Cohabitation of insulators and silencing elements in yeast subtelomeric regions

Genevieve Fourel, Emmanuelle Revardel¹, Catherine Elaine Koering and Eric Gilson²

Laboratoire de Biologie Moléculaire et Cellulaire, Ecole Normale Supérieure de Lyon, UMR5665 CNRS/ENSL, 69364 Lyon Cedex 07, France

¹Present address: Laboratoire de Biologie Cellulaire et Moléculaire du Développement des Plantes, Université de Bordeaux I, 33405 Talence Cedex, France

²Corresponding author
e-mail: Eric.Gilson@ens-lyon.fr

G.Fourel and E.Revardel contributed equally to this work

In budding yeast, the telomeric DNA is flanked by a combination of two subtelomeric repetitive sequences, the X and Y' elements. We have investigated the influence of these sequences on telomeric silencing. The telomere-proximal portion of either X or Y' dampened silencing when located between the telomere and the reporter gene. These elements were named STARs, for subtelomeric anti-silencing regions. STARs can also counteract silencer-driven repression at the mating-type *HML* locus. When two STARs bracket a reporter gene, its expression is no longer influenced by surrounding silencing elements, although these are still active on a second reporter gene. In addition, an intervening STAR uncouples the silencing of neighboring genes. STARs thus display the hallmarks of insulators. Protection from silencing is recapitulated by multimerized oligonucleotides representing Tbf1p- and Reb1p-binding sites, as found in STARs. In contrast, sequences located more centromere proximal in X and Y' elements reinforce silencing. They can promote silencing downstream of an insulated expressed domain. Overall, our results suggest that the silencing emanating from telomeres can be propagated in a discontinuous manner via a series of subtelomeric relay elements.

Keywords: insulator/silencer/telomere/*TBF1/REB1*

Introduction

Eukaryotic genomes are thought to be organized into chromatin domains in which a number of processes such as transcription, replication and splicing are regulated autonomously and locally (reviewed in Lamond and Earnshaw, 1998). Chromosomal domains may simply arise as a consequence of their autonomous function or may be delimited actively by specialized boundary elements. While abundant evidence supports the existence of such elements in *Drosophila* and mammals (reviewed in Gerasimova and Corces, 1996), boundary elements have not been described as yet in yeast. A most conspicuous

feature of the yeast genome compared with that of multicellular organisms is its compactness (Dujon, 1996). Non-coding DNA occupies very little space and genes lie in apparent promiscuity. Independence in gene regulation appears to rely on the strict confinement of chromatin changes to the transcription unit (reviewed in Wentz *et al.*, 1997). However, recent data supporting cooperativity between mating-type silencers (Boscheron *et al.*, 1996), as well as the capacity of some mammalian transcription factors to function in yeast (Kunzler *et al.*, 1994), suggest that yeast gene regulation may be more susceptible to long-range chromatin interactions than previously thought. Therefore, yeast may also have evolved efficient mechanisms for insulating genes from each other.

Insulators have been operationally defined as sequences capable of protecting transgenes from a variety of position effects, including those mediated by enhancers and silencers in euchromatin, centric heterochromatin and telomeric chromatin (Roseman *et al.*, 1995; Gerasimova and Corces, 1996). Importantly, insulators do not abrogate the activity of a given enhancer or silencer but rather prevent its communication with the shielded target (Cai and Levine, 1995). In addition, they operate only when interposed between the enhancer/silencer and the target, and they can be inserted in either orientation (Kellum and Schedl, 1992; Chung *et al.*, 1993). Thus, an insulator is a sequence that prevents activation or repression from extending across it to a promoter. Accordingly, insulators display properties of boundary elements.

Artificial transposition of a euchromatic gene into a heterochromatic environment leads to a shutdown of its expression in some of the cells in a clonal and heritable manner, a phenomenon known as position effect variegation (PEV) in *Drosophila* and mammals (reviewed in Wakimoto, 1998). Maintenance of the silenced state of homeotic genes in *Drosophila* similarly occurs through a generalized repression phenomenon and involves specialized proteins belonging to the Polycomb group (reviewed in Pirrotta, 1997). In *Saccharomyces cerevisiae*, telomeres, silent mating-type loci (*HM*) and arrays of rDNA repeats share many of the features of heterochromatin (Hecht *et al.*, 1995; Braunstein *et al.*, 1996; Fritze *et al.*, 1997). Hence, a reporter gene placed near a telomere is expressed in a variegated manner similar to PEV (telomeric position effect or TPE) (Gottschling *et al.*, 1990).

Key components of silenced chromatin in *S.cerevisiae* are the silent information regulators Sir2p, Sir3p and Sir4p, as well as histones H3 and H4 (for reviews, see Sherman and Pillus, 1997; Lustig, 1998). Silenced chromatin assembly is initiated at *HM* silencers composed of specific combinations of binding sites for Abf1p, origin replication complex (ORC) and Rap1p, and at telomeric repeats composed of multiple Rap1p-binding sites. These act as a 'surface' for Sir interaction, either directly or

through Sir1p, as in the case of ORC (Chien *et al.*, 1993; Moretti *et al.*, 1994; Triolo and Sternglanz, 1996). This Sir recruitment appears to nucleate the cooperative assembly of a larger complex through homo- and heterotypic interactions, leading to polymerization of Sirs outward along chromatin (Cockell *et al.*, 1995; Hecht *et al.*, 1996; Triolo and Sternglanz, 1996; Moazed *et al.*, 1997). This model of continuous, linear propagation of silenced chromatin away from silencers has found some elegant support in studies of TPEs (Renauld *et al.*, 1993). More recent physico-chemical analysis suggested a folding back of the telomere to the subtelomeric region, which would stabilize the interactions between Rap1p, Sir proteins and histones (Strahl-Bolsinger *et al.*, 1997).

Two silencers, called E and I, flank each of the *HM* loci and cooperate to repress transcription efficiently (Shei and Broach, 1995; Boscheron *et al.*, 1996). Whereas individual Abf1p-, ORC- or Rap1p-binding sites are incapable of conferring silencing alone, these sites enhance the effect of a distant silencer at *HML* and have been called proto-silencers by analogy with proto-enhancers (Boscheron *et al.*, 1996). Silencers do not function efficiently unless positioned in the vicinity of a telomere. Telomeres are thought to act as sinks regulating the availability of Sir proteins in the nucleus (Maillet *et al.*, 1996; Marcand *et al.*, 1996a,b). It is likely that high local concentrations of silent chromatin components at telomeres boost silencer function; however, a more specific type of telomere-silencer cooperation in inducing a silencing-competent state of Sir-containing chromatin cannot be excluded.

Adjacent to telomeric repeats in *S.cerevisiae* is a mosaic of middle repetitive elements that exhibits a high degree of polymorphism, both among individual strains and among different chromosome ends (Louis, 1995). All chromosome ends have an X region or a Y' element abutting the telomere repeats. All X regions share a core sequence of ~470 nucleotides (core X) which contains an ARS consensus sequence (also named ACS) and, in most cases, an Abf1p-binding site. The STR regions (for subtelomeric repeats, also called J regions) consist of one or more of several small elements designated STR-D, -C, -B and -A. The highly conserved Y' element is found at some chromosome ends in up to four tandem copies (Louis and Haber, 1992). The Y' elements contain two large open reading frames (ORFs) which are expressed during meiosis (Burns *et al.*, 1994; Louis, 1995) and an ACS sequence. When Y' elements are present, they are combined with a core X sequence on their centromeric side. The junctions between the core X and the Y' element usually have variable numbers of the STR-D, C, B and A elements, and can also contain internal telomeric repeats. Although X and Y' do not exhibit sequence homology, they share binding sites for essential yeast proteins, including an ACS bound by the multiprotein complex ORC involved in replication initiation and silencing, as well as several sites for Tbf1p and Reb1p in between the junction with telomeric repeats and the first ACS encountered from the telomere (Chasman *et al.*, 1990; Liu and Tye, 1991; Brigati *et al.*, 1993). Interestingly, the DNA-binding domain of Tbf1p is related to that of various telomeric proteins, including TRF1 and TRF2 in mammals and Taz1p in fission yeast (reviewed in Brun *et al.*, 1997).

The function of Tbf1p is unknown. Reb1p is a weak transcription factor whose binding sites are found in a variety of yeast promoters throughout the genome (Liaw and Brandl, 1994; McLean *et al.*, 1995). Overall, the presence of these conserved binding sites in the immediate subtelomeric region of both X- and Y'-containing ends suggests that they may influence some properties of chromosome ends.

In the present work, we show that both X and Y' elements contain terminal sequences capable of blocking silencing. The anti-silencing activity of these sequences is counteracted by silencing elements located more centromere proximal in X and Y' elements. These results provide evidence that boundary-like elements, analogous to those in mammals and *Drosophila*, also exist in yeast.

Results

To investigate the involvement of the X and Y' subtelomeric elements in TPE, we constructed a series of isogenic strains with a fragmented chromosome VII (Gottschling *et al.*, 1990) carrying various portions of X or Y' sequences inserted between the terminal telomeric DNA repeats and a *URA3* reporter gene (Figure 1A). Cells with a repressed *URA3* were identified as those able to form colonies in the presence of 5-fluoro-orotic acid (5-FOA), a compound toxic for cells expressing a functional *URA3* gene product (Boeke *et al.*, 1984). In order to assess *URA3* expression, we also evaluated the fraction of normal sized colonies growing on a synthetic medium without uracil (SC-U). In addition, 5-FOA-resistant cells were checked for viability by growth on a medium lacking uracil to ensure that 5-FOA resistance was due to silencing and not to mutation of *URA3*.

The most telomere-proximal portions of X and Y' elements can interfere with telomere-driven silencing: STAR sequences

The silencing of *URA3* strikingly decreased from 80 to 3% of 5-FOA-resistant colonies when a 0.3 kb STR sequence corresponding to the most distal part of the subtelomeric X element was interposed between telomere repeats and *URA3* (Figure 1B, compare GF1 with GF2 and GF3). The same result was obtained with STR sequences originating from either the II-L or the XI-L chromosome ends. Since the strength of TPE is expected to decrease with distance from the telomere (Renauld *et al.*, 1993), silencing may function very poorly in our experimental system at a distance of 0.3 kb from the telomere. To address this possibility, we inserted a 0.3 kb sequence isolated from the Y' open reading frame (hereafter called the Y'-ORF) between the telomere and *URA3*. This insertion resulted in no more than a 2-fold decrease of *URA3* silencing (Figure 1B, compare GF1 and GF4). A similar slight decrease in TPE was observed upon insertion of a 120 bp fragment carrying multiple Gal4p-binding sites (4×UAS), or of a 300 bp fragment from pBR322 (data not shown). Insertion of an STR sequence between the Y'-ORF segment and telomere repeats, however, led to a strong 10- to 100-fold decrease of TPE, comparable with that seen upon insertion of an STR alone (Figure 1B, compare GF1 and GF2 with GF4 and GF5). The simplest interpretation of this result is to consider the

Y'-ORF sequence as neutral or passive with respect to TPE. In contrast, the STR sequence appears specifically to counteract TPE.

An anti-silencing effect also was observed when analyzing the telomere-proximal region of a Y' element. Insertion of a 0.14 kb Y' sequence isolated from the most telomere-proximal region of the XII-L chromosome end resulted in a >50-fold drop in *URA3* silencing (Figure 1C, GF1 and GF7). This steep decrease was not adversely affected by further insertion of the Y'-ORF sequence (Figure 1C, GF8).

Results qualitatively similar to these were obtained at the truncated chromosome VII in another strain (S288C), showing that the anti-silencing due to X and Y' regions is not a background-specific phenomenon (data not shown).

In summary, a sharp drop in TPE is associated with sequences naturally located immediately adjacent to the telomeric DNA. Based on their shared ability to abrogate TPE, the STR sequences of the X element and the 0.14 kb 3'-terminal Y' segment were collectively dubbed STARs for subtelomeric anti-silencing regions. The 0.14 kb 3'-terminal Y' segment is named Y'-STAR in the rest of the text.

STARs can block silencing at *HML*

We next investigated whether STARs can also interfere with repression of a reporter gene mediated by silencers at the *HML* locus. A series of yeast strains was generated in which most of *HML* was replaced by *URA3*, flanked by various combinations of *HML-E*, *HML-I*, deletion derivatives and STR sequences.

Control strains bearing either no insert or the 120 bp 4×UAS stuffer fragment displayed a very strong *URA3* silencing in the presence of both native silencers (Figure 2, GF11, GF12 and GF14). In contrast, deletion of both silencers resulted in an apparent total loss of silencing, i.e. no 5-FOA-resistant colony could be observed (Figure 2, GF28–GF33). Silencing diminished upon deletion of either silencer, but this decrease was more pronounced upon deletion of the promoter-proximal silencer (Figure 2, compare GF16 and GF17 with GF22 and GF23; and GF19 and GF20 with GF25 and GF26). This effect is in full agreement with previous reports using a *LEU-lacZ* reporter gene (Boscheron *et al.*, 1996).

Insertion of the STAR sequence from XI-L between *HML-E* and *URA3* did not have a marked effect on the fraction of 5-FOA-resistant colonies in the context of both native silencers (Figure 2, GF13 and GF15). However, a substantial increase in the fraction and size of colonies growing on a medium lacking uracil was observed, suggesting a slight derepression (data not shown). Remarkably, *URA3* silencing was totally abolished upon insertion of STR and deletion of *HML-I*, irrespective of the orientation of *URA3* (Figure 2, GF18 and GF21). In contrast, upon deletion of *HML-E*, STR had almost no effect on residual silencing due to *HML-I* (Figure 2, GF22–GF27). Only a modest decrease in silencing was observed when *URA3* was orientated such that STR lies next to the *URA3* promoter (Figure 2, GF27). We conclude that STR blocks *HML-E* silencer-driven silencing when interposed between the silencer and the promoter.

STR-mediated anti-silencing results from the cooperation of the STR-A, -B and -D submotifs

To localize the sequences responsible for the anti-silencing effect of STR sequences, we inserted between the telomere and *URA3* a series of fragments corresponding precisely to the STR submotifs defined by Louis *et al.* (1994). Of the four STR submotifs initially tested (D, C, B and A; respectively 145, 43, 57 and 32 bp long), only B and D induced a slight derepression of *URA3* on their own (Figure 3, compare GF1 with GF34–GF37). Interestingly, the A submotif synergized with the anti-silencing due to B, while exhibiting no effect on its own (Figure 3, compare GF34, GF35 and GF38). More generally, increasing the inserted fragment by one submotif improved anti-silencing, except for C (Figure 3, GF34–GF42). However, none of the tested STR subregions (BA, CB, DC, CBA and DCB) reached an anti-silencing activity comparable with that conferred by the full-length, 300 bp STR element. These results suggest that the A, B and D submotifs of STR cooperate in STR-mediated anti-silencing.

Multiple binding sites for Tbf1p or Reb1p recapitulate STAR activity

The DNA sequences of STR and Y'-STAR contain several putative binding sites for the proteins Tbf1p (Liu and Tye, 1991; C.E.Koering and E.Gilson, in preparation) and Reb1p (Liaw and Brandl, 1994) (summarized in Figures 1 and 3D). These sites indeed appear to be recognized *in vitro* by their cognate protein. Thus, Reb1p binds DNA fragments containing STR-D and Y'-STAR (Chasman *et al.*, 1990) and Tbf1p binds DNA fragments containing STR-A (Liu and Tye, 1991), Y'-STAR, STR-B and STR-D but not STR-C (data not shown). The correlation between the presence of Tbf1p and/or Reb1p sites and the anti-silencing properties of STR-A, STR-B, STR-D and Y'-STAR suggested that Tbf1p and Reb1p might be involved in anti-silencing. Therefore, we addressed whether anti-silencing could be recapitulated from STR-derived Tbf1p- or Reb1p-binding sites. Multimers of the STR-A submotif (STR-A) and of an internal region of the STR-D region (STR-d), previously demonstrated each to harbor Tbf1p and Reb1p sites, respectively (Chasman *et al.*, 1990; Liu and Tye, 1990), were generated. Whereas STR-A and STR-d had no activity on their own when inserted as monomers between the telomere and the *URA3* gene (Figure 3B, GF72, GF73, GF75 and GF76), a strong anti-silencing effect was observed with multimers (Figure 3B, GF74 and GF77). It is worth noting that the decrease in silencing in both GF74 and GF77 is much more pronounced than in the distance control GF4 (Figure 1B), although the size of the 4×UAS sequences is approximately half of that of the Y'-ORF sequence used in GF4. This further supports the idea that the anti-silencing induced by the STR sequence is not due to distance effects between the telomere and the reporter gene, but rather reflects the existence of specific elements able to restrain silencing. Thus, the anti-silencing capacity of STAR can be recapitulated from short, reiterated motifs able to bind Tbf1p or Reb1p proteins.

At this point, it was conceivable that additional proteins binding to the 40 bp STR-A and STR-d oligonucleotides or some special feature of these sequences themselves, and not the binding of Tbf1p and Reb1p, were responsible

Fig. 2. STR can overcome silencing at *HML*. The *HML*-E and -I silencers are drawn as boxes, black when intact and white when carrying the e1 and i (Δ^{242}) deletions, respectively (see Materials and methods). Most of the *HML* locus was replaced by *URA3* inserted in either orientation as indicated by an arrow. Fragments were inserted between *HML*-E and *URA3*, the STR derived from telomere XI-L or a 120 bp fragment containing 4 $\times$ UAS GAL4 used here as a stuffer. Pictures of a dilution series from a representative culture are shown for each strain, aligned with the corresponding histogram bar. From left to right: undiluted culture, three 10 $\times$ dilutions, and two 3 $\times$ dilutions. When not a single colony grew out on SC+5-FOA from undiluted culture, the fraction of 5-FOA-resistant cells was estimated to be <0.0005% as indicated. The details are otherwise the same as in the legend for Figure 1.

for the anti-silencing. We therefore engineered similar constructs with Tbf1p- and Reb1p-binding sites of different origins. We used sequences isolated by affinity selection of random oligonucleotides using bacterially produced Tbf1p (C.E.Koering and E.Gilson, in preparation) and Reb1p proteins (Liaw and Brandl, 1994), and a repetition of 10 TTAGGG motifs known to bind *in vitro* up to four Tbf1p molecules (Liu and Tye, 1991; Billaud *et al.*, 1996). It is worth noting that the consensus sequences for the Tbf1p and Reb1p sites share a common GGG core sequence, which might bear on the fact that both proteins harbor a DNA-binding domain related to the Myb family (reviewed in Brun *et al.*, 1997). Therefore, the sequences of the oligonucleotides containing a Tbf1p and a Reb1p site were chosen so that they specifically bind their cognate protein, as checked by *in vitro* binding assays (data not shown). A single binding site for either Tbf1p or Reb1p had no detectable effect (Figure 3C, GF78, GF79, GF82 and GF83) while three tandemly inserted Reb1p or Tbf1p sites, as well as the (TTAGGG)₁₀ fragment, recapitulated anti-silencing (Figure 3C, GF80, GF81, GF84, GF85 and GF86). Noticeably, STR-A and Tbf1p sites proved less efficient than STR-d and Reb1p sites, respectively (Figures 3B, compare GF74 with GF77; Figure 3C, compare GF80–GF81 with GF84–GF85). Combinations of Tbf1p and Reb1p sites did not reveal any significant synergy as compared with the insertion of the same number of only one type of site (data not shown). These data strongly

suggest that Tbf1p and Reb1p proteins binding to multiple sites, such as those in STARs, largely contribute to the anti-silencing phenomenon.

STAR-mediated anti-silencing is counteracted by core X and internal Y' sequences

In contrast to the above results obtained using short, subtelomeric sequences directly abutting telomere repeats, silencing of *URA3* was not altered upon insertion of a 0.9 kb full-length X element from II-R (Figure 1B, GF6) or from XI-L (data not shown). Substantial silencing levels of *URA3* were also restored upon insertion of 0.16 or 0.76 kb of more internal Y' sequences in addition to the short terminal 0.14 kb fragment (Figure 1C, GF9 and GF10, respectively). The 0.16 kb Y' fragment, centromere-proximal to Y'-STAR, contains the Y' ARS element. However, these Y' constructs did not attain the degree of silencing seen in strain GF1 in which *URA3* lies next to telomere repeats. Silencing of *URA3* in strains GF6, GF9 and GF10 was lost upon disruption of the *SIR4* gene (data not shown). These data suggest that the anti-silencing activity of STARs is abrogated by core X and internal Y' sequences, by restoring predominant Sir-mediated silencing.

We next asked whether core X can exhibit silencing activities on its own, when dissociated from STR or in its absence. When separated from STR by *URA3*, core X was still able to overcome the anti-silencing effect of STR

Fig. 3. Sequences involved in STR-mediated anti-silencing. (A) The STR region from telomere XI-L was dissected into submotifs as defined in Louis *et al.* (1994) [see also Materials and methods and (D)]. The details are otherwise the same as in the legend for Figure 1. (B) Single and multimerized oligonucleotides corresponding to the STR-A submotif and to STR-d, a small portion of STR-D containing Reb1p-binding sites [see (D)], were inserted between *URA3* and the telomere. The triangle indicates the 5' to 3' orientation of the sequence. (C) Single or multimerized oligonucleotides corresponding to binding sites for Tbf1p and Reb1p isolated by affinity selection were inserted between *URA3* and the telomere. The symbols corresponding to Tbf1p or Reb1p sites are below the line when their orientation is different from the orientation in the natural subtelomeric regions. (D) Sequences of the XI-L STR region and of the XII-L Y'-STAR. The sequences corresponding to the STR-A and STR-d oligonucleotides are shaded in gray. The gray boxes over the sequence indicate putative Reb1p sites as defined by the core consensus sequence 5'-CGGGTAA, allowing a single mismatch outside the three consecutive G residues (Liaw and Brandl, 1994). The Reb1p sites in STR-D and in Y'-STAR are bound *in vitro* by Reb1p (Chasman *et al.*, 1990). The empty boxes under the sequence correspond to putative Tbf1p sites as defined by the consensus 5'-TAGGGTT, allowing a single mismatch outside the three consecutive G residues (C.E.Koering, in preparation). The presence of Tbf1p-binding sites in STR-D, STRB, STR-A and Y'-STAR DNA fragments has been demonstrated *in vitro* (Liu and Tye, 1991; data not shown).

(Figure 4A, compare GF3, GF6 and GF44). The same type of result was obtained by replacing STR with the Y'-STAR (Figure 4A, compare GF7 with GF46). Moreover, the insertion of core X on the centromeric side of *URA3* in the absence of STR resulted in an increase of *URA3* silencing, revealed by a slightly lower frequency of colonies growing to a large size on medium lacking uracil as compared with GF1 (Figure 4B, compare lines 2 and 4). Altogether, these data suggest that subtelomeric core X sequence exhibits bidirectional silencing activities. Hence, core X and the 0.76 kb internal Y' fragment will be referred to as 'subtelomeric silencing elements'.

The relative position of STARS and subtelomeric silencing elements determine their effect

Strikingly, the insertion of the full-length X element in reverse orientation, between *URA3* and the telomere, resulted in a low level of silencing that compared with that achieved using the STR region alone (Figure 5A,

compare GF6, GF51 and GF3). Similarly, inversion of the 0.9 kb Y' 3'-terminal fragment yielded silencing levels that were far lower than those obtained with the 0.14 kb Y'-STAR region alone (Figure 5B, compare GF10, GF57 and GF7).

We then investigated which portion of the inverted X element is responsible for its anti-silencing activity. An inverted core X sequence does not function as an anti-silencer by itself when inserted between *URA3* and the telomere (Figure 5A, compare GF1, GF53 and GF54). In addition, the same inverted core X sequence introduced between STR and *URA3* was still able to counteract the anti-silencing activity of STR, like the wild-type X sequence (Figure 5A, compare GF6, GF55 and GF56). Furthermore, STARS displayed anti-silencing activity in either orientation (Figure 5, compare GF1 with GF3 and GF52; GF7 and GF58). This strongly suggests that the anti-silencing observed upon inversion of X and Y' sequences is due to the inverted STARS. Therefore, the

Fig. 4. STARs can insulate *URA3* from both telomeric and core X silencing activities. Fragments were inserted not only between *URA3* and telomere repeats as in previous figures, but also at a *Bgl*III site located on the centromeric side of *URA3* and separated from the *URA3* segment by a 240 bp stuffer sequence (see Materials and methods). **(A)** Two STARs bracketing *URA3* protect its expression from the action of surrounding silencing elements. **(B)** Core X displays genuine silencing activity. Colonies growing to a large size on synthetic medium without uracil were counted (SC-Ura). Core X silencing activity was revealed as both a slight increase in the fraction of 5-FOA-resistant cells and a decrease in the SC-Ura/SC ratio. The details are otherwise the same as in the legend of Figure 1.

order in which STAR and subtelomeric silencing elements are arranged appears critical for the silencing of an internally located reporter gene. These data are consistent with a model in which STARs act as a barrier to silencing, whereas subtelomeric silencing elements may promote silencing downstream of STARs.

Since STARs can counteract TPE in either orientation and also restrain *HML*-E-driven silencing (see above and Figure 2), we asked whether STARs are able to antagonize the silencing effect associated with a core X sequence positioned on the centromeric side of *URA3*. Bracketing *URA3* between two STR sequences restored predominant *URA3* expression as compared with a strain in which the STR located between core X and *URA3* is absent (Figure 4A, GF44 and GF45). In addition, flanking *URA3* by one STR and one Y'-STAR sequence essentially abrogated its silencing, resulting in 0.005% of the cells able to form colonies on 5-FOA-containing medium (Figure 4A, GF47). Since this insulating effect is far stronger than with two STRs, we asked whether a 'super' STAR sequence is created by combining an STR and a Y'-STAR. However, inserting an STR and a Y'-STAR adjacent to one another between *URA3* and the telomere repeats led to *URA3*

silencing levels that were not lower than with either sequence alone (Figure 4A, compare GF49 with GF3 and GF7), and combining a Y'-STAR with a full-length X element did not counteract core X silencing activity (Figure 4A, compare GF6 and GF48). In addition, placing an STR sequence next to a *URA3* promoter did not alter *URA3* silencing (Figure 4A, GF86). Therefore, STAR sequences flanking *URA3* operationally define an internal region largely resistant to silencing.

Subtelomeric silencing elements and STARs operate independently

The above results are consistent with a model in which non-adjacent STAR sequences specifically cooperate to delimit an intervening domain that is shielded from silenced chromatin. STARs may act as a blockade to the spreading of silenced chromatin, without affecting the activity of surrounding silencers. Alternatively, STARs may impair specifically the function of subtelomeric silencer elements. To address this possibility, we generated a yeast strain in which both STAR-mediated anti-silencing and the silencing activity of core X could be assessed at the same time. This strain carried an internally located

Fig. 5. STARs and subtelomeric silencing elements operate in an orientation-independent but position-dependent manner. An arrow indicates the orientation of individual subtelomeric fragments. The details are otherwise the same as in the legend of Figure 1.

URA3 as a reporter of core X silencing and a *TRP1* reporter gene bracketed by two STARs as a reporter of anti-silencing (Figure 6A and B, GF61). As controls, we generated strains without core X (Figure 6B, GF60) and without both core X and an STR (Figure 6B, GF59).

The *TRP1* gene was found essentially expressed in all three strains, with rates of SC-W/SC colonies reaching levels close to 100% in strains GF60 and GF61 (Figure 6B, lines 1, 4 and 7). In the absence of the telomere-proximal Y'-STAR sequence, *TRP1* is repressed, confirming the expectation that *TRP1* is no longer subjected to TPE when it is separated from the telomere by a STAR sequence (data not shown). The fraction of *URA3* OFF cells dropped from 3 to 0.06% upon insertion of an STR sequence between *URA3* and *TRP1* (Figure 6B, compare lines 2 and 5; Figure 6C, compare GF59 with GF60). Thus two STARs inserted at some distance from each other have additive effects. It is worth noting that not a single colony emerged on SC-W+5-FOA from GF59 cultures, showing that the 3% fraction of colonies in which *URA3* is OFF corresponds to colonies in which *TRP1* is also silenced (Figure 6B, line 3; Figure 6C, GF59). This tight coupling of *TRP1* and *URA3* expression is in full agreement with a continuous propagation of the repressed telomeric chromatin, as previously demonstrated (Renauld *et al.*, 1993). In contrast, the presence of an

STR sequence between *URA3* and *TRP1* in strain GF60 allowed the growth of almost all of the 0.06% 5-FOA-resistant cells on SC-W+5-FOA (Figure 6B, compare lines 5 and 6; Figure 6C, GF60). Colonies growing on SC-W+5-FOA were of the same size as those growing on SC+5-FOA (Figure 6C, GF60), excluding a rapid switch between ON and OFF states for *TRP1* that may have allowed some growth on SC-W+5-FOA medium. That growth on SC-W+5-FOA did not arise as a result of genomic rearrangement was checked by Southern blot analysis of individual colonies (data not shown). Since most of the *URA3* OFF colonies of GF60 contain a *TRP1* gene ON, we conclude that an intervening STR sequence can apparently decouple the silencing status of two neighboring genes and block the propagation of repressed telomeric chromatin. This observation is consistent with a model in which STARs can act as boundary elements delimiting domains of independent gene expression.

We then asked whether the silencing activity of core X can accommodate the TPE blockade observed in the *URA3*–STR–*TRP1*–Y'-STAR segment. The further insertion of a core X fragment between *URA3* and STR led to ~50% of the cells forming colonies on 5-FOA-containing medium, and these cells all had the *TRP1* gene ON (Figure 6B, lines 8–9; Figure 6C, GF61). Therefore, core X appears to retain the function of a subtelomeric silencing

Fig. 6. STARs uncouple silencing of two adjacent genes. The strains presented in this figure carry both *URA3* and *TRP1* reporters at telomere VII-L. They allow us to address specifically whether the centromere-proximal *URA3* gene can be repressed (cross) at the same time as when the *TRP1* gene is active (arrow), upon growth on SC-W+5-FOA. The details are otherwise the same as in the legends of Figures 1 and 2.

Fig. 7. Silencing properties of core X at *HML*. The details are otherwise the same as in the legends of Figures 1 and 2.

element in strain GF61, despite the presence of an interposed, transcriptionally active domain between itself and the telomere.

Core X does not act as a silencer at *HML*

The finding that core X reinforces silencing downstream of a transcriptionally active area could indicate that core X is a bona fide silencer. To address this question, core X was inserted at the *HML* locus in a system similar to that described above for assaying STAR activity. Not a single colony grew on 5-FOA-containing medium in the context of both *HML-E* and *-I* silencers deleted, regardless of the presence or absence of core X sequences (Figure 7, GF31, GF62 and GF63). This result contrasts with the

substantial levels of *URA3* silencing observed when either *HML-E* or *HML-I* silencer was intact (Figure 7, compare GF19 and GF25 with GF48). Thus core X does not function as a classical silencer element at *HML*.

However, core X largely compensates for the loss of *HML-E* in the presence of *HML-I*, and this effect was observed with core X inserted in either orientation (Figure 7, compare GF64 and GF65 with GF48). Therefore, core X element behaves at *HML* like a proto-silencer that enhances the action of a distant silencer without acting as a silencer on its own (Boscheron *et al.*, 1996). This proto-silencer activity was predicted due to the presence of both an ACS and an Abf1p-binding site in core X (Louis, 1995). It is worth noting that the part of Y' which partially

Fig. 8. Partial dependence of core X-associated silencing upon *SIR1* and *HDF1*. The details are otherwise the same as in the legend of Figure 1.

counteracts the Y'-STAR anti-silencing activity (Figure 1, compare GF7 with GF9) also contains an ACS. Therefore, the ability of internal subtelomeric elements to counteract STAR-associated anti-silencing appears to be related to the presence of combinations of proto-silencers in the X and Y' subtelomeric elements.

Differential requirements for TPE and core X silencing for *HDF1* and *SIR1*

The presence of ACS in core X and Y' elements suggests that Sir1p function, which was assumed so far to be specific to silencing at the *HM* loci (Aparicio *et al.*, 1991; Triolo and Sternglanz, 1996), may also be involved in subtelomeric silencing. The *URA3* gene remained silenced upon *sir1* mutation when placed either immediately next to telomere repeats or with core X sequences inserted in between (Figure 8A, lines 1–4 and 7–10). However, a significant derepression was observed in the *sir1* strain when a full-length X element was inserted, which is more clearly visible on medium lacking uracil than on 5-FOA-containing medium (Figure 8B, lines 11 and 12). Thus, core X silencing activity is partially compromised upon mutation of *SIR1*.

In contrast to Sir1p, Ku appears to be involved in TPE when assayed in the absence of X or Y' elements, but not in mating-type silencing (Boulton and Jackson, 1998; Gravel *et al.*, 1998; Laroche *et al.*, 1998; Nugent *et al.*, 1998). Since core X-dependent TPE appears to be closely related to that of mating-type silencing, we decided to investigate its dependence on the *HDF1* gene product. Silencing of *URA3* inserted next to telomere repeats dropped by four orders of magnitude to barely detectable levels upon deletion of *HDF1* (Figure 8B, compare GF1 and GF66). However, it decreased only from 90 to 3% when a core X sequence was inserted between *URA3* and the telomere (Figure 8B, compare GF44 and GF67). A

minority of 5-FOA-resistant colonies from GF67 grew to large sizes (data not shown). However, these did not grow on a medium without uracil, and therefore appear to result from mutations in the *URA3* gene. Accordingly, these large colonies were not taken into account in the presented data. We conclude that in the context of a deleted *HDF1* gene, core X silencing capacity at telomeres is at most partially affected. Inserting a full-length X element revealed a decrease in silencing as compared with a core X insert, dropping from 3 to 0.5% of 5-FOA-resistant colonies (Figure 8A, compare GF67 and GF68). Thus, the effect of deleting the *HDF1* gene on TPE is less critical for a telomere containing X subtelomeric sequences, as previously described with strains carrying reporter genes next to telomere repeats.

Altogether, the partial dependency of X-associated telomeric silencing on *SIR1* and *HDF1* demonstrates that silencing driven by natural telomeres displays some distinct requirements when compared with classically defined TPE observed with a reporter gene inserted next to telomere repeats.

Discussion

Current models invoke the spreading of silenced chromatin at yeast telomeres continuously outwards from a telosome (Renaud *et al.*, 1993; Hecht *et al.*, 1996). Many mutations affecting the integrity of the telomeres (reviewed in Sherman and Pillus, 1997; Lustig, 1998), as well as transcription through the telomeric tract (Sandell *et al.*, 1994), have been shown to result in the dramatic reduction of TPE. In contrast, few alterations in subtelomeric regions have been reported to cause a similar effect, such as the induced transcription of genes artificially placed in a subtelomeric position (Aparicio and Gottschling, 1994).

Here we show that sequences which are naturally found

immediately abutting telomere repeats in both X and Y' subtelomeric elements are endowed with anti-silencing properties and appear to act as barriers to the spreading of silenced chromatin. We named these sequences STAR for subtelomeric anti-silencing region. They correspond to the STR sequence previously defined as a variable portion of an X repeat and to a 145 nucleotide sequence of Y'. That STAR-mediated anti-silencing has gone undetected so far (Renauld *et al.*, 1993; Hecht *et al.*, 1996) is best explained by the presence in both X and Y' of sequences which are directly adjacent to the STARs on their centromere side and which act as subtelomeric silencer elements. STARs and subtelomeric silencer elements operate independently although they are naturally found juxtaposed. Both types of elements displayed similar properties at another normally repressed region, the *HML* silent mating-type locus (Figures 2 and 7; G.Fourel, E.Revardel, E.Lebrun and E.Gilson, in preparation).

The characteristic properties of STAR and subtelomeric silencing elements were defined through a number of genetic combinations that were assayed for silencing at the VII-L telomere. Some of these constructs mirror natural telomeric settings, and our results are consistent with data presented in Pryde and Louis (1999). Thus, although individually using STR and core X sequences in telomere fragmentation constructs allows the detection of STAR (Figures 1 and 3) and silencing effects (Figures 4 and 5), inserting *URA3* on either side of a full-length X element (Figures 1 and 4) or between core X and STR sequences (Figure 4) results in high silencing levels. These subtelomeric configurations are summarized schematically in Figure 9A. In addition, the very weak silencing of a *URA3* gene inserted within a variety of Y' elements (Louis and Haber, 1990; Louis *et al.*, 1994; Kim *et al.*, 1995; Pryde and Louis, 1999) could be accounted for by the capacity of the Y'-STAR and the STR of an X sequence located on the centromeric side of Y' to create an intervening area protected from silencing (Figure 9A, g). A *TRP1* or a *URA3* gene thus bracketed by two STARs mimicked such a situation (Figures 4 and 6). Protection from telomeric silencing by STARs may permit transcription of Y'-ORFs at meiosis (Louis, 1995) or in survivors defective for telomerase (Yamada *et al.*, 1998), as well as the expression of genes belonging to the *SUC* and *RTM* families, found in analogous positions, between X and Y' elements (Carlson *et al.*, 1985; Ness and Aigle, 1995). The presence of a subtelomeric silencing element in the terminal 0.9 kb of Y' nevertheless suggests that some silencing may occur within full-length Y' elements, which is in agreement with the immunoprecipitation of Y' sequences by anti-Sir3p antibodies (Gotta *et al.*, 1997) and the reduced level of histone acetylation of nucleosomes located within Y' elements (Braunstein *et al.*, 1993; Rundlett *et al.*, 1998). Alternatively, Sir-containing chromatin over Y' sequences might display an altered competence for silencing, thus allowing nearly normal expression of an inserted reporter gene but exhibiting some aspects of silent chromatin.

The apparent resumption of silencing on the centromeric side of Y' elements observed at a variety of telomeres (Pryde and Louis, 1999) was predicted previously, based on the lack of repression of reporters embedded in Y's (Carlson *et al.*, 1985; Louis and Haber, 1990; Louis *et al.*,

Fig. 9. Properties of STAR and subtelomeric silencing elements. (A) Summary of the silencing properties of *S.cerevisiae* subtelomeric sequences described in this work. As initially determined for telomeric position effect (TPE), silenced chromatin emanating from a telomere extends no more than a few kilobases towards the centromere, as indicated by a black triangle (a). Specific subtelomeric silencing elements (hatched oval) may act as a relay prolonging silencing propagation (b). STAR sequences (box), on the contrary, may limit the spreading of silenced chromatin, whether placed in the immediate proximity to telomere repeats (c) or at some distance (d). This STAR effect can be overcome by the presence of subtelomeric silencing elements (e and f). However, two distant STAR sequences operationally define an intervening domain that is insulated from the influence of surrounding silencing elements, whose activity is nevertheless not compromised (g). It is not known whether the extent of the spreading of silenced chromatin is similar when emanating from the telomere in TPE (a) or when associated with a subtelomeric silencing element (b and e–g). (B) Our results predict that an array of subtelomeric elements can propagate silencing in a discontinuous manner through multiple silencing relays over a much longer distance than the one measured in the absence of any subtelomeric element.

1994; Kim *et al.*, 1995) and on the efficient spreading of silencing beyond Y', as initially reported for a single telomere (Renauld *et al.*, 1993). The hypothesis that a specific element might be involved in the reinitiation of silencing (Renauld *et al.*, 1993) is now confirmed with the silencing activity of core X operating in the presence of an interposed transcribed domain between itself and the telomere (see Figure 9A, g). The SIR3-dependent silencing of the retrotransposon Ty5-1 naturally found in this context at telomere III-L (Vega-Palas *et al.*, 1997) is also readily accounted for by the presence of an adjacent core X sequence.

STARs as insulators

In this work, we demonstrated that STARs protect a reporter gene from the repressing effects of neighboring silencing elements without notably affecting their integrity and potency. This operates only when STAR sequences are interposed between the gene and the silencing element(s). Yeast STARs may thus be considered as insulators, although their activity in an enhancer-blocking assay has not been examined. Furthermore, an intervening STAR was found to uncouple the silencing of neighboring genes in a manner similar to the chicken β -globin insulator (Pikaart *et al.*, 1998). STARs may thus act as boundaries of functionally independent chromatin domains. The existence of sequences able to constrain telomeric silenced chromatin to limited areas was suggested previously in *Schizosaccharomyces pombe* (Nimmo *et al.*, 1994). The

apparent orientation dependence of *HM* silencer activity may be accounted for by the existence of insulators, similar to STARs, bordering *HM* loci (Shei and Broach, 1995).

Similarly to other described insulators (Chung *et al.*, 1997), a high degree of redundancy was observed in STARs: (i) progressive deletions in STR had a corresponding decrease in anti-silencing (Figure 3A); (ii) the multimerization of short STR submotifs (STR-A, STR-d) recapitulated anti-silencing activity, whereas a single submotif had no such effect (Figure 3B); and (iii) tandemly repeated binding sites for Tbf1p and Reb1p, as found arranged in arrays in STR-A, STR-B and STR-D, antagonize TPE (Figure 3C). In agreement with an important role for Tbf1p- and Reb1p-binding sites in STR anti-silencing, the STR-C submotif which apparently does not contribute to anti-silencing appears to be devoid of Tbf1p and Reb1p sites (data not shown and Figure 3D). Interestingly, STR-C is not always found in STR regions (E.Louis, personal communication), suggesting that the anti-silencing activity of STARs is of biological significance.

Reb1p is known as a weak transcription factor (see, for example, Liaw and Brandl, 1994; McLean *et al.*, 1995). Therefore, one cannot exclude that transcriptional activation of the reporter gene promoter may contribute to the apparent anti-silencing effect in configurations where STARs and promoter are juxtaposed. However, this is clearly not the case in the majority of constructions which are presented here. Other reported cases where insulation turned out to involve transcriptional elements (Corces and Geyer, 1991; Glover *et al.*, 1995; Ohtsuki and Levine, 1998) suggest that both processes might be more intricate than originally thought.

STARs might delimit a very terminal telomeric compartment protected from influences of the rest of the genome. Silenced chromatin would nevertheless spread efficiently outside of this compartment due to the additional presence of subtelomeric silencing elements, such as core X (Figure 9). However, the local effects of subtelomeric silencing elements appears restricted to limited domains (Pryde and Louis, 1999) which may be accounted for by the presence of additional insulators.

Core X and *HM* silencers as relays of telomere-driven silencing

We recently showed that an oligonucleotide representing either an ARS sequence or an Abf1p-binding site, such as that found in core X, was sufficient to recapitulate some subtelomeric silencing activity, and that both types of proto-silencers additively contribute to core X silencing activity (G.Fourel, E.Lebrun, E.Revardel and E.Gilson, in preparation). An ARS sequence is also found in the Y' silencing element. Thus, natural TPE is likely to involve the binding of ORC and Abf1p to subtelomeric silencing elements, in addition to the binding of Rap1p to telomeric repeats. It is remarkable that the same three factors are responsible for *HM* silencer activity. Silencing at *HM* loci and natural telomeres accordingly was found to share many more similarities than previously suspected, such as a partial requirement for Sir1p and only a weak dependence on the *HDF1* gene product.

Although core X mediates silencing when located in the vicinity of a telomere (Figure 6, summarized in Figure 9), it does not behave as a silencer at *HML* (Figure

7). This is reminiscent of the previously demonstrated dependence of silencer efficiency on the distance from the telomere (Maillet *et al.*, 1996; Marcand *et al.*, 1996a). It is thus tempting to speculate that core X, and possibly also the Y' silencing elements, are weak silencers, incapable of autonomous function at *HML*, but boosted in an immediate telomeric environment. Supporting this hypothesis is a mutation in histone H4, that was found to weaken *HMR* silencing in its natural location but had almost no effect when *HMR* was placed in the immediate vicinity of the telomere (Thompson *et al.*, 1994).

Subtelomeric silencing elements appear to act as relays of telomere-driven silencing by prolonging silenced chromatin propagation into more internal regions of the chromosome (Figure 9B). This might reflect some continuity of silenced chromatin emanating from telomere repeats in spite of permitted discontinuity in the silencing phenomenon. The immunoprecipitation of subtelomeric chromatin using anti-Rap1p antibodies strongly suggests a physical interaction between core X and the telosome (Strahl-Bolsinger *et al.*, 1997). This interaction may play a key role in core X silencing activities and may explain the requirement for telomere proximity. Conversely, bona fide *HM* silencers, the capacity of which to function at internal sites can be strongly enhanced by adjoining supplementary silencing elements (Shei and Broach, 1995; Maillet *et al.*, 1996), may be viewed as potent relay elements partially relieved of their requirement for telomere proximity. A model involving relay elements was also proposed recently in vertebrate to account for the expression pattern of homeobox genes (*Hox*), which are activated in temporal and spatial sequence co-linear with the position of the genes within their clusters. Relay elements would become progressively inoperative in the course of development, allowing the directional opening of chromatin (Kondo *et al.*, 1998).

Subtelomeric regions consolidate telomere functions

A functional interaction between telomere repeats and core X involving silenced chromatin was invoked previously on the basis of the ability of core X to enhance the role of telomeric repeats in improving plasmid partitioning in a Sir-dependent manner (Enomoto *et al.*, 1994). This interaction appeared to be distance and orientation independent, predicting that these sequences could interact when separated by one or more Y' (Longtine *et al.*, 1993). Strikingly, core X-mediated improvement of plasmid partitioning and core X silencing activity display common determinants and thus appear to be a single phenomenon. Cooperation with core X may recruit additional Sir proteins as compared with the telomere alone. This is expected to promote and/or stabilize telomere associations and targeting to subnuclear compartments, hence improving chromosome end stability (Gotta *et al.*, 1996; Maillet *et al.*, 1996; Marcand *et al.*, 1996a,b). Overall, the subtelomeric regions appear to interact functionally with telomeres to extend and consolidate essential functions of chromosome ends.

Why are insulators and silencers together?

Boundary elements were described previously in *Hox* clusters in *Drosophila* and found associated with Polycomb

Table I. Oligonucleotides used in the PCR amplification of natural subtelomeric regions

Primer	Sequence
19A1	5'-GCGCAGATCTGTATGACTATAGAGTACAG
19B2	5'-CGCGGATCCATCGTAAATAATACATACATAC
19C1	5'-GCGCAGATCTTAACGTTTCAATATGGAGG
19C2	5'-GCGGGATCCACACACCCTAACACAATCCTAAC
89A1	5'-GCGCAGATCTGCATATATTCTAATGTATG
89B2	5'-GCGGGATCCTAATACATATACTTACCC
89C1	5'-GCGCAGATCTCGATTATTTGTAACTTTTC
89C2	5'-GCGCGGATCCACACCCACTACTCTAACCC
42A1	5'-GCGCAGATCTGAAAGTTGGAGTTTTCAGCG
42A2	5'-GCGGGATCCAACCACACCTCCGAAATCTGC
42B1	5'-GCGCAGATCTAAACATAAAATAAAGGTAG
42B2	5'-CGCGGATCCCACACTTTTACATCTACCTCTAC
42B3	5'-TCCTAGATCTGTTGAACATCCGGG
STRA-BgBis	5'-TCCTAGATCTACACCCACTACTCTAACCC
STRB-Ba	5'-TCCTGGATCCGGGCGAGGTAAC
STRB-Bg	5'-TCCTAGATCTAACCTGTCTC
STRC-Ba	5'-TCCTGGATCCAGTAGAGAG
STRC-Bg	5'-TCCTAGATCTCACTCAACCATAC
STRD-Ba	5'-TCCTGGATCTTTGTAAACG
STRD-Bg	5'-TCCTAGATCTTACCAGCCC
STRA+	5'-GATCTGATCAGGGTTGGATTAGAATAGGGTTAGAGTAG
STRA-	5'-GATCCTACTCTAACCTATTCTAATCCAACCTGATCA
STRd+	5'-GATCTGGATGGTAGGTAATGGCAGGTAAGTGGTAGG
STRd-	5'-GATCCCTACCCTTACCCTGCCATTACCCTACCATCCA
TBF1S+	5'-GATCTTTCAGTAGGGTTGGATCG
TBF1S-	5'-GATCCGATCCAACCTACTGCAAA
REB1S+	5'-GATCTCACGCGGCGGTAAGAGGG
REB1S-	5'-GATCCCTCTTACCAGCCGCGTGA

response elements (Mihaly *et al.*, 1997). Such a combination strikingly mirrors the arrangement of STAR and silencing elements in *S.cerevisiae* subtelomeric regions. The boundary elements were hypothesized to ensure both the autonomous activity of adjacent *cis*-regulatory domains and to restrict silencing and activation maintenance to the appropriate domain. If such apparently antagonistic sequences were to be found combined more generally, it might also point to common functional determinants. Yeast now provides the possibility of genetic and molecular approaches for dissecting the mechanisms by which insulators and silencing elements operate, as well as their putative interactions.

Materials and methods

Plasmid constructions

Molecular biology techniques were performed as described in Sambrook *et al.* (1989). The Expand HiFi system (Boehringer Mannheim) was employed to carry out PCRs according to the manufacturer's recommendations.

Plasmids used in end replacements at telomere VII-L were obtained as follows. The reference construct containing the *URA3* reporter gene abutting telomere repeats (pURTEL, used to generate the GF1 strain) was obtained by deleting the *ADE2* gene as a *Bam*HI fragment from pADADE2(+) (Gottschling *et al.*, 1990). In order to introduce *Bgl*III-compatible fragments 5' of *URA3*, we used pUASURTEL which carries a fragment of 240 nucleotides inserted at the *Hind*III site of pURTEL and containing a *Bgl*III site, a HO recognition sequence and four GAL4 UAS-binding sites (S.Marcand, E.Revardel, G.Fourel and E.Gilson, unpublished plasmid). In this plasmid, the *Bgl*III site is located 460 bp away from the *URA3* ORF. Various types of fragments carrying terminal *Bam*HI and/or *Bgl*III sites were then introduced either at the *Bam*HI site located 3' of *URA3* in pURTEL or at the *Bgl*III site located 5' of *URA3* in pUASURTEL. These fragments were obtained by PCR amplification of natural subtelomeric regions, carried out using plasmids p19X10, p89H9 and p42H2 [containing respectively chromosome II-R, XI-L and

XII-L end (Louis and Borts, 1995), kindly provided by E.Louis] as templates and the oligonucleotides listed in Table I.

The PCR amplification reactions used to generate the various X and Y' portions were performed with the following pairs of primer: core X (II-R), 19A1 + 19B2; STR (II-R), 19C1 + 19C2; X (II-R), 19A1 + 19C2; core X (XI-L), 89A1 + 89B2; STR (XI-L), STRD-Ba + STRA-BgBis; X (XI-L), 89A1 + 89C2; Y'-ORF (XII-L), 42A1 + 42A2; Y'-STAR (XII-L), 42B3 + 42B2; 0.16 kb + Y'-STAR (XII-L), 42B1 + 42B2; 0.76 kb + Y'-STAR (XII-L), 42A1 + 42B2; STR-B (XI-L), STRB-Ba + STRB-Bg; STR-C (XI-L), STRC-Ba + STRC-Bg; STR-D (XI-L), STRD-Ba + STRD-Bg; STR-BA (XI-L), STRD-Ba + STRA-BgBis; STR-CB (XI-L), STRC-Ba + STRB-Bg; STR-DC (XI-L), STRD-Ba + STRC-Bg; STR-CBA (XI-L), STRC-Ba + STRA-BgBis; and STR-DCB (XI-L), STRD-Ba + STRB-Bg. The STR-A and STR-d submotifs from XI-L (Figure 3D), and binding sites for Tbf1p and Reb1p, as well as their multimer derivatives, were obtained through annealing of STRA+ and STRA-, STRd+ and STRd-, TBF1S+ and TBF1S-, and REB1S+ and REB1S- oligonucleotides, respectively. All the PCR DNA fragments or annealed oligonucleotides inserted into plasmids were checked by sequencing.

The construction of plasmids containing both the *URA3* and *TRP1* reporter genes was performed in the following steps: (i) insertion in reverse orientation into the *Bam*HI site of pURTEL of the Y'-STAR PCR fragment (see above); (ii) the resulting pURSTel plasmid was used to insert into the reconstituted *Bam*HI site either a *TRP1* gene as a 0.85 kb *Bgl*III fragment derived from pFL39 (Bonneaud *et al.*, 1991) or a core X or a X PCR DNA fragment from II-R (see above), resulting in pURTRPSTel, pURcXSTel and pURXSTel, respectively; (iii) the 0.85 kb *Bgl*III *TRP1* fragment was inserted into the reconstituted *Bam*HI site of pURcXSTel and pURXSTel, resulting in pURcXTRPSTel and pURXTRPSTel, respectively. The plasmids pURTRPSTel, pURcXTRPSTel and pURXTRPSTel were used to generate the yeast strains GF59, GF60 and GF61, respectively.

A plasmid was also constructed that harbored the *TRP1* gene, orientated such that transcription proceeds away from telomere, in place of *URA3* in pURTEL (pTPtel). It was obtained through a blunt end ligation replacing the *Hind*III-*Bam*HI *URA3* fragment by the 1.1 kb *Hind*III-*Cla*I *TRP1* fragment of pFL39 (Bonneaud *et al.*, 1991). Plasmids used to modify the *HML* locus were all derived from a pE-i plasmid which contains a wild-type *HML*-E silencer and the I^{Δ242} deletion of the *HML*-I silencer (Mahoney and Broach, 1989) carrying single *Kpn*I and *Bcl*II

sites between the two *HML* fragments (an unpublished construct from our laboratory). *Bam*HI–*Bgl*III PCR fragments representing various subtelomeric regions as described above or a 4×UAS *GAL4 Bam*HI–*Bgl*III fragment were inserted at the *Bcl*I site and *URA3* was inserted at the *Kpn*I site following excision from pAF101 (Thierry *et al.*, 1990).

Yeast strains, media and methods

Standard genetic manipulations of yeast and growth in rich or selective media were as described in Rose *et al.* (1990). Yeast transformations were done according to a PEG/lithium acetate protocol (Elbe, 1992) using 2 ml of an exponentially growing yeast culture. All telomere fragmentations and gene disruptions were confirmed by Southern blot analysis.

Yeast strains described in this study harboring a modified VII-L telomere are derivatives of W303-1a (*MATa ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can 1-100*) and those with a modified *HML* locus have an S150-2B background (*MATa leu2-3, 112 ura3-52 trp 1-289 his3D gal2*). The chromosome VII-L end was first fragmented using either pTPtel or pADADE2. The resulting strains were then transformed with digested *URA3*- or *URA3*- plus *TRP1*-containing constructs, and Ura+Trp- or Trp+Ade- colonies were selected, respectively. Two rounds of non-selective growth in 2 ml of YPD were then carried out to favor the loss of episomes that may have arisen from the circularization of transformed DNA fragments, the partitioning of which is expected to be improved by the presence of subtelomeric sequences (Longtine *et al.*, 1992).

Similarly, we screened for loss of the *LEU2''lacZ* marker following transformation of our *HML* constructs as *Not*I fragments in EG42 (Boscheron *et al.*, 1996) through a filter-based β-galactosidase color assay. Recombination between the *HML*-E and the *HML-I*^{Δ242} (named i in Figure 2) silencer alleles of the transfected constructs and the *HML*-e1 and *HML*-I alleles in the recipient strain yielded all four expected combinations that were detected using *Eco*RV and *Bgl*III digestion in Southern blot analysis as described in Boscheron *et al.* (1996).

The primers used to introduce a null mutation of *HDF1* by the KanMX method (Wach *et al.*, 1994) were provided by Ed Louis. A partial disruption of *SIR1* was obtained using pJ123.2 which leads to deletion of the 3' half of the ORF, reported to confer the same phenotype as a null mutation (Stone *et al.*, 1991).

Analysis of *URA3* expression

The variegated expression of *URA3* was monitored by scoring the fraction of yeast cells from a culture pre-grown in non-selective conditions either expressing or not the *URA3* gene. Cells with a repressed *URA3* gene were identified as those able to form colonies in the presence of 5-FOA, a compound toxic for cells expressing a functional *URA3* gene product (Boeke *et al.*, 1984). Evaluating the fraction of normal sized colonies growing on a synthetic medium without uracil (SC-Ura) also assessed *URA3* expression. For each strain, at least three independent transformants, the structure of which had been verified previously by Southern blot analysis, were first streaked on synthetic complete medium (SC) and grown for 3 days at 32°C. For each transformant, cells from at least two isolated colonies were then inoculated into SC liquid medium (20 μg/l uracil). After overnight growth to saturation, 10 μl drops of serial dilutions were plated onto SC, SC-Ura and SC+5-FOA (1 g/l), as well as onto SC-Trp, SC-TrpUra and SC-Trp+5-FOA for strains carrying both *URA3* and *TRP1* reporters. Following growth for 36 h at 32°C, colonies were counted at the appropriate dilution where they were in the range of 15–50. 5-FOA-resistant cells were checked occasionally for viability upon transfer to a medium lacking uracil in order to ensure that 5-FOA resistance was due to silencing and not to mutation of *URA3*. It is noteworthy that the *sir1* null phenotype induced upon use of the pJ123.2 construct was observed to be associated with a general increase in telomeric silencing upon growth at 30 but not at 32°C (data not shown).

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