-marked telomere; Figure 4B, line 3) and AJL 459 (containing two elongated telomeres, one with a VIIL:: *URA3*/*ADE2*-marked telomere and the second with a VIIL:: *ura3*/*ADE2*-marked telomere; Figure 4B, line 2), FOA$^r$ white colonies were initially identified. For both strains, given the unidirectionality of silencing, white FOA$^r$ cells would be indicative of repression at the *ADE2* gene at VIIL in homolog 1 and derepression in homolog 2 (Figure 4B, lines 2 and 3). The degree of switching between the derepressed/repressed and repressed/repressed states was determined by measuring the percentage of white FOA$^r$ cells that switch to FOA$^r$ red cells after ≈20 generations of growth on nonselective media. This assay therefore measures switching only at the VIIL:: *ura3*/*ADE2*-marked telomere.

BL4-3 contains one VIIL:: *URA3*/*ADE2*-marked telomere, with a 25% chance that a second unmarked telomere is elongated. Recently derived white colonies were picked and standard fluctuation analysis was performed after ≈20 generations of growth. The degree of switching between the derepressed and repressed states was determined by measuring the percentage of derepressed (white) cells that switch to the repressed (red) state after ≈20 generations of growth on nonselective media (see Figure 4B, line 1; materials and methods).

Our results indicate that BL4-3 and AJL459 have similar high levels of switching (Figure 4B, lines 1 and 2). However, when a short homolog is paired with an elongated telomere (YP8), red FOA$^r$ cells are produced at wild-type values 50-fold lower than AJL459 (Figure 4B, line 3). Hence, the degree of switching is not substantially influenced by the size of its homolog, which further suggests an *in cis* effect.

Fluctuation analysis of a diploid strain containing elongated and wild-type-length VIIL:: *URA3*/*ADE2*-marked telomeres (AJL421; Figure 4B, line 4) demonstrated near-wild-type levels of silencing. Hence, the elongated telomere did not improve the low efficiency of silencing conferred by the wild-type-length telomere. Strains containing two elongated VIIL:: *URA3*/*ADE2*-marked telomeres (AJL419; Figure 4B, line 5), while yielding values ≈500-fold higher than AJL 421, did not display a positive effect on silencing relative to the efficiency of a single elongated telomere (Figure 4B, lines

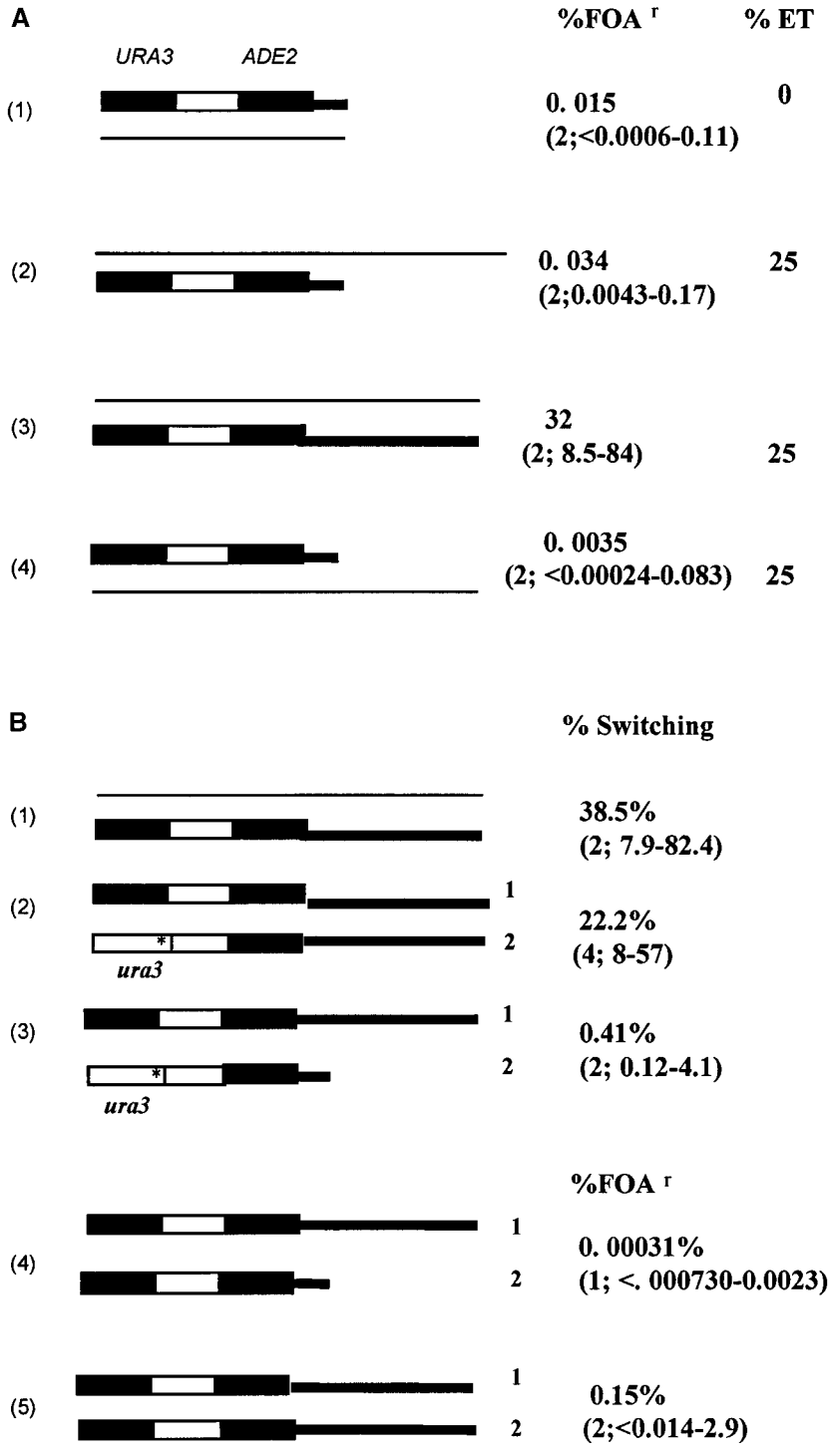


Figure 4.—Telomere elongation-mediated hyperrepression acts *in cis.* (A) Relationship between hyperrepression and overall length of the telomeric tract. Diploid strains containing a single wild-type length VIIL:: *URA3*/*ADE2*-marked telomere and either 0 (AJL 425; line 1) or 25% (AJL 427 and AJL 428; line 2) elongated telomeres were constructed as indicated (Table 1). BL4-3 (line 3) contained 25% elongated telomeres with an elongated VIIL:: *URA3*/*ADE2*-marked telomere. The strain BL4-2 (line 4) is isogenic to BL4-3 except for the deletion of VIIL:: *URA3*/*ADE2* to wild-type tract size. The average median is presented together with the sample sizes and the total range of values in the distribution, in parentheses. The thin line represents a potential nonhomologous chromosome that might pair and influence the silencing of the wild-type length VIIL:: *URA3*/*ADE2*-marked telomere. With the exception of BL4-3, which contained a marked telomere of 650–1100 bp, the remaining VIIL:: *URA3*/*ADE2* telomeres were wild type in length (300 bp). %ET refers to the expected percentage of elongated telomeres. (B) Interactions among telomeres of homologous chromosomes. The degree of switching from the derepressed state to the repressed state was assayed. BL4-3 (line 1), which was used as a hyperrepression control, contains telomeres of 1.1 kb. The second strain, AJL 459 (line 2), contains two elongated VIIL telomeres, one of which contains a VIIL:: *ura3*/*ADE2*-marked telomere (homolog 2) and the other a VIIL:: *URA3*/*ADE2*-marked telomere (homolog 1). AJL 459 telomeres consisted of two elongated forms: 1.3 and 1.6 kb. In the experiments assayed, each of the seven AJL 459 colonies from the fluctuation assay contained two telomeres between 1.1 and 1.4 kb. The third strain, YP8 (line 3), is identical to AJL459 except that the VIIL:: *ura3*/*ADE2* (homolog 2) telomere is wild type in length, while the lengths of the marked elongated telomeres in two experiments were ≈1.1 and 1.5 kb, respectively. In the fluctuation assays, each of the samples contained telomeres from 0.9 to 0.95 and 1.25 to 1.5 kb, respectively. In both A and B, the 5-FOA$^r$ values are presented as percentages. The asterisks refer to mutations within the *URA3* gene. White boxes represent mutant *ura3* genes, while black boxes refer to the wild-type gene. The fourth strain, AJL 421 (line 4), contains two VIIL:: *URA3*/*ADE2*-marked telomeres, one of wild-type length and a second elongated telomere of >700 bp. The fifth strain, AJL419 (line 5), contains two elongated marked telomeres with telomere sizes of >700 bp. In both cases, silencing was measured by standard fluctuation analysis and the frequency of FOA$^r$ cells was determined (*i.e.*, FOA$^r$/total cells). The data were converted into a percentage value.

4 and 5). Taken together, these data indicate the absence of interaction among the telomeres of homologs that could promote the repressed state and provide an additional compelling argument that the heritability of the repressed state functions intramolecularly (*in cis*) to promote hyperrepression.

**Telomere elongation overcomes the requirement for yCAF-1:** Recent experiments have demonstrated that mutations in any one of the three subunits (*CAC1*, *CAC2*, and *CAC3*) of the yeast chromatin assembly factor I (yCAF-1) cause a significant reduction in telomeric silencing (Enomoto *et al.* 1997; Kaufman *et al.* 1997;

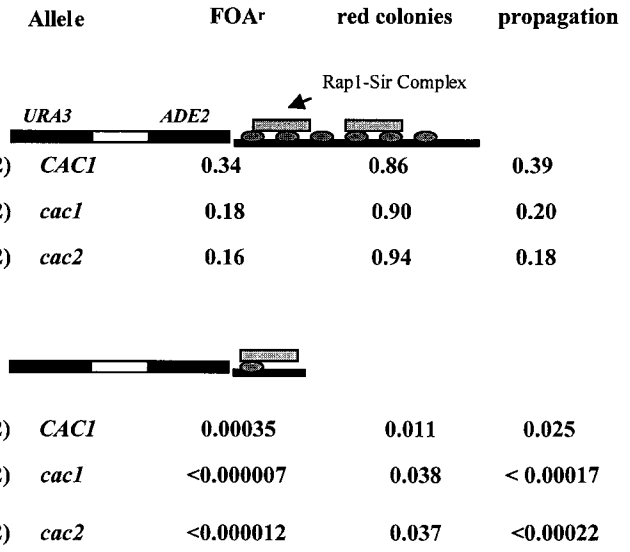


Figure 5.—Telomere elongation overcomes the requirement for yCAF-1 in the propagation of silencing. Wild-type, *cac1*, and *cac2* strains containing a VIIL:: *URA3*/*ADE2*-marked telomere with or without elongated telomeres were derived from sporulation of the diploids YP1, YP2, YP3, and YP4. The appropriate spore colonies were assayed for the fraction of red and FOA$^r$ colonies in wild-type, *cac1*, and *cac2* backgrounds. The number of fluctuation analyses carried out is shown in parentheses. We note that all elongated telomeres in this experiment ranged from 1.9 to 2.6 kb. In each case, the average of the medians of different experiments is shown. In the diagram of the elongated and wild-type-length marked telomere, black boxes indicate the *URA3* and *ADE2* genes, gray ovals represent Rap1p, and Sir3p is denoted by a rectangular gray box.

Monson *et al.* 1997). At a VIIL:: *URA3*/*ADE2*-marked telomere, each of the *cac* mutants abrogate the formation of wild-type-size 5-FOA$^r$ colonies, but produce 5-FOA$^r$ microcolonies at a frequency of 0.25% (Kaufman *et al.* 1997; data not shown). A previous study using single-cell analysis indicated that *cac1* mutants have a decreased probability of inheritance of the repressed state from preexisting repressed cells (Monson *et al.* 1997). The *cac* mutants can also produce an unstable repressed state at *HML* that is poorly maintained. This is a probable reflection of a CAF-1 requirement in maintaining the continual deposition of histones during heterochromatin formation (Enomoto *et al.* 1997; Kaufman *et al.* 1997).

Is elongation-mediated heritability of the repressed state dependent on yCAF-1? To test this, we first examined the effect of the *cac1* and *cac2* null mutations on telomeric silencing at the VIIL:: *URA3*/*ADE2*-marked telomere. Interestingly, unlike the effect of this mutation at other sites, silencing at the *ADE2* gene adjacent to both wild-type-length and elongated telomeres is *CAC1*- and *CAC2*-independent (Figure 5). Rather, the extent of propagation of silencing from the *ADE2* gene into the *URA3* gene (2.5%) in wild-type cells carrying wild-type-length telomeres was reduced ≈150-fold in *cac1* and *cac2* cells. In contrast, *CAC1* cells containing elongated telomeres displayed 39% propagation, which decreased only 2-fold in *cac1* and *cac2* mutant cells (Figure 5). Hence, propagation of the hyperrepressed state due to telomere elongation is *CAC1*- and *CAC2*-independent. In addition, microcolonies observed in *cac1* and *cac2* cells are abrogated in the presence of the elongated VIIL:: *URA3*/*ADE2*-marked telomere.

## DISCUSSION

In these studies, we have provided the first evidence for a role of telomere structure in cellular memory, specifically in the heritability of the "closed chromatin" adjacent to telomeres. This hypothesis is consistent with previous reports indicating that the *HML* silencer is required for heritability and repressed chromatin states in yeast (Holmes and Broach 1996; Bi and Broach 1997; Ansari and Gartenberg 1999). We have also found that the degree of propagation is associated with the increased heritability of the repressed state, suggesting that heritability and spreading may be functionally interrelated rather than distinct steps in silencing.

The system that we used to assay heritability and propagation was a comparison of these parameters in cells containing wild-type-length or elongated VIIL:: URA3/ADE2-marked telomeres. Using this assay, we found that telomere elongation leads to an increase in both heritability and propagation. This process acts on the elongated telomere *in cis* and, unlike wild-type-length telomeres, is independent of CAF-1.

The possibility that elongated telomere-mediated hyperrepression operates in a distinct pathway of telomeric silencing is highly unlikely, as silencing remains dependent on the Rap1 C terminus, Sir2p, Sir3p, and Sir4p (Li 1998). High levels of silencing are not sufficient to promote heritability and propagation, because tethering of LexA-Sir3p at the subtelomeric/telomeric junction did not display either phenotype. Thus, telomere length is likely to regulate the factors and/or structures that are necessary for both heritability and propagation of the repressed state.

This increased inheritance and propagation appears to require the continued presence of the elongated telomere. This conclusion is based on the finding that rapid deletion of a VIIL:: *URA3*/*ADE2*-marked telomere to wild-type length also abrogates hyperrepression (see Figure 4B; Li and Lustig 1996). Rapid deletion is an intrachromatid recombination process that can delete elongated telomere tract sequences to near wild-type length in <20 generations (Li and Lustig 1996).

An alternative, albeit less likely possibility, is that the experimental procedure for measuring the stability of the repressed state may produce a bias for a subset of cells. Because propagation is measured as the percentage of repressed telomere-proximal genes (*i.e.*, *ADE2* genes) that are also repressed in distal genes (*i.e.*, *URA3*

genes), growth on 5-FOA media in the heritability assays may select for those cells that are propagated to greater distances. In this scenario, the high levels of 5-FOA resistance in the absence of subsequent selection would suggest the presence of a molecular memory of the propagated state.

Our data suggest that telomere length affects the stability of the repressed state *in cis* rather than *in trans.* This study therefore argues against any strong negative transvection effect at the telomere that may occur through the communication between homologs or nonhomologs. It also argues against titration of a limiting factor at the telomere.

These *in cis* effects are particularly intriguing given the numerous instances in which telomeric processes (acting *in trans*) are dependent upon associations with either other telomeres or telomere-associating factors (Cockell *et al.* 1998; Lustig 1998). For example, recombinational telomeric rapid deletion displays a strong dependence on the length of other telomeres in the cell (Li and Lustig 1996). In addition, silencing appears to require sequestration of telomeres and silencing factors to a specific nuclear location mediated through *in trans* effects among telomeres and telomeric factors (Gotta *et al.* 1996; Maillet *et al.* 1996; Marcand *et al.* 1996). In addition, the creation of double-strand DNA breaks induces the exchange of silencing factors from the telomeres to double-strand breaks (Martin *et al.* 1999; Mills *et al.* 1999). The *in cis* effect that we observe contrasts with the *HMR* locus in diploid cells. At *HMR*, the level of repression appears to be controlled by cell type rather than by a mechanism acting independently on the two homologs (Sussel *et al.* 1993).

In our experiments, *cac1* and *cac2* mutants had no effect on the repression of the telomere-proximal *ADE2* gene on the left arm of chromosome VII. The major effects of mutations in *cac1* and *cac2* cells are on the propagation of silencing from the *ADE2* gene into the *URA3* gene at a wild-type-length VIIL:: *URA3*/*ADE2* as well as the formation of microcolonies. The reason for the diminished effects of *cac* mutations on telomeric silencing mutants, observed in other studies (*e.g.*, Kaufman *et al.* 1997), is as yet unclear. However, it has been previously noted that telomeric silencing is context- and gene-specific, and possibly dependent on the chromatin structure of each terminus (Gottschling *et al.* 1990; Monson *et al.* 1997).

How can telomere length influence transcriptional heritability? Our data at present appear to best fit a structural model for the heritability of the repressed state. In this model, a telomeric structural switch, formed *in cis* late in DNA replication, triggers the formation of highly stable and specialized subtelomeric chromatin that may confer the greater heritability of the repressed state. We propose that structures produced by elongated telomeres in yeast may form such a structural switch efficiently. Hence, the relative stability of telomeric length through DNA replication may provide the cellular memory in silencing. The extent of the telomere-induced stability on subtelomeric chromatin may then promote the propagation of silencing. The structural switch model is also consistent with the lack of a linear correlation between silencing and telomere length that would be expected from a purely length-mediated process (Kyrion *et al.* 1993).

One of the subtelomeric structures that may be formed is a fold-back structure, mediated through interactions between telomeric and subtelomeric factors (Grunstein 1997). Clearly, other more complex telomeric/subtelomeric associations are also conceivable. Regardless, the formation of this stable structure could act as an intramolecular "lock," preventing association and dissociation of silencing factors, as well as preventing further competition with activators in the G2/M phase of the cell cycle (Aparicio and Gottschling 1994). A similar scenario may occur at wild-type telomeres, except that the rate and/or stability of the structure may be significantly reduced. In this model, elongated telomeres and yCAF-1, possibly targeted by PCNA (Krude 1999; Shibahara and Stillman 1999), might act redundantly late in DNA replication in the formation or stabilization of the optimum structure for silencing (Ferguson *et al.* 1991; Ferguson and Fangman 1992).

In summary, the structure of telomeres plays a major role in modulating the heritability of adjacent closed chromatin states. This may well reflect a more general role for the structure of repetitive regions in the formation of heterochromatin. This stands in contrast to some alternative forms of cellular memory in higher eukaryotes that involve the covalent modification of DNA (Martienssen and Richards 1995). The behavior of telomeres in higher eukaryotic cellular memory will be an important area for future investigation.

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