The EMBO Journal Vol.18 No.9 pp.2538–2550, 1999

Limitations of silencing at native yeast telomeres

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Silencing at native yeast telomeres, in which the subtelomeric elements are intact, is different from silencing at terminal truncations. The repression of *URA3* inserted in different subtelomeric positions at several chromosome ends was investigated. Many ends exhibit very little silencing close to the telomere, while others exhibit substantial repression in limited domains. Silencing at native ends is discontinuous, with maximal repression found adjacent to the ARS consensus sequence in the subtelomeric core X element. The level of repression declines precipitously towards the centromere. Mutation of the ARS sequence or an adjacent Abf1p-binding site significantly reduces silencing. The subtelomeric Y′ elements are resistant to silencing along their whole length, yet silencing can be re-established at the proximal X element. Deletion of *PPR1*, the transactivator of *URA3*, and *SIR3* overexpression do not increase repression or extend spreading of silencing to the same extent as with terminally truncated ends. *sir1*Δ causes partial derepression at X-ACS, in contrast to the lack of effect seen at terminal truncations. *orc2-1* and *orc5-1* have no effect on natural silencing yet cause derepression at truncated ends. X-ACS silencing requires the proximity of the telomere and is dependent on *SIR2*, *SIR3*, *SIR4* and *HDF1*. The structures found at native yeast telomeres appear to limit the potential of repressive chromatin.

Keywords: native telomeres/proto-silencers/silencing/X elements/Y′ elements

Introduction

Epigenetic ‘silencing’ of genes occurs at a number of different genomic locations in the yeast *Saccharomyces cerevisiae*: the silent mating-type loci *HML* and *HMR*, close to telomeres, and within the tandem rDNA array (see Sherman and Pillus, 1997; Lustig, 1998). Transcriptional repression appears to be due to a specialized chromatin structure in these regions. Several proteins are involved in repressive chromatin, including the histones H3 and H4, their associated acetylases and deacetylases, the silent information regulators *SIR1–SIR4* and the yeast origin recognition complex (ORC). Some proteins have roles in all of the domains, while others are more specific to particular regions (reviewed in Sherman and Pillus, 1997).

Silencing at telomeres, or telomere position effect (TPE), was first described using a terminal truncation that places *URA3* adjacent to a newly formed $TG_{1–3}$ tract, resulting in a deletion of all of the subtelomeric sequences normally found at yeast chromosome ends (Gottschling *et al*., 1990). The best studied of these is at *ADH4* on chromosome VII-L. The main features of silencing at this truncation and others of a similar nature include variegated expression; repression spreading continuously from the terminus, diminishing with increasing distance from the terminus; dependence on many of the genetic components of silencing at *HML* and *HMR*; lack of dependence on *SIR1*; sensitivity to the overexpression of *SIR3* and other silencing factors; and sensitivity to loss of the *URA3* transactivating factor *PPR1* (Gottschling *et al*., 1990; Aparicio *et al*., 1991; Renauld *et al*., 1993; Aparicio and Gottschling, 1994). Several models have been proposed to explain TPE. Most involve the interaction of Sir2p/3p/4p complexes with Rap1p at the $TG_{1–3}$, and with phased nucleosomes in the adjacent sequences (Grunstein, 1997, 1998; Wotton and Shore, 1997). Propagation of Sir3p and subsequently other silencing factors along the adjacent sequences, and the lability of this chromatin state, result in the variegated expression of genes in the vicinity. Recent work indicates a role for non-homologous end-joining proteins (Hdf1p, Hdf2p, Rad50p, Mre11p and Xrs2p) in TPE (Boulton and Jackson, 1998; Laroche *et al*., 1998) and/or telomere maintenance (Porter *et al*., 1996; Gravel *et al*., 1998; Nugent *et al*., 1998; Polotnianka *et al*., 1998).

Native chromosome ends in *S.cerevisiae* have a number of subtelomeric repeat elements, which vary between ends and strains. These include the Y′ element, several small elements designated subtelomeric repeats (STRs) A, B, C and D, and the 473 bp core X element (Louis *et al*., 1994; Pryde *et al*., 1995). Core X is the only repeat sequence that is found at all ends and, interestingly, it contains several potentially functional sequences. These include an ACS (ARS consensus sequence, the binding site for the yeast ORC) in all cases, and an Abf1p-binding site at 31 out of 32 ends. These are two of the three elements found at the *HM* silencers, with the third element (Rap1p-binding sites) being found within the telomeric repeat sequences nearby. In addition to the known elements of *HM* silencers, there are also binding sites for the telomere-binding protein Tbf1p in the STR elements (Brun *et al*., 1997).

The question remains as to the nature of telomeric silencing at native ends. Is it the same as that observed with modified truncated ends or, given the presence of core X, is it perhaps more like that found at the *HM* loci? A number of genes involved in carbon source utilization, such as the *SUC*, *MAL* and *MEL* gene families, are subtelomeric in location and are expressed (reviewed in Pryde and Louis, 1997). Previous studies in this laboratory found very little, if any, silencing within the Y′ elements

(Louis and Haber, 1990; Louis, 1995). However, a recent study suggests that natural telomeric silencing can occur (Vega-Palas *et al*., 1997, 1998). A Ty-5 element that is located 1.8 kb from the telomere of chromosome III-L is expressed at very low levels in wild-type strains, with the repression being *SIR3* dependent.

The work presented here describes the nature of telomeric silencing at native ends. *URA3* was introduced at several subtelomeric locations on different chromosome ends and levels of silencing determined at each of these locations. The effects of mutations within the core X-ACS and Abf1p-binding site are determined and the role of *SIR*s, *ORC2*, *ORC5* and *HDF1* are examined. In conjunction with Fourel *et al*. (1999), we can conclude that there are proto-silencers in the native subtelomeric regions that limit the domain and level of repressive chromatin at yeast telomeres, as well as potential insulating elements.

Results

Substantial silencing is seen at some ends which do not have a Y′ element, while others exhibit little repression

A series of isogenic strains was created, in which *URA3* was integrated into the subtelomeric region of a number of chromosome ends which do not have a Y′ element (hereafter termed X-only ends). In each case, *URA3* is orientated such that transcription proceeds towards the telomere. Transcriptional repression of the marker gene was determined by measuring the fraction of cells that were able to grow on plates containing 5-fluoro-orotic acid (5-FOA). Cells that are expressing the *URA3* gene product are killed when grown on 5-FOA, thus selecting for those cells in which the gene is not functioning (Boeke *et al*., 1984). Only those 5-FOA-resistant colonies that were able to grow when replica plated onto media lacking uracil, confirming that the resistance phenotype was due to silencing rather than mutation of the *URA3* gene, were counted. Telomeric repression was also measured in a control strain containing *URA3* at a terminally truncated chromosome VII-L. The average frequency of silencing observed in this strain was 58%, which is similar to that observed in previous studies (Gottschling *et al*., 1990; Renauld *et al*., 1993).

The levels of silencing observed at each chromosomal position are presented in Figure 1A and B. Also plotted on Figure 1A are the frequencies of 5-FOA resistance observed at V-R and VII-L in previous studies (taken from Gottschling *et al*., 1990; Renauld *et al*., 1993) and the gradients of repression at each end. The V-R and VII-L ends are truncated and lack native subtelomeric sequences. The results indicate that there are two classes of telomeric silencing at X-only ends. Three out of six ends (X-R, IV-L and III-R) which have *URA3* integrated at position 1 displayed levels of silencing of <1% (Figure 1B). At position 1, the promoter is ~1.75 kb centromere-proximal to the $TG_{1–3}$ sequence and ~1 kb centromere-proximal to the core X-ACS sequence. However, at the remaining three ends (XIII-R, XI-L and II-R), levels of silencing at this position are ~100-fold higher, similar to the levels observed at terminal truncations. A high level of repression is also seen on chromosome XIV-R when *URA3* is integrated at position 2, indicating that this end is also a member of the strongly silenced class.

There is a limited domain of repression centering on the X-ACS

In contrast to silencing at truncated ends, the region of repression at native telomeres is very limited, with silencing being maximal at position 1 (the X-ACS). 5-FOA resistance is undetectable at position 3 with weakly silenced ends, and either very low (<0.002%) or undetectable at position 4, with strongly silenced ends (Figure 1A and B). At terminal truncations, there is a log-linear drop in silencing of ~1–2 orders of magnitude per kilobase from the peak of silencing towards the centromere, in both wild-type and *ppr1*Δ strains (see Figure 1A). At native ends, there is a much more precipitous drop. For example, at XI-L it is 4–5 orders of magnitude per kilobase, such that silencing becomes undetectable by 2 kb from the X-ACS. This was true even when the *URA3* promoter was weakened using a *ppr1*Δ mutation (see below and Figure 2C).

Ends with both core X and Y′ elements also exhibit silencing near the X-ACS, with low levels of repression throughout the Y′ element

A series of isogenic strains was created in which *URA3* was integrated at several positions on Y′-containing chromosome ends (hereafter termed X–Y′ ends); within the Y′ element itself and at increasing distances internal to the element. The relationship between the location of the *URA3* reporter gene and levels of silencing is presented in Figure 1C.

In most strains, frequencies of 5-FOA resistance of <1% were observed when *URA3* was integrated at the X-ACS. The level decreases with increasing distance centromere-proximal to core X, and is undetectable by position 4. The only exception is chromosome IX-L, which displayed a level of silencing at X-ACS similar to that observed at strongly silenced X-only ends. This end is different from all other X–Y′ ends in that it contains a short stretch of mitochondrial intron DNA at the junction between core X and the Y′ element, instead of the STR elements which normally are found within this region (Louis and Haber, 1991).

Little or no silencing was observed when *URA3* was integrated immediately internal to the $TG_{1–3}$ telomere repeat sequences, placing the promoter ~1 kb from the telomere (position 9). This structure is essentially the same as the VII-L terminal truncations previously studied, with the exception of the presence of the native subtelomeric sequences. Frequencies of 5-FOA resistance were low (<0.01%) or undetectable when the marker gene was integrated at several other positions within the Y′ element. Similar results were observed at a number of Y′-containing ends, including IX-L. This indicates that the strong transcriptional repression seen at IX-L is restricted to the region immediately centromere-proximal to core X and that Y′ elements are protected from strong repression.

X-ACS silencing is dependent on sequences within the core X element

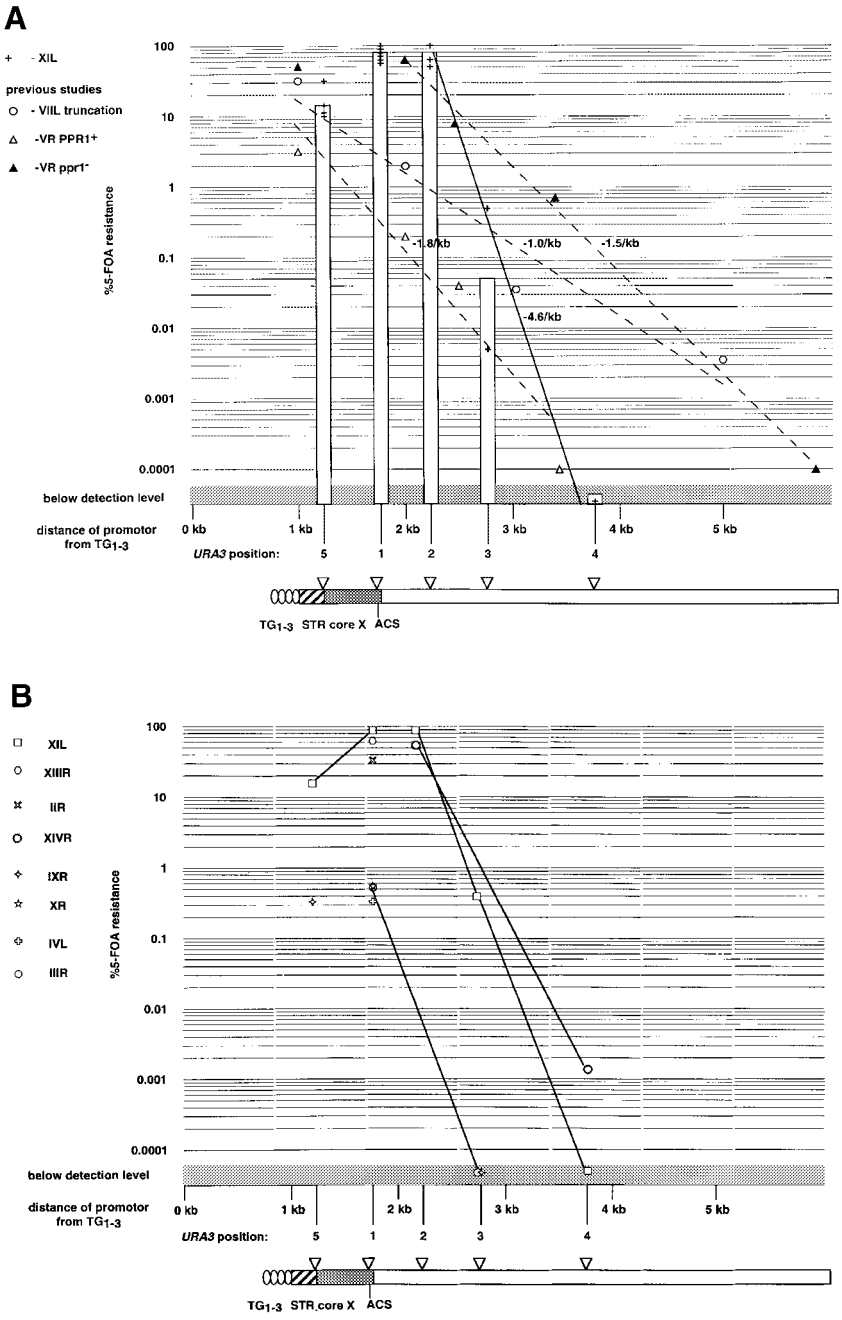
Core X elements contain two of the three sequences found within HM silencers, namely the ARS consensus sequence

and Abf1p-binding site. In order to determine if these sequences are required for repression at native telomeres, silencing was measured in isogenic strains carrying either intact or mutated core X elements, in the strain which contains *URA3* adjacent to the X-ACS of chromosome XI-L. The mutations introduced an *Nde*I restriction site into the ACS or an *Sph*I site into the Abf1p-binding site. Disabling the core X element significantly affected levels of silencing, with the Abf1p-binding site mutation resulting in a 26-fold reduction in repression, and mutation of the ACS reducing silencing by two orders of magnitude (Figure 2A).

Under the hypothesis that proteins essential for native silencing bind to core X, we tested whether large numbers of extrachromosomal core X elements could titrate these proteins and reduce levels of silencing. A high copy number plasmid containing core X and STR sequences from XI-L (pFEP33) was transformed into strains containing *URA3* either at the X-ACS on XI-L or at a terminally truncated VII-L end. Increased dosage of core X caused no differences in levels of silencing between the wild-type and plasmid-carrying strains in either case (data not shown).

One subtelomeric region appears to be inaccessible to transplacement recombination in a SIR-dependent manner

Initial attempts to integrate *URA3* into the core X–STR junction (position 5) resulted in no correct transformants. Those transformants that were recovered included conversion of the *ura3*Δ to *URA3* and integration at a number of genomic locations with rearrangements. This problem



was overcome by transforming into a *sir3::kanMX4* strain and subsequently mating with a *SIR3* wild-type strain, by protoplast fusion. *SIR3*$^+$ segregants were obtained for measurement of silencing. Frequencies of silencing at the X–STR junction were slightly lower than, but of the same order as, those observed at the X-ACS at any particular end (Figure 1B and C). Again, both strong and weak silencing were observed at X-only ends (Figure 1B, XI-L versus IX-R).

X-ACS silencing is dependent on SIR1 as well as on SIR2, -3 and -4

Mutations in *SIR2*, *-3* and *-4* completely abolish silencing at *HML*, *HMR* and terminally truncated telomeres (Rine and Herskowitz, 1987; Aparicio *et al*., 1991). Mutations in *SIR1* result in partial derepression at the *HM* loci (Rine *et al*., 1979) but have no effect at truncated telomeres (Aparicio *et al*., 1991). However, telomeric silencing is improved when Sir1p is targeted to the telomere (Chien *et al*., 1993). The involvement of the Sir proteins in native telomeric silencing was determined by measuring frequencies of 5-FOA resistance in isogenic wild-type or *sir*Δ strains. These strains contain *URA3* integrated at the X-ACS on chromosome XI-L. Mutations in *SIR2* and *SIR4* completely abrogated silencing, within the limits of the assay (0.00005%), whilst the *sir3*Δ mutation reduced the level of silencing by 2.5×10^4-fold (Figure 2A). Unlike the terminal truncations, *sir1*Δ also caused slight derepression, reducing the level of silencing by ~2-fold (Figure 2A), as seen by Fourel *et al*. (1999). Although the absolute 5-FOA resistance values are variable over different experiments, a 2-fold reduction was observed consistently between wild-type and *sir1*Δ pair-wise measurements. The *sir1*Δ and *sir3*Δ mutations were also introduced into the VII-L truncated strain. In agreement with previous studies, *sir1*Δ had no effect on silencing, while *sir3*Δ caused complete loss of repression (Figure 2A).

X-ACS silencing is dependent on HDF1 but not on ORC2 and ORC5

HDF1, the yeast homologue of mammalian *KU70*, is involved in the maintenance and subnuclear organization of telomeres (Porter *et al*., 1996; Laroche *et al*., 1998; Nugent *et al*., 1998). It has been shown recently that mutation of *HDF1* results in a disruption of telomere architecture in yeast, with the foci being spread randomly throughout the nucleus rather than at their normal peripheral locations (Laroche *et al*., 1998). Silencing of *URA3* at the X-ACS on XI-L was measured in isogenic wild-type and *hdf1*Δ strains. The deletion reduced silencing by ~5×10^3-fold (Figure 2B), indicating that *HDF1* is required for native telomeric silencing. It caused a similar level of

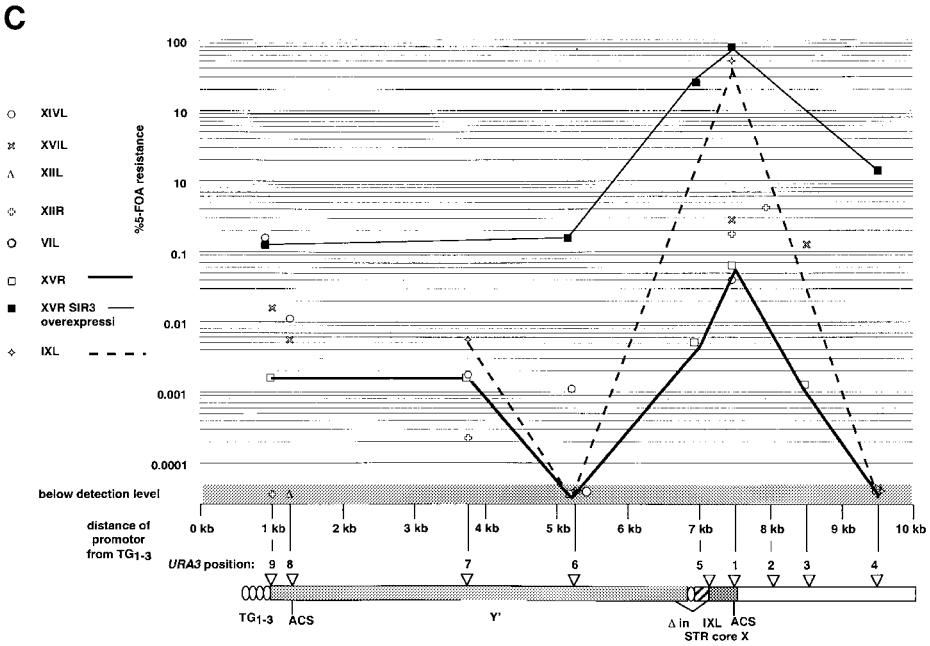


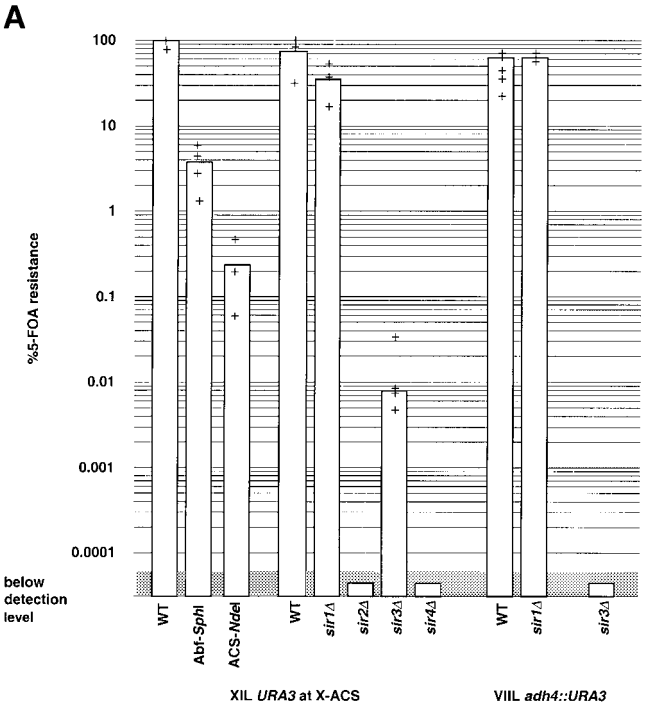
Fig. 1. Silencing measured at native subtelomeric locations. For measurement of silencing, cells were grown on non-selective plates and 10-fold serial dilutions plated onto synthetic complete medium (SC) and medium containing 5-FOA. Examples are presented in Figure 3. The structure of the subtelomeric region and the locations of the *URA3* insertions are shown for X-only and X–Y′ ends. Data points are connected for ends that have *URA3* insertions at most locations. (**A**) Silencing at the left end of XI (vertical bars) is compared with previous TPE studies at terminal truncations (Gottschling *et al*., 1990; Renauld *et al*., 1993). Maximal repression is seen at the XI-L X-ACS and 500 bp centromere-proximal to this location. Repression is reduced towards the telomere and drops off precipitously towards the centromere compared with artificial truncations. The gradient of repression at XI-L (solid line) is significantly steeper than those at terminal truncations (dashed lines). (**B**) Eight different X-only ends were marked at various locations with *URA3*. They appear to fall into two classes, with one set exhibiting substantial repression at the X-ACS region, as with XI-L. The other set exhibit <1% silencing, with repression again being maximal at the X-ACS. (**C**) X–Y′ ends were marked at the same locations as the X-only ends, as well as at several sites within the Y′ element. Very little repression is seen within Y′s even adjacent to the terminus. Silencing increases near the X-ACS as seen in X-only ends. The left end of IX, which has an unusual subtelomeric structure at the X–Y′ junction, exhibits substantial silencing at the X-ACS, 7.5 kb from the terminus, while exhibiting very little within the Y′ element, indicating discontinuous silencing at this native end. *SIR3* overexpression increases silencing within the core X domain at XV-R, but the spread of repressive chromatin is limited (see text and Figure 2D).

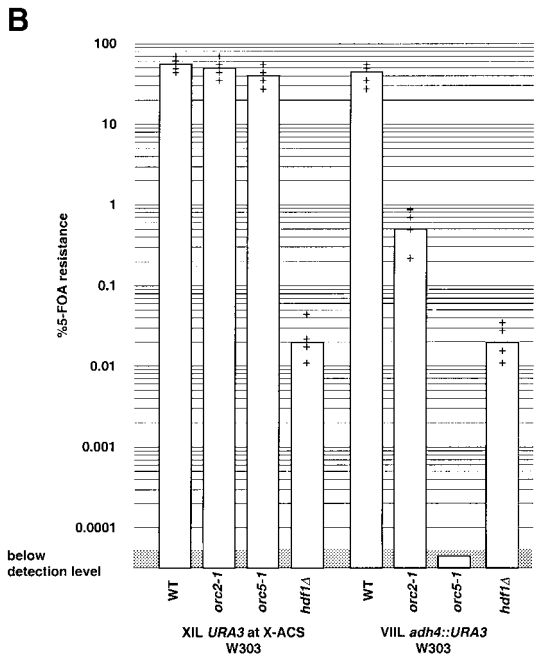
derepression at a terminally truncated VII-L end, as has been reported previously (Boulton and Jackson, 1998; Gravel *et al*., 1998; Laroche *et al*., 1998; Polotnianka *et al*., 1998).

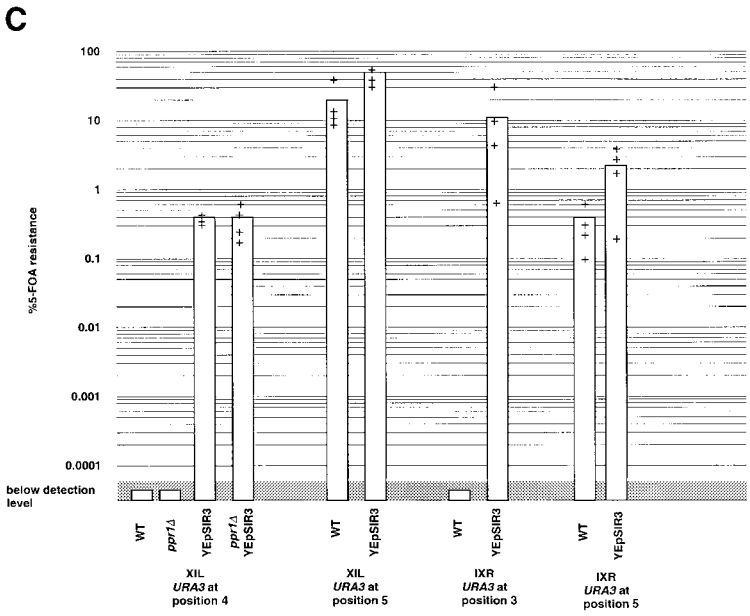
Silencing was also measured in a series of isogenic wild-type, *orc2-1* or *orc5-1* strains containing *URA3* integrated at position 1 on chromosome XI-L. Neither of the *ORC* mutations had a significant effect on silencing at native telomeres (see Figure 2B). However, as previously reported (Fox *et al*., 1995), *orc2-1* and *orc5-1* mutations resulted in severe derepression in strains containing the VII-L truncation.

ppr1 mutation and SIR3 overexpression have variable effects on native telomeric silencing

Previous studies utilizing terminal truncations have shown that a *ppr1*Δ mutation enhances telomeric repression of *URA3* by ~10^2- to 10^3-fold, and extends the spread of silencing (Renauld *et al*., 1993; Aparicio and Gottschling, 1994). To determine if native telomeric silencing was affected in the same way, a *ppr1*Δ version of a strain in which *URA3* is integrated at position 4 on XI-L was constructed. The *ppr1*Δ mutation did not extend the spread of repression, with 5-FOA resistance still being undetectable at position 4, which places the promoter only







1.5 kb proximal to the region of maximal repression (Figure 2C). The *ppr1*Δ mutation was also introduced into strains which contain *URA3* immediately internal to the telomere (position 9) or adjacent to the X-ACS (position 1) on XV-R. The level of silencing measured at position 9 remained at ~0.001%, but at position 1 it increased to 10% (data not shown).

Overexpression of *SIR3* has been shown to significantly increase silencing close to the telomere (up to 1.5×10^5-fold), and to extend silencing (>1%) to 16–22 kb from the telomere at a truncated end (Renauld *et al*., 1993), indicating that silencing would be measurable up to 35 kb from the end. To determine whether increased dosage of *SIR3* could extend silencing at native telomeres, a high copy number plasmid carrying *SIR3* (YEpSIR3) was introduced initially into strains which contained *URA3* at positions 5 or 4 on XI-L. *SIR3* overexpression increased the frequency of 5-FOA resistance by 2-fold at position 5, the X–STR junction, and from undetectable to 0.4% at position 4 (Figure 2C). 5-FOA resistance was also measured in a *ppr1*Δ version of the latter strain, with the same level of repression being detected. Extrapolation of this frequency from the point of maximum repression indicates that silencing would be undetectable by 7 kb from the telomere. *SIR3* overexpression therefore does not spread silencing at strongly silenced native ends to the same extent as at terminal truncations.

YEpSIR3 was also introduced into a series of strains in which *URA3* is located at varying positions on XV-R, a Y′-containing end (Figures 1C and 2D). Within the Y′ element, *SIR3* overexpression increased silencing, although the frequency of 5-FOA resistance remained <1%. However, when *URA3* was located at the X–STR junction or adjacent to the X-ACS, silencing increased to the level that is seen at strongly silenced ends. At position 4 (placing the *URA3* promoter 9.5 kb from the telomere, 2 kb from the X-ACS), *SIR3* overexpression increased 5-FOA resistance to ~1%. Extrapolation of this frequency from the point of maximum repression at core X indicates that the region of detectable silencing would be extended to 15 kb from the telomere, 7.5 kb from X-ACS. *SIR3* overexpression had a similar effect at IX-R, a weakly silenced X-only end, and IX-L, the strongly silenced X–Y′ end (see Figure 2C and D).

The X element does not silence independently of the telomere

It was determined whether the X element could act independently as a silencer and promote silencing of genes located at internal locations. PCR fragments containing the XI-L core X and associated STR sequences, marked with *URA3*, were targeted to either *LYS4* or *LEU2*, placing core X ~400 or 100 kb from the telomere, respectively. At *LYS4*, the X element was integrated in the same orientation with respect to the telomere as it is at the ends, with the X-ACS centromere proximal to the Abf1p-binding site. At *LEU2*, core X was integrated in either the same or opposite orientation with respect to the telomeres. In all three cases, 5-FOA-resistant colonies were undetectable (data not shown).

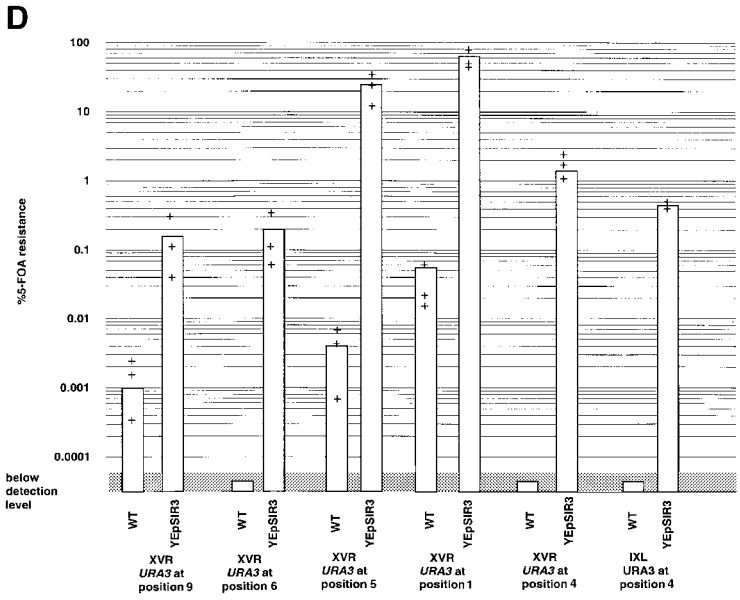


Fig. 2. The genetic requirements of native silencing. Silencing at the X-ACS on XI-L was measured in strains which carried mutations of the ACS- or Abf1p-binding site in core X. Silencing was also measured in *sir1*Δ, *sir2*Δ, *sir3*Δ, *sir4*Δ, *hdf1*Δ, *orc2-1* and *orc5-1* genetic backgrounds and compared with the VII-L terminal truncation in the same backgrounds. The effect of *ppr1*Δ and *SIR3* overexpression was also determined at various locations. (**A**) Mutation of the ACS or Abf1p-binding site reduce native silencing by up to two orders of magnitude, with the ACS mutation having the greater effect. As with the previous TPE studies, native silencing requires *SIR2*, *SIR3* and *SIR4*. In contrast to the artificial truncations (VII-L), native silencing is also affected by a mutation in *SIR1*, which reduces silencing by 2-fold. (**B**) Silencing at both ends was greatly reduced in the *hdf1*Δ background. Native silencing is not affected by the *orc2-1* and *orc5-1* mutations, while artificial TPE is severely affected in these strain backgrounds. (**C**) Increased expression of *SIR3* and deletion of *PPR1* had little effect on silencing at XI-L. *SIR3* overexpression increases the level of silencing seen at IX-R, a weakly silenced end, but not to the same extent as is seen at terminal truncations. (**D**) *SIR3* overexpression greatly increases the level of silencing seen at a number of positions on XV-R (an X–Y′ end), and at position 4 on IX-L, although again not to the same extent as with terminal truncations. Silencing within the Y′ element remains <1%. Native ends are more resistant to propagation of the repressive state than previously seen in terminal truncation studies.

Discussion

Much of what is known about silencing at yeast telomeres has been determined using terminal truncations of a specific end, with a tract of newly synthesized $TG_{1–3}$ formed adjacent to a marker gene. This has been a valuable tool for determining a great deal about telomere biology and chromosome architecture; however, nothing about the function of silencing or the extent of silencing on a whole genome level has been elucidated. There are several paradoxes concerning silencing at yeast telomeres. One is that the chromosome ends are utilized for gene amplification in certain situations, and it seems counterproductive to increase expression by amplification while at the same time repressing these genes. TPE is highly variable and unstable, and there does not appear to be any regulation of the silenced state, unlike that at *HML* and *HMR*. What would be the advantage of silencing if within a few generations the culture derived from a silenced cell gives rise to a large fraction of expressing cells and vice versa? Another paradox is the previous observations that markers embedded in a subtelomeric Y′ element were not subject to silencing (Louis, 1995), while markers more proximal had silencing enhanced if there was an adjacent Y′ (Renauld *et al*., 1993). This may indicate that some subtelomeric elements are resistant to silencing without affecting the adjacent regions.

In this study, we look at the properties of silencing at native chromosome ends in order to address some of these paradoxes. Our results show that there is silencing at native chromosome ends but the domains and levels of silencing are different from those for artificial terminal truncations. Native telomeric silencing also differs from that seen at *HML* and *HMR*.

Some ends exhibit very little silencing while others have substantial silencing

Since core X is structurally similar to the E and I sites of *HML* and *HMR*, we might expect to see high levels of silencing in this region. In X-only ends, there appear to be two classes of silencing, with maximal repression occurring at the X-ACS. Some of these ends exhibit substantial repression of *URA3* adjacent to the core X-ACS, with levels at least as high as those seen for the VII-L terminal truncation. Another set of ends exhibit lower levels at this position (<1% of a culture silenced). In X–Y′ ends, this location generally exhibits <1% of the culture being silenced, with one exception, IX-L, which is discussed more fully below. The differences are not an artefact of transformation since multiple transformants were obtained for most locations, with similar levels of repression being observed for each set of transformants. It is unclear as to why there are two classes of ends, one of which is protected from high levels of transcriptional repression. One possibility is that transcription of a nearby gene is preventing the spread of silent chromatin at the weakly silenced ends. However, there is no correlation between the distance of the closest open reading frame (ORF) to the telomere and levels of *URA3* repression. A second possibility could be the presence of anti-silencing elements at some ends. The STR elements have been found to prevent silencing from propagating proximally at an artificial VII-L truncation (Fourel *et al*., 1999). This effect could be ascribed to the binding of Tbf1p. All of the ends which were marked in this study were therefore compared for the presence of binding sites for a number of telomeric proteins, including Rap1p and Tbf1p. No major differences were found between the two classes of ends in terms of the number or location of binding sites. It is possible that there are yet to be identified anti-silencing elements centromere-proximal to core X at some ends.

The domain of silencing around the X-ACS is constrained, with silencing being dependent on sequences within core X

In both X-only and X–Y′ ends, the levels of silencing decrease both proximally and distally to the X-ACS (see Figure 1A–C). This is in contrast to the models of repression in which the repressive chromatin is propagated continuously from the telomere. In addition, the precipitous drop of 4.5–5 orders of magnitude in silencing per kilobase seen at native ends contrasts sharply with the 1–2 orders of magnitude per kilobase drop seen in previous studies with VII-L and V-R terminal truncations. It appears that there is establishment of a silenced domain at the X-ACS, which is more limited than that seen for terminal truncations. This may be due to the similarity of core X to the E and I silencers of the truly repressed *HML* and *HMR* loci. Indeed, mutation of either the ACS or the Abf1p-binding site within the core X element led to a reduction in silencing at the XI-L X-ACS marked end, with the ACS mutation having the greater effect. This observation indicates that silencing at native ends involves the interaction of proteins with the core X element. Previous studies have shown that the inclusion of the X element on a plasmid containing telomere repeat sequences improved plasmid segregation by ~3 fold. Mutation of the Abf1p-binding site resulted in a partial reduction of the increase in stability. Mutation of the ACS converts core X from a segregation-enhancing element to one which interferes with plasmid segregation (Enomoto *et al*., 1994).

It has been shown that the yeast Ty-5 retrotransposons integrate preferentially within or adjacent to the *HM* silencers and adjacent to the X-ACS (Zou *et al*., 1996), suggesting a similarity between the chromatin in each of these domains. Most of the subtelomeric Ty-5s, or their long terminal repeats (LTRs), lie within 0.8 kb of the X-ACS, with the remainder falling within 1.5 kb. This observation supports the proposal that there is a limited domain of silencing at native ends, centering on the X-ACS. The *SIR3*-dependent repression of Ty-5-1 (Vega-Palas *et al*., 1997, 1998) is consistent with these results since its promoter lies 0.75 kb from the III-L X-ACS, within the domain of repression.

Despite this similarity to *HML* and *HMR* in structure and limitations of repression, the silencing seen at native ends is still variegated in the sense that the cells appear to be unstable in their ON or OFF states and can switch between states.

The silencing at X-ACS requires the proximity of the telomere

In order to test whether the X region has autonomous silencing activity, the *URA3*-marked X-ACS from the strongly silenced XI-L end, along with flanking sequences

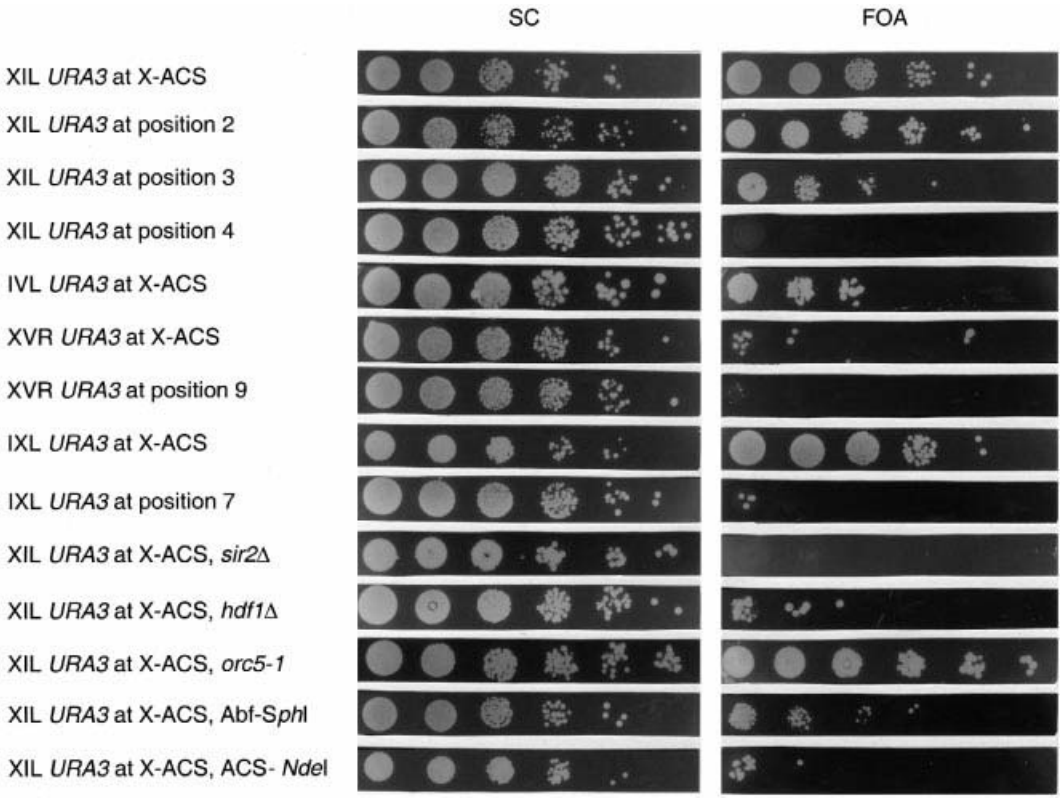


Fig. 3. Measurement of repression of *URA3* at various locations. Ten-fold serial dilutions of cells were plated onto SC media and medium containing 5-FOA.

including all of the STR elements, was inserted at internal locations on two chromosomes. In all cases, there was no measurable repression. Unlike the E and I elements, the X region is not a silencer in its own right and requires the proximity of the telomere. This could be due to the presence of Rap1p at the telomeres but not at the internal X regions. Core X had been shown to behave as a proto-silencer at *HML* in conjunction with *HML*-I (Fourel *et al*., 1999).

Y′s are resistant to silencing

Repression within Y′s was either very low (a maximum of 0.1% of a culture being 5-FOA resistant) or undetectable at all the locations studied. Minimal repression was even observed adjacent to the Y′-ACS or at the terminus of the Y′ element, with *URA3* the same distance from the $TG_{1–3}$ as in the terminal truncations. At IX-L, where repression at the X-ACS is at the same level as the strongly silenced class of X-only ends, the Y′ is still resistant to silencing. Previous studies have shown that Sir2p co-precipitates with Y′ DNA (Gotta *et al*., 1997). These results would therefore suggest that the binding of Sir proteins to the Y′ element is not sufficient to promote high levels of silencing at native ends. Alternatively, Sir proteins may not bind to all Y′ elements and the ends which were studied here may be those that lack the proteins, although this seems less likely. The ACS in Y′s is different from the one in core X in that it lies in the opposite orientation, and has no associated Abf1p-binding site. The distal end of Y′s contain a potential Tbf1p-binding site which may act as an anti-silencer. The flanking of Y′ elements by anti-silencing sequences could therefore explain their resistance to repression. However, this would not account for the low level of silencing seen within the Y′ element at IX-L, as this end lacks most of the STR sequences (Louis and Haber, 1991). This loss of potential STR-associated anti-silencers may, however, explain the high levels of repression seen at the X-ACS of this end.

The pattern of silencing seen at X–Y′ ends further indicates that repression at native ends is discontinuous, with repressive chromatin being able to assemble centromere-proximal to a region that is expressed. The strong repression observed at the IX-L X-ACS indicates that the low level of silencing observed at the X-ACS of most X–Y′ ends is not due to the distance of the marker from the telomere. Overexpression of *SIR3* has less of an effect on silencing when *URA3* is embedded in the Y′ element than when it is not. This again suggests that sequences embedded within Y′s are protected from high levels of repression, whilst stronger silencing can establish telomere-distal, at the X-ACS. A similar discontinuity in silencing has been noted by Fourel *et al*. (1999) who have observed the expression of a *TRP* gene adjacent to the telomere, while a *URA3* gene more distal to the terminus was repressed.

The genetic control of native telomere silencing is different from other silencing

Native silencing has some of the same genetic components as artificial TPE, such as derepression of the XI-L highly silenced end by *sir2*Δ, *sir3*Δ, *sir4*Δ and *hdf1*Δ. However, unlike artificial TPE, the *sir3*Δ mutation does not completely abrogate silencing. It has also been observed that *sir3*Δ does not completely derepress the *HMR* locus in

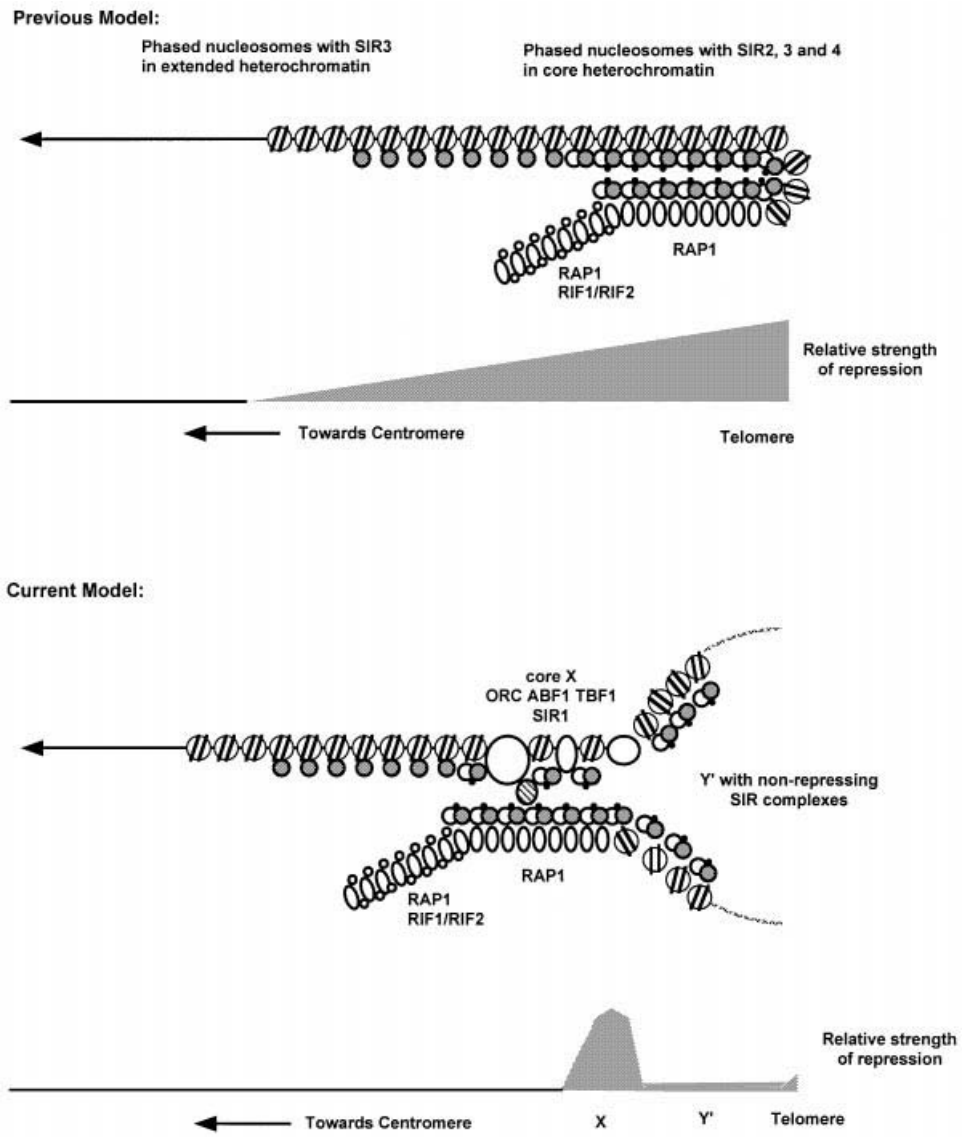


Fig. 4. A model for formation of repressive chromatin at native telomeres. Previous models propose that interactions of Sir complexes with histones and Rap1p cause telomeric DNA to fold back on itself, creating core heterochromatin. This core heterochromatin is able to spread on overexpression of *SIR3*. The new model proposes that it is the interaction of telomere-associated Rap1p–Sir complexes with proteins bound to core X that forms a tight domain of repressive chromatin at native telomeres. The Y′ elements are excluded from this domain. Overexpression of *SIR3* can enhance interactions within the domain but only extends the spread of silent chromatin by several kilobases.

Y55 strains (E.Louis, personal observations). In addition *sir1*Δ appears to have an effect on native silencing (2-fold derepression) while having no effect on the VII-L truncation construct. The effect of the X-ACS mutation and the involvement of *SIR1* suggest that native silencing may involve the ORC, which has an important role in silencing at *HML* and *HMR* (see Loo and Rine, 1995). The ORC has been shown to have a role in artificial TPE (Fox *et al*., 1997), which is surprising given that the artificial telomere constructs used in such studies lack the ORC-binding sites which normally are present in native subtelomeres (within the core X and Y′ elements). It has been proposed that the presence of the ORC bound to an adjacent chromosome end may be sufficient to promote silencing at a truncated end. As was previously reported, *orc2-1* and *orc5-1* result in derepression at the VII-L terminal truncation. Surprisingly, they had no effect on the highly repressed XI-L X-ACS marked end, although a similar decrease in repression to that seen with the *sir1*Δ mutation was expected. This paradox has yet to be resolved. Models of altered concentrations of silencing factors, or the association of truncated telomeres with native telomeres, cannot explain these data. It is also unclear how *SIR1* might be acting at native telomeres if it is not being tethered by the ORC, as is the case at *HML* and *HMR* (Chien *et al*., 1993; Triolo and Sternglanz, 1996; Fox *et al*., 1997). Two classes of *ORC5* alleles have been identified, which are either silencing or replication defective. The two types of alleles complement, suggesting that different ORC species function at different origins within the genome (Dillin and Rine, 1997). These results could therefore be explained by an alternative ORC species functioning at the X-ACS.

Silencing at native ends is less sensitive than artificial TPE to weakened marker promoters and to increased expression of *SIR3*. Although increased expression of

Table I. Oligonucleotides used for insertion of *URA3* at various locations

Positions based on XI-L		
1	S1	ATATTAAGGAACTTTAAGTTAATGATACCATGATAGTATTAAGACgcttttcaattcaattcatc
	S2	AAATATTCTATTCTTCAACCATAATACATAAACACACTTAATTGCaaatcattacgaccgagatt
2	S1	CTGAAAATATCAAAATTTCTGGGTTGCGATAGTTTTTGTGTAACCgcttttcaattcaattcatc
	S2	TTATTGTTGATAGAACACTAACCCTTCAGCTTTATTTCTGGTTACaaatcattacgaccgagatt
3	S1	AAGGACGGTATCTACACTATCGCAAATTAGAGACAAACGCCAATTgcttttcaattcaattcatc
	S2	TATTTTGTTCGTTAATTTTCAATGTCTATGGAAACCCGTTCGTAAaaatcattacgaccgagatt
4	S1	GTTACAATTTTATTTTTATCATACACGATTCTTTGAACATCAACTgcttttcaattcaattcatc
	S2	GCAATTACGTAATTGTAGCCGCTGAAGGCGGATGGTATTGAGAGAaaatcattacgaccgagatt
5	S1	ATGGCATGTGATGTGTTGGTGGGATTAGAGTGGTAGGGTAAGTATgcttttcaattcaattcatc
	S2	CCCTCCATATTGAAACGTTAACAAATAATCGTAAATAATACACATaaatcattacgaccgagatt
Positions based on XV-R		
1	S1	ATTAAGGAACTTTTACGTTAATGACGTCATGGTAGTGCTCGTACTgcttttcaattcaattcatc
	S2	AAATATTCCATTCTTCAACAATAATACATAAACATATTGACTTGAaaatcattacgaccgagatt
2	S1	AAACCCAAAATAGAAACTTCTCTTTTGCGTCACTGTTCTGGAAAAgcttttcaattcaattcatc
	S2	TATTGCTTATTGGACATACCCTATTAGCTTTATTACCATGCACCCaaatcattacgaccgagatt
3	S1	GCCGCTGCTCCAGCCACTACCACTCTATCTCCATCTGACGAAAGAgcttttcaattcaattcatc
	S2	AGCTCTGATATCGGAGACGTAAACACCCAATTCGACCAAGTTGACaaatcattacgaccgagatt
4	S1	TTTTTCAGCTTTCTGATTAATCTCTTCGGTTTAAATTTTTTAGCAgcttttcaattcaattcatc
	S2	GATTTGATACCAACCGCGTTATAAGGGTTACTAGAAAGTAATAGCaaatcattacgaccgagatt
5	S1	TACGGCATGTGGTGGTAGGGTAAGTATATGTGTATTATTTACGATgcttttcaattcaattcatc
	S2	CACCATACTGTTGTTCTACCCACCATGTTGAAACGTTAACAAATGaaatcattacgaccgagatt
6	S1	ACGTAGATGAGCTATCGATTTTTTCTGCATACCAAGCAAGTTTACgcttttcaattcaattcatc
	S2	ATAGATCACGCTTCAGCCGCTCTGTGTCGACTTTCTTTTCGCCAGaaatcattacgaccgagatt
7	S1	TTAGCCCTCTTTGAAATTGAACCAGAGTCGAAGGCCATTGTAGTTgcttttcaattcaattcatc
	S2	TCTCCAAGAGCAGGCCAATTCTTCCACTTCGTTGGTTGTGCTTGCaaatcattacgaccgagatt
8	S1	GTATTTCACTGTTTTGATTTAGTGTTTGTTGCACGGCAGTAGCGAgcttttcaattcaattcatc
	S2	CTTTTTATAGATTGTCTTTTTATCCTACTCTTTCCCACTTGTCTCaaatcattacgaccgagatt
9	S1	AGAACAGGGTTTCATTTTCATTTTTTTTTTTTAATTTCGGTCAGAgcttttcaattcaattcatc
	S2	CTCTCACATCTACCTCTACTCTCGCTGTCATACCTTACCCGGCTTaaatcattacgaccgagatt

S1 is the centromere proximal side and S2 is the distal side of the site of insertion. Lower case bases are the sequences homologous to *URA3*.

Table II. Oligonucleotides used for complete ORF replacement by *kanMX4*

HDF1	S1	ATGATTTGTTAAGTGACTCTAAGCCTGATTTTAAAACGGGAATATTcgtacgctgcaggtcgac
	S2	AAATATTGTATGTAACGTTATAGATATGAAGGATTTCAATCGTCTAtcgatgaattcgagctcg
SIR1	S1	GAATTTGGGCACATGTGACCCGGAATGTATATTGAGTAATATAAGAcgtacgctgcaggtcgac
	S2	CACCCGCTTATATGTTGGTATCCATAACTGATAATCTTACCAACTAtcgatgaattcgagctcg
SIR2	S1	TAGACACATTCAAACCATTTTTCCCTCATCGGCACATTAAAGCTGGcgtacgctgcaggtcgac
	S2	ATTGATATTAATTTGGCACTTTTAAATTATTAAATTGCCTTCTACAtcgatgaattcgagctcg
SIR3	S1	AGAGGTTTAAGAAAGTTGTTTTGTTCTAACAATTGGATTAGCTAAAcgtacgctgcaggtcgac
	S2	GTACATAGGCATATTTATGGCGGAAGTGAAAATGAATGTTGGTGGAtcgatgaattcgagctcg
SIR4	S1	AAGGAAGCTTCAACCCACAATACCAAAAAAGCGAAGAAAACAGCCAcgtacgctgcaggtcgac
	S2	AACAGGGTACACTTCGTTACTGGTCTTTTGTAGAATGATAAAAAGAtcgatgaattcgagctcg

S1 is the upstream end and S2 is the downstream end of the genes. Lower case bases are the sequences homologous to the pFA6a-*kanMX4*.

SIR3 has a significant effect at weakly silenced X-only ends and X–Y′ ends, the increase in repression is still orders of magnitude less than that seen with terminal truncations. The spread of repressive chromatin appears to be being blocked. In general, *ppr1*Δ strains do not exhibit an increased repression, indicating that resistance to spread of silencing at native ends is not due to the transcription factors of the marker gene blocking repressive chromatin.

The sequences at *HMR* and *HML* are resistant to integration due to either the inability of sequences to recombine or the lack of expression of inserted sequences (R.Borts, personal communication). In contrast, most telomeric sequences are easily targeted by transformation, with the exception of the X–STR junction. This resistance to transplacement into the X–STR junction was relieved in *sir3*Δ strains, where the insertion of *URA3* at the junction was easily recovered. However, in *SIR3* derivatives of the X–STR insertions, the level of silencing was no higher than that seen at the X-ACS. It is possible that the *URA3* insertion altered the chromatin structure at this location such that it now exhibits variegated expression rather than complete repression.

Model for repression at native telomeres

Previous studies have mapped Sir2p, Sir3p and Sir4p along telomeric heterochromatin and have shown the association of these proteins with telomeric DNA to reflect the gradient in silencing observed at truncated telomeres (Hecht *et al*., 1996; Strahl-Bossinger *et al*., 1997). Models to explain artificial TPE (reviewed in Grunstein, 1998) propose that the folding back of telomeric DNA allows interaction between Sir protein complexes bound to Rap1p, and those associated with the more internal histones (see Figure 4). These complexes form core heterochromatin which can spread through interactions between Sir3p and the histones. A similar looping model has been proposed to explain silencing in *Drosophila*, with interactions between

Table III. Oligonucleotides used for confirming *sir1::kanMX4* and *hdf1::kanMX4*

HDF1	A1	ACAACAGGTCACTTCTGCAAG
	A4	GGGACCCACAAAGTAATTGTC
SIR1	A1	CCTCAAGCGAATGGTGGATTCCTT
	A4	TTAACGGTACTACAGTACGGCTCG
kanMX4	K2	TTCAGAAACAACTCTGGCGCA
	K3	CATCCTATGGAACTGCCTCGG

Polycomb group proteins leading to the formation of a core silencing complex (reviewed in Pirrotta, 1997). The results presented here indicate that these interactions alone are not sufficient for silencing at native telomeres, since Y′ elements are resistant to high levels of repression and the domain of silencing is limited at all native ends. In an alternative model, we propose that silencing at native ends requires the interaction of telomere-associated Rap1p/Sir2p,3p,4p complexes with proteins bound at core X, leading to the formation of a region of highly repressive chromatin. The folding process required for these interactions to occur may account for the physical inaccessibility observed at the X–STR junction. The folding back of the telomere would also result in the looping out of the Y′ elements, removing them from the condensed region (Figure 4). In most cases, the presence of a Y′ element causes a low level of silencing at the X-ACS. This is presumably due to a weakening of the protein interactions at core X, although the reason behind this has yet to be determined. Unlike artificial TPE, the core heterochromatin is limited in its ability to spread internally. Overexpression of *SIR3* increases the level of repression at weakly silenced ends to that of strongly silenced ends, within the core X domain, possibly by enhancing the interactions at core X. However, in all cases, the domain of repression can be extended by only a few kilobases as opposed to the tens of kilobases seen with terminal truncations. The structures found at native telomeres prevent the propagation of silent chromatin as seen at terminal truncations. The question as to why some X-only ends are resistant to high levels of silencing remains to be answered. It is possible that those ends which exhibit minimal silencing are not bound by an ORC, or are bound by a different ORC species that is not competent for repression. As discussed above, there may also be anti-silencing elements at some ends.

Is native telomere silencing for repression or is it architectural?

It seems unlikely that the function of TPE is for repression of nearby genes, due to the instability of the repressive state and the fact that the subtelomeric region is used for gene amplification in some circumstances. It is possible that repression is in fact secondary to the true role of the sequences and proteins involved in silencing, such as in the nuclear localization of the telomeres. A barrier to recombination between sequences at telomeric and internal locations has been found (A.C.Timbrell, T.C.Huckle, A.P.Underwood, D.E.Eyre, H.C.Gorham, R.H.Borts and E.J.Louis, unpublished). A structure involving the proteins bound to telomeric and subtelomeric sequences may result in such a barrier and, indeed, *HDF2*, the yeast *KU80* homologue, was recovered in a screen for genes involved in the separation of telomeric and internal domains (A.C.Timbrell, T.C.Huckle, A.P.Underwood, D.E.Eyre, H.C.Gorham, R.H.Borts and E.J.Louis, unpublished). The possible connection between the recombinational barrier, nuclear architecture and silencing is seen in the disruption of telomeric localization (Laroche *et al*., 1998) and abrogation of silencing in *hdf1*Δ strains.

Materials and methods

Plasmid constructions

Plasmid pEL89H contains the terminal *Hin*dIII fragment from the left arm of chromosome XI, subcloned into the polylinker of pGEM3Z^{f} (–). Plasmid pFEP22 was created by ligating an oligonucleotide containing a *Bgl*II and a *Sal*I restriction site (upstream oligo, 5′-AATTAGATCTGT-CGACGT-3′; downstream oligo, 5′-AATTACGTCGACAGATCT-3′) into the *Mun*I site of pEL89H. The *Bgl*II site lies 5′ to the *Sal*I site in pFEP22. A *Bam*HI-linkered *URA3* fragment was ligated into the *Bgl*II site of pFEP22, creating plasmid pFEP24.

Plasmid pFEP33 was constructed by the insertion of a core X PCR fragment, carrying *Bgl*II linkers, into the *Bam*HI site of plasmid YEpFAT10 (Runge *et al*., 1989). Plasmid pFA6a-*kanMX4* (Wach *et al*., 1994) was used for the disruption of *SIR1–4* and *HDF1*. Plasmids YEpSIR3 (Renauld *et al*., 1993) and pΔPPR1::HIS3 (Renauld *et al*., 1993) were used for the *ppr1*Δ and *SIR3* overexpression studies, respectively.

PCR amplification of URA3

Unless otherwise indicated, *URA3*-marked strains (see Figure 1) were created using a PCR amplification method based on that of Baudin *et al*. (1993). PCR fragments were generated using primers as indicated in Table I. PCR amplification was performed in a total volume of 50 µl, containing 100 ng of plasmid pFEP24, 200 µM of each dNTP, 5 µl of 10× reaction buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris–HCl pH 8.8, 0.1% Tween-20], 1.5 mM $MgCl_2$, 1 µM of each primer and 2.5 U of Biotaq polymerase. The reaction conditions were: 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min 30 s at 72°C. In the final cycle, the extension step was 2 min.

Mutation of sequences within core X

*Nde*I and *Sph*I restriction sites were introduced into the core X ARS consensus sequence and Abf1p-binding site, respectively, using the Stratagene QuikChange™ Site-Directed Mutagenesis Kit. Oligonucleotides were as follows: ACS-*Nde*I, GTTGAAGAGTAGAATATTCATAT-GTTTAGGTAATTTTAGTGG and its complement; AbfI-*Sph*I, GCT-GAGGCAAGTGCCGTGCATGCTGATGTGAGTGCATCG and its complement. Plasmid pFEP24 was used as template.

Disruption of SIR1–4 and HDF1 (yKu70) with kanMX4

SIR1–4, and *HDF1* were disrupted with *kanMX4* by the method of Wach *et al*. (1994). PCR fragments were generated using primers as indicated in Table II. Reaction volumes and conditions were as above, using plasmid pFA6a-*kanMX4* as the template. All *kanMX4*-containing transformants were selected on plates containing 400 mg/l G418. *SIR1*- and *HDF1*-disrupted strains were confirmed by colony PCR as described below, and *SIR2*, *-3* and *-4* disruptions by loss of mating type.

Verification of sir1::kanMX and hdf1::kanMX transformants by colony PCR

Correct disruption of *SIR1* and *HDF1* was confirmed by colony PCR using flanking oligonucleotides (A1 and A4) in combination with internal oligos (K2 and K3) from *kanMX4* (Table III). A small amount of cells were picked from a fresh overnight colony on a YEPD plate into 5 µl of H_2O and overlaid with a drop of mineral oil. The mixture was heated at 95°C for 3.5 min prior to adding the remainder of the reaction mix (see PCR amplification of *URA3*) to a total volume of 50 µl. Reaction conditions were as described above, but with an annealing temperature of 58°C.

Measurement of silencing

Haploid cells were grown for 2–3 days on complete synthetic media at 30°C. Single colonies were resuspended in 100 µl of H_2O and 10-fold serial dilutions made. Then 10 µl of each dilution was plated onto complete synthetic media and medium containing 5-FOA. For the *SIR3*

and core X overexpression studies, the cells were plated onto media lacking leucine to maintain the plasmids. The percentage of cells giving rise to 5-FOA-resistant colonies after 2–3 days growth at 30°C was calculated. Examples from a number of the strains used in this study are presented in Figure 3. In addition, for the *sir1*Δ studies, single colonies as described above were resuspended in 350 µl of H_2O and 10-fold serial dilutions made. Then 100 µl of these cell suspensions were spread onto complete synthetic media and medium containing 5-FOA. This gave more accurate measurements of the percentage of 5-FOA-resistant colonies.

Yeast methods and strains

URA3 was inserted at the X-ACS of IX-L, XI-L and XIII-R by transformation of the S288C strain FYBL1-8B (*MAT***a**, *ura3Δ851, leu2Δ1, his3Δ200, lys2Δ202*) with 1 µg of *Sac*I-digested pFEP24. *URA3* was integrated at this and all other subtelomeric locations by transformation with appropriate PCR fragments (Table I). Strains containing terminal truncations at VII-L were created by transformation of FYBL1-8B with pADH-UCAIV (Gottschling *et al*., 1990), digested with *Eco*RI and *Sal*I. All transformations were performed using a modified lithium acetate procedure (Gietz *et al*., 1992).

Mating of SIR3::kanMX4 strains by protoplast fusion

Initial spheroplasting was carried out as described previously (Larin *et al*., 1996). Subsequent protoplast fusion was performed according to Curran and Bugeja (1996).

Other methods

The chromosomal location of the integrated *URA3* was confirmed by CHEF gel and Southern analysis. Genomic DNA was prepared in agarose plugs for electrophoresis by CHEF as previously described (Louis, 1998). Chromosomes were separated in a 1% agarose gel using standard conditions. DNA for Southern analysis was prepared as previously described (Borts *et al*., 1986), and digested with *Stu*I. In both analyses, DNA was transferred onto Hybond N^+ and probed with fluorescein-labelled (Amersham) *URA3*. Transformants were verified further by crossing with strains marked with *URA3* at known ends. The resultant diploids were sporulated, dissected and the segregation of *URA3* determined.

Acknowledgements

The authors would like to thank Rhona Borts and other members of the laboratory for helpful discussion and comments, Eric Gilson for discussion and sharing unpublished results, Bernard Dujon for the parental strains, and Dan Gottschling and Virginia Zakian for providing several of the plasmids used in this study. This work was funded by The Wellcome Trust.

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Received November 10, 1998; revised and accepted March 2, 1999