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Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression

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Transcriptional repression at the silent mating-type loci in yeast requires the targeting of silent information regulator (Sir) proteins through specific interactions formed at cis-acting silencer elements. We show here that a reporter gene flanked by two functional silencers is not repressed when integrated at >200 kb from a telomere. Repression is restored by creation of a new telomere 13 kb from the integrated reporter or by elevated expression of *SIR1*, *SIR3*, and/or *SIR4*. Coupled expression represses in an additive manner, suggesting that all three factors are in limiting concentrations. When overexpressed, Sir3 and Sir4 are dispersed throughout the nucleoplasm, in contrast to wild-type cells where they are clustered in a limited number of foci together with telomeres. Efficient silencer function thus seems to require either proximity to a pool of concentrated Sir proteins, that is, proximity to telomeres, or delocalization of the silencing factors.

[Key Words: Silencing; *SIR*; yeast mating type; telomere position effect; subnuclear organization]

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The organization of eukaryotic DNA within the interphase nucleus must facilitate the maintenance, replication, recombination, and coordinated expression of the genetic material. Several lines of evidence suggest that chromosomal domains are organized within the interphase nucleus. First, in situ hybridization with whole chromosome probes has demonstrated that mammalian chromosomes occupy specific territories and are not intertwined in the nucleoplasm (Cremer et al. 1993). Second, specific domains, such as telomeres and centromeres, often show nonrandom subnuclear distribution. For instance, telomeres appear to be adjacent to the nuclear envelope in polytene nuclei and embryonic cells of *Drosophila* (Mathog et al. 1984) and appear clustered in foci in budding yeast (Klein et al. 1992; Palladino et al. 1993; Cockell et al. 1995; Gotta et al. 1996). In fission yeast and in mammalian tissue culture cells, cell cycle-dependent positioning of telomeres has been observed (Funabiki et al. 1993; Vourc'h et al. 1993) and most species show a clustering of telomeres at the nuclear enve-

lope in the "bouquet" stage of meiosis (for review, see Gilson et al. 1993).

In several instances the subnuclear localization of a chromosome or a chromosomal domain could be closely correlated with its transcriptional state. For instance, it has been observed that electron-dense, negatively stained heterochromatin remains highly condensed and localized at the nuclear periphery in differentiated interphase cells (e.g., see Rae and Franke 1972; Mathog et al. 1984), as is the inactive X chromosome of mammalian females (Walker et al. 1991). In addition, the inactive, centromeric heterochromatin of *Drosophila* salivary gland nuclei coalesces into a single chromocenter (Heitz 1934). In brief, a variety of long-range interactions between chromosomal regions can be detected, which may also involve interactions between chromatin and elements of nuclear substructure. However, despite extensive correlations, the functional relationships between subnuclear organization and mechanisms that regulate gene expression remain unknown.

Local chromatin organization, usually envisaged as domain structure or as a conformation propagated along the nucleosomal fiber, is also responsible for the activation and inactivation of genes. In flies this is true for

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certain developmental programs of gene expression (Paro 1993), for centromeric position-effect variegation (for review, see Karpen 1994), and it is reflected in the variable expression of foreign genes integrated into mammalian chromosomes (e.g., Butner and Lo 1986). In the yeast *Saccharomyces cerevisiae*, gene repression at the silent mating-type loci (*HML* and *HMR*, collectively termed the *HM* loci) correlates with a general reduction in accessibility of the repressed domain to the yeast endonuclease HO and other DNA modifying enzymes (for review, see Laurenson and Rine 1992). In a similar fashion, polymerase II genes positioned adjacent to the poly(TG₁₋₃) tracts at yeast telomeres were found to succumb to a heritable but reversible transcriptional inactivation (Gottschling et al. 1990), which appears to spread inward from the telomere (Renauld et al. 1993).

The nucleation of the repressed chromatin state at telomeres requires a *cis*-acting DNA element, the telomeric TG₁₋₃ repeat, to which bind multiple copies of repressor activator protein 1 (Rap1; Gottschling et al. 1990; Liu et al. 1994; Stavenhagen and Zakian 1994; Buck and Shore 1995). At *HML* and *HMR*, short *cis*-acting silencer elements serve the same purpose, and, like enhancers, these silencers function at variable distances to repress targeted promoters (Brand et al. 1985, 1987; Mahoney and Broach 1989). Motifs within the *cis*-acting silencers (called **E** or **I**) are again recognized by *trans*-acting factors needed for the establishment of silencing, namely Rap1 (Shore and Nasmyth 1987), Abf1 (ARS binding factor 1; Buchman et al. 1988), and the origin recognition complex (ORC; Micklem et al. 1993), a six-polypeptide complex that recognizes the ARS consensus (Bell et al. 1993).

Sites for any two of the three silencer binding factors are sufficient for silencer function, at least in the context of the *HM* loci where a second partially functional silencer is present (i.e., *HMR-I* or *HML-I*; Brand et al. 1985, 1987; McNally and Rine 1991; Boscheron et al. 1996), and genetic results show that silencing at *HM* loci and telomeres requires the carboxy-terminal 130 amino acids of Rap1 (Sussel and Shore 1991; Kyrion et al. 1993; Liu et al. 1994). When long stretches of the (TG₁₋₃)_n telomeric repeat are integrated within the genome, they also confer a Sir-dependent repression of adjacent promoters (Stavenhagen and Zakian 1994), although a minimum of 828 bp (containing >40 potential Rap1-binding sites) are required. The fact that *HM* silencers repress genes more efficiently than internal telomeric tracts is thought to reflect the juxtaposition of Rap1 to ORC and Abf1 sites but may also reflect the chromosomal context of *HM* loci (see below).

In addition to the *cis*-acting sequences and their ligands, both telomeric and mating-type silencing require the silent information regulator genes, *SIR2*, *SIR3*, and *SIR4*, the *NAT1/ARD1* amino-terminal acetylase, and the amino termini of histones H3 and H4 (for review, see Laurenson and Rine 1992). Differences in the mechanisms of *HM* and telomeric silencing have also been noted. Namely, *sir1* mutations weaken silencing at *HML*, rendering it metastable (Pillus and Rine 1989), al-

though it has no effect at telomeres (Aparicio et al. 1991). Nonetheless, an artificially targeted Sir1 can enhance telomere-proximal silencing (Chien et al. 1993). Conversely, overexpression of the *TLC1* gene, encoding the RNA component of telomerase, derepresses at telomeres but has little effect on *HM* loci (Singer and Gottschling 1994).

Several lines of evidence suggest that Sir3 and Sir4 are structural components of repressed chromatin. First, they were shown to bind the amino termini of histones H3 and H4 *in vitro* (Hecht et al. 1995). Furthermore, deletions that derepress silencing *in vivo* both disrupt the *in vitro* binding and result in an altered localization of Sir3 and Sir4 in yeast nuclei, suggesting direct interactions with nucleosomes *in vivo* (Hecht et al. 1995). Second, overexpression of Sir4 or the carboxy-terminal domain of Sir4 derepresses both mating-type and telomeric silencing (Marshall et al. 1987; Cockell et al. 1995), whereas overexpression of Sir3 extends repression inwards from a marked telomere (Renauld et al. 1993). Third, Sir3 and Sir4 interact in two-hybrid assays with themselves, with each other, and with Rap1 (Chien et al. 1991; Moretti et al. 1994), and Sir4 and Rap1 coprecipitate in a DNase-insensitive complex from yeast nuclear extracts (Cockell et al. 1995). Finally, Rap1, Sir3, and Sir4 proteins all localize by immunofluorescence to a limited number of foci, many of which appear to be near the nuclear periphery (Palladino et al. 1993). Recently, combined immunofluorescence and *in situ* hybridization studies have shown that Rap1, Sir3, and Sir4 immunofluorescence signals coincide with hybridization signals of subtelomeric repeats (Gotta et al. 1996). The correlation of repression with the concentration of Rap1 and Sir3 in foci have led to the hypothesis that transcriptional silencing may be facilitated by the juxtaposition of telomeres with each other and/or with the nuclear envelope (Palladino and Gasser 1994; Hecht et al. 1995), although such clustering is clearly not sufficient for the establishment or maintenance of repression (Cockell et al. 1995).

The presence of the silent mating-type loci near the telomeres of chromosome III (~13 kb for *HML* and 25 kb for *HMR*) has led to the speculation that this particular chromosomal location of these loci may also contribute to the *HM* silencing process (Gilson et al. 1993). Indeed, moving the *HM* loci away from the telomere was shown to affect their silencing properties (Thompson et al. 1994; Shei and Broach 1995). Here, we further investigate the role of chromosomal context in silencing by integrating a reporter gene flanked by complete *HML-E* and *HML-I* silencers to quantify repression at various chromosomal sites. We demonstrate that proximity to telomeric repeat sequence is necessary for the repression of our reporter construct, although this requirement can be overcome by an elevated expression of Sir1, Sir3, and Sir4 proteins. These factors are thus limiting for some sites in the chromosome but not for others, suggesting the existence of concentration gradients or pools of silencing factors within the wild-type yeast nucleus. We speculate that this unequal distribution of Sir proteins

essentially creates compartments within the nucleus that can influence the function of Rap1 and Abf1 as either activators or repressors of transcription.

Results

HML silencer-mediated repression is dependent on chromosomal location

We have established a rapid, quantitative assay for silencer- and Sir-mediated gene repression in yeast, based on a reporter construct in which the $\alpha 1$ and $\alpha 2$ coding regions at *HML* are replaced by a minimal *LEU2* promoter fused to the bacterial *lacZ* gene (the *LEU2''lacZ* gene). This construct is flanked by >1 kb of sequence from each side of the *HML* locus, including the *E* and the *I* silencer elements (the *E>I* construct in Fig. 1A). When integrated into the genome, this construct allows us to monitor silencing quantitatively using a soluble assay for β -galactosidase. We have demonstrated previously that cells carrying the *LEU2''lacZ* integrated at the *HML* locus have very low β -galactosidase activity (0.07 Miller unit, standardized as 1; see Fig. 1B, strain EG5; Boscheron et al. 1996). Expression levels increase by 40- to 60-fold in the absence of silencers or in strains lacking *sir1* or *sir3*, and, like natural *HM* silencing, it is independent of the promoter orientation with respect to the silencers (Boscheron et al. 1996). Because inactivation of Sir3 or Sir4 results in the complete derepression of *HML α and *HMR* α (Klar et al. 1981; Nasmyth et al. 1981; Ivy et al. 1986), we assume that the β -galactosidase activity measured in *sir3* or *sir4* cells corresponds to the fully derepressed level of the reporter cassette.*

To examine whether *HM* silencing is influenced by its natural chromosomal location near the left end of chromosome III, we inserted the *LEU2''lacZ* gene flanked by the two functional *HML* silencers at four internal chromosomal loci: within the *LYS2* gene, located 342 kb from the right end of chromosome II; adjacent to the *HIS3* gene, ~250 kb away from the right telomere of chromosome XV; downstream of the *KEX2* gene, ~200 kb away from the left telomere of chromosome XIV; and downstream of the *SIN4* gene, at ~5 kb centromere proximal from the *KEX2* insertion. In all cases the recipient chromosomes are significantly larger than chromosome III. The insertional events were confirmed by both PCR and Southern blot restriction site analysis (Materials and methods; data not shown).

The expression level of the *LEU2''lacZ* gene flanked by two functional silencers (the *E>I* construct in Fig. 1A) increases 40- to 43-fold when inserted at *LYS2*, *HIS3*, *KEX2*, or at *SIN4*, as compared with insertion at *HML* (cf. Fig. 1B, strains EG5, EG59, EG112, LM2, and LM3). All values for β -galactosidase activity are standardized to the expression level of the same construct inserted at *HML*. Deletion of the *E* silencer at *LYS2* (the *e₁>I* construct in Fig. 1A and strain EG36 in Fig. 1B) results in a fully derepressed level of the reporter gene expression because the β -galactosidase activity is equivalent to that ob-

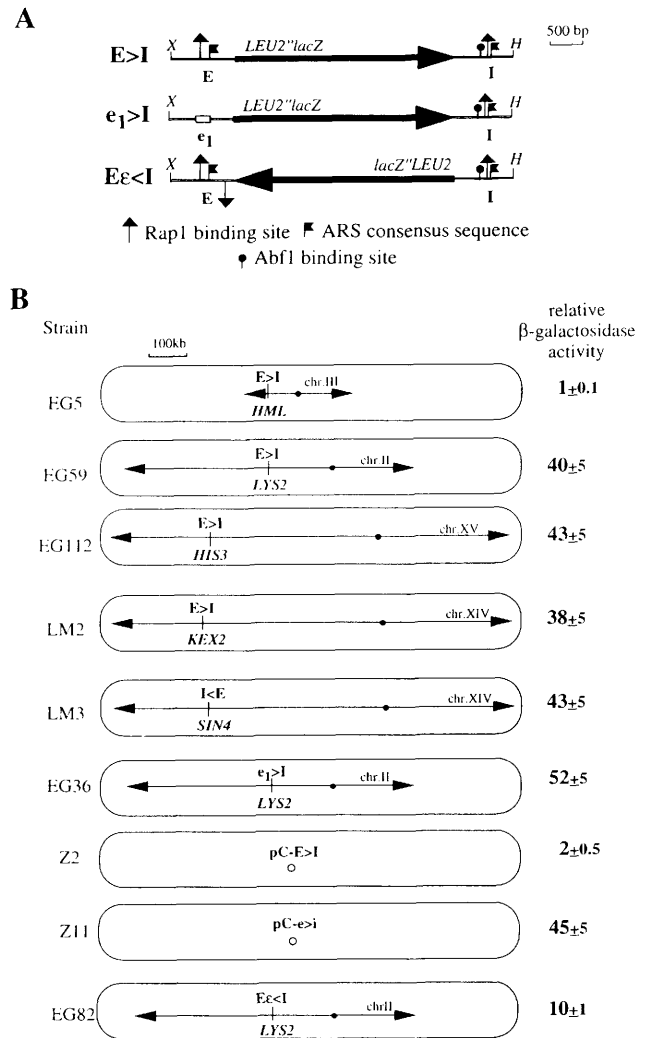


Figure 1. The chromosomal context influences the expression of a *LEU2''lacZ* reporter flanked by *HML-E* and *HML-I*. (A) Diagram of the *LEU2''lacZ* silencing cassettes. The 3.4-kb *LEU2''lacZ* reporter gene is expressed under the control of a minimal *LEU2* promoter. The symbols representing the mapped Rap1, Abf1, and ARS consensus sequences (ACS) are shown at the bottom. *E>I* indicates that both *E* and *I* silencers are present and that the promoter of the reporter gene is located near *E*, *e₁>I* is deleted for *E* and *Ee<I* includes a RAP1-binding site in between *E* and the 3' end of the reporter gene. (X) *Xba*I, (H) *Hind*III. (B) The relative β -galactosidase activities produced in yeast strains carrying the indicated *LEU2''lacZ* silencing cassettes are given to the right of the strain tested. All strains are isogenic except for the integration or plasmid indicated in the oblong circles, and all carry a *LEU2*-expressing vector. The standard deviation of the mean was calculated from the results of at least four independent trials using two parallel reactions for each point; the standard deviation is indicated. The strain EG5 carries the *E>I* cassette at *HML* and produces 0.07 Miller unit (Boscheron et al. 1996) that was standardized to 1. Arrows represent telomeres and dots indicate the position of the centromere. The position of the targeted silencing cassette is shown by a vertical bar. Replicative plasmids (pC-E>I and pC-e>i) are represented (O).

served for the construct at *HML* in cells mutant for *sir1*, *sir3*, or *sir4* (Fig. 2A, columns 2–4). The expression of the reporter gene at *LYS2* is only slightly increased by mutations in the *SIR* genes (Fig. 2B, columns 2–4) or by

overexpression of the Sir4 carboxy-terminal domain (data not shown), which fully derepresses the *E>I* construct integrated at *HML* (Boscheron et al. 1996). These results suggest that *HML* silencer function depends at

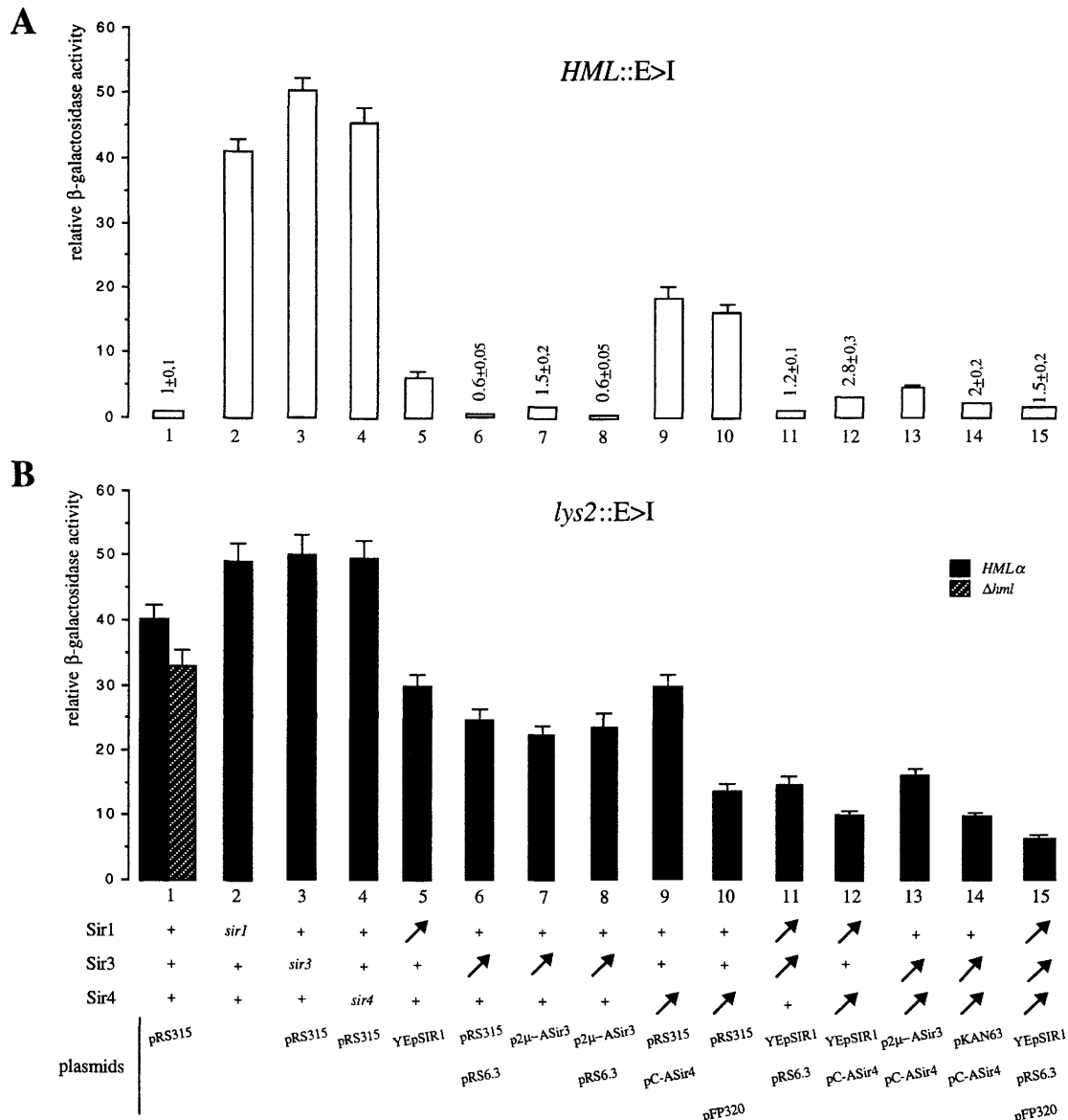


Figure 2. Silencer-mediated repression at the *LYS2* locus requires elevated concentrations of Sir proteins. The dependence of the *LEU2''lacZ* expression on silencing factors is illustrated in EG37 (*hml::E>I*; A) and EG70 (*lys2::E>I*; B) by the disruption of *sir1* by *LEU2*, *sir3* by *TRP1*, and *sir4* by *HIS3* (columns 2, 3, and 4, respectively) or by transforming with plasmids overexpressing the genes encoding Sir1 (YEpSIR1) or Sir3 (pKAN63, p2 μ -ASir3, pRS6.3) or Sir4 (pC-ASir4, pFP320). All the cells contain a functional *LEU2* gene either in the chromosome (*sir1::LEU2*) or on a plasmid (pRS315, YEpSIR1, p2 μ -ASir3, or pKAN63) to allow growth in media selective for leucine. The *sir* disruptions and plasmids are listed below the columns and are applicable to both A and B. Plasmids in the *top* row all carry *LEU2* and in the *middle* carry *URA3*, and pFP320 carries *TRP1*. When two or three plasmids were carried by the same strain, they are listed vertically. All measurements were made on media selective for leucine and, when necessary, for tryptophan and/or uracil. EG37 and EG70 carrying any combination of one, two, or three of the following parental plasmids without *SIR* gene inserts produced similar amounts of β -galactosidase: pRS315 (CEN-ARS *LEU2*), pAAH5 (2 μ -*LEU2*), pRS314 (CEN-ARS *TRP1*), pRS316 (CEN-ARS *URA3*). The comparison of β -galactosidase activities from *LEU2''lacZ* reporter at *LYS2* (EG59) in the presence and absence of an intact *HML* locus is shown by the solid and hatched bars in column 1, respectively. In all cases the activity is presented relative to that obtained with EG5 carrying pRS315 (A, column 1, 0.07 Miller unit standardized to 1). The error bars represent the standard deviation of the mean. Very low values from the insert at *HML* are indicated above the bars.

least partially on chromosomal context; that is, constructs that repress at *HML* do not necessarily do so at internal loci.

To verify that the translocated fragment of *HML* is indeed sufficient to confer silencing, the *HML::LEU2''lacZ* DNA (i.e., E>I) that was inserted at internal locations was cloned on a CEN-containing plasmid. The expression of the reporter gene is now repressed by the flanking silencer sequences, as observed at their native location at *HML* (strain Z2; Fig. 1B). Again, repression is fully dependent on intact silencer sequences, and the expression level of the plasmid-borne construct without silencers is equivalent to the intact construct inserted at internal loci (strain Z11; Fig. 1B). This shows that all the genetic information necessary for silencing is contained within the *HML* DNA fragments inserted at *LYS2*, *HIS3*, *KEX2*, and *SIN4*. Moreover, this result suggests that the factors or conditions that restrict repression at internal loci are not applicable to a plasmid-borne construct.

To exclude that the absence of silencer-mediated repression at *LYS2* is a peculiarity of the *LEU2''lacZ* reporter, we have inserted the *URA3* or the *ADE2* gene flanked by *HML* silencers at various loci. When integrated at *HML*, variegated expression of *URA3* was visualized by the growth of ~10% of the plated cells on 5-fluoro-orotic acid (5-FOA) and by sectored pink/red colonies for the *ADE2* construct (data not shown). In contrast, no repression was monitored when either construct was integrated at *LYS2* (data not shown). This position-dependent silencer function is not strain specific, because the insertion of the same *URA3* silencing construct at *HML* or at *LYS2* in a diploid strain of a different parental background shows repression at *HML* but not at *LYS2* (data not shown). Interestingly, when integrated at the *SUC2* locus found at ~25 kb from the left end of chromosome IX (Carlson et al. 1985), the *URA3* silencing construct is repressed to a level similar to that observed at the natural *HML* locus (data not shown). Although this repression might reflect the late timing of replication that characterizes telomere-proximal sequences (Ferguson and Fangman 1992), we were unable to measure repression when the silencer-flanked reporter was integrated at *KEX2* and *SIN4*, two internal locations reported to be late replicating (Fig. 1B; cited in Diller and Raghuraman 1994). These results suggest that proximity to a telomere, not simply sequence context, influences silencer-mediated repression.

The formation of a new telomere in cis restores HML silencer function at LYS2

To test whether telomere proximity can improve silencer function at *LYS2*, we have fragmented the distal part of the chromosome that carries the *HML::LEU2''lacZ* DNA at *LYS2* in a diploid strain (Fig. 3A). In diploid cells, the *HML::LEU2''lacZ* construct inserted at *LYS2* is derepressed, with a level of β -galactosidase 43-fold higher than that measured when the same construct is inserted at *HML* (Fig. 3B, cf. EG84 and EG85). Truncating chromosome II places the reporter construct 13 kb from the

newly formed telomere and results in full repression of the reporter gene (Fig. 3B, cf. EG84 and EG86). To demonstrate that this is not owing to a reduction in the dosage of a gene carried on the distal arm of chromosome II, we have performed a similar truncation on the chromosome II homolog lacking the reporter construct. In this case no repression is observed (Fig. 3B, strain EG87), demonstrating that it is formation of a new telomere in *cis* that influences silencer function at *LYS2*. This also confirms that no sequence within the 13 kb distal of *LYS2* inherently interferes with silencing. Thus, we conclude that the lack of silencer-mediated repression at *LYS2* in its normal chromosomal position probably reflects the large distance that separates this locus from its most proximal telomere.

Internal silencing is not attributable to propagation from the proximal telomere

We observe that the *HML::LEU2''lacZ* is efficiently repressed when positioned within 13 kb of a telomere (i.e., at *HML* or near a fragmented end of chromosome II). It is conceivable that this organization allows the linear propagation of telomeric silencing from the end of the chromosome to the reporter construct, rather than promoting the de novo establishment of repression by the adjacent silencers. To test this we inserted the *URA3* gene 3.1 kb away from the TG₁₋₃ repeats at the left telomere of chromosome III in a strain carrying *LEU2''lacZ* at *HML* (strain EG47; Fig. 4). Under these conditions *URA3* exhibits the expected variegated pattern of expression, producing ~5% 5-FOA^R colonies (data not shown). By growing these strains in the presence of 5-FOA or in the absence of uracil we place a continual selection on the cells for either the repression of *URA3* or its transcription at a level sufficient to support uracil biosynthesis. By comparing the two conditions of growth, we can monitor expression of the *HML::LEU2''lacZ* construct in relation to the repressed or active state of the telomere-proximal *URA3*.

During 10 generations of growth on either 5-FOA or uracil-deficient media, the expression of the *LEU2''lacZ* gene flanked by both E and I at *HML* is unchanged, remaining at a fully repressed level (strain EG47; Fig. 4). This stable, repressed level of *HML::LEU2''lacZ* expression is also observed when *URA3* is inserted at the telomere of another chromosome, allowing us to conclude that neither growth conditions nor the transcriptional state of a telomere-proximal *URA3* has an effect on expression at *HML* (strain EG93; Fig. 4). We were able to extend this analysis to the *HML::LEU2''lacZ* reporter inserted at *LYS2*, because the insertion of the telomeric repeat 13 kb away from *LYS2* coincided with the integration of an intact *URA3* gene immediately adjacent to the telomere. Again, the tight repression of the *HML::LEU2''lacZ* reporter is shown to be independent of the transcriptional state of *URA3* modulated by growth on either uracil-deficient or 5-FOA media (strain EG86; Fig. 4). This indicates that silencer-mediated repression at *HML* and at other internal sites is indepen-

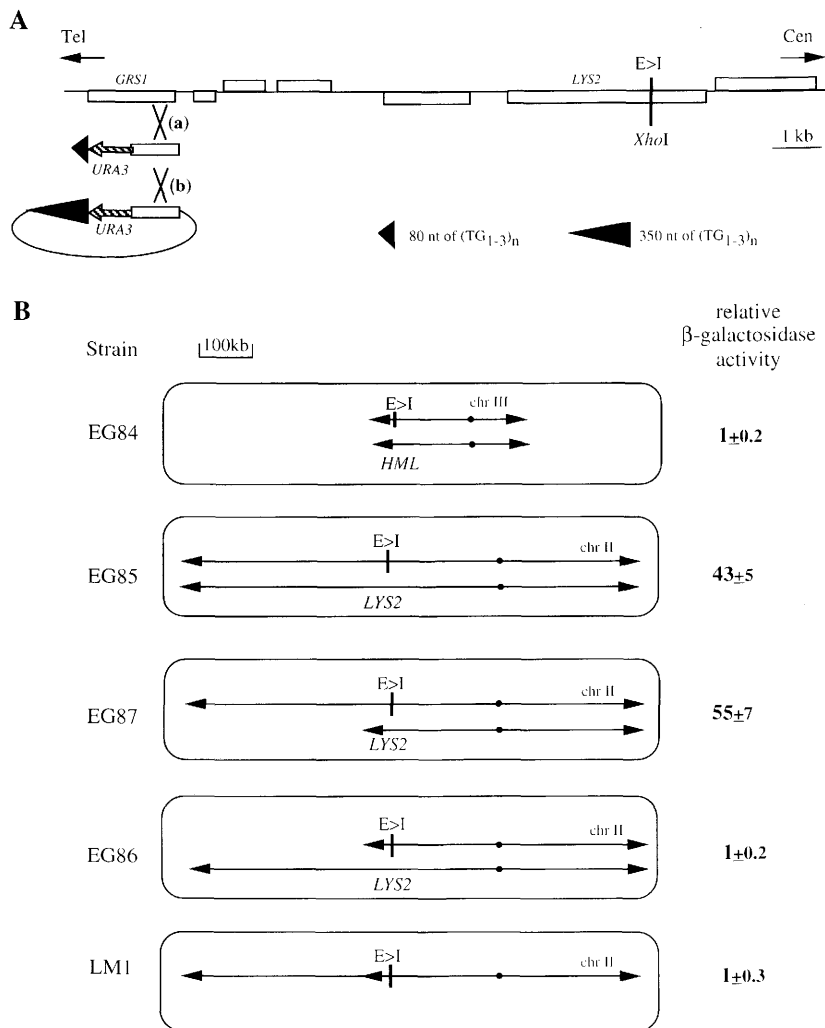


Figure 3. Insertion of yeast telomeric repeats 13 kb from *LYS2* restores repression. (A) The integration of yeast telomeric repeats at the *GRS1* locus on chromosome II is depicted on the genetic map of the *GRS1*–*LYS2* region. Hanging rectangles are ORFs transcribed toward the centromere, and upright rectangles are those transcribed toward the telomere. The site of integration of the E>I silencing cassette at *LYS2* is represented by a vertical bar. X indicates the site of integration in *GRS1* of either (a) a linear DNA fragment derived from pURATel80LYS exposing at one end 80 nucleotides of $(TG_{1-3})_n$ DNA or (b) the plasmid pURATel350LYS cut at a unique site within the *GRS1* region, which inserts 350 nucleotides of $(TG_{1-3})_n$ without creating a telomeric end. Integration a was done in the diploid strain EG85 and Southern blots were done to confirm whether the integration was in the *lys2::LEU2''lacZ* marked chromosome (creating EG86) or in the other homolog (creating EG87). Integration b was done in the haploid strain EG59 creating strain LM1. (B) The diploids EG84 and EG85 are described in Materials and methods, and EG87, EG86, and LM1 are described in A. β -Galactosidase activity and calculation of relative activity is as described in Fig. 1.

dependent of the state of expression at the immediately proximal telomere, even though proximity to a telomeric repeat does facilitate the establishment of repression. The independence of these two related silencing events is underscored by the fact that repression of the *HML::LEU2''lacZ* reporter inserted at *HML* is derepressed in *sir1* cells (Fig. 2A, column 2; Boscheron et al. 1996), whereas the telomeric silencing of the *URA3* gene located in between the left telomere of chromosome III and *HML* is not (strain EG47; Fig. 4; data not shown).

Additional Rap1 sites allow repression of *HML::LEU2''lacZ* at *LYS2*

At *HML* the insertion of an additional Rap1-binding site 3' of the reporter gene (the Rap1 consensus from the $\alpha 2$ promoter) enhances the efficiency with which the double silencer construct represses *LEU2''lacZ*, although the binding site does not act as a silencer on its own (the E ϵ <I construct in Fig. 1A; see also Boscheron et al. 1996). We show here that it can also partially restore repression of the reporter gene at its internal *LYS2* site (strain EG82;

Fig. 1B). Moreover, insertion of a series of Rap1 sites, in the form of 350 bp of TG_{1-3} repeat at a distance of 13 kb (Fig. 3A), also confers repression on the *lys2::HML::LEU2''lacZ* reporter, even without truncating the chromosome (Fig. 3B, strain LM1). By performing this insertion in a haploid cell, we ensure that the distal 342 kb of chromosome II, which encodes several essential genes, is not eliminated. Southern blot and PCR analyses confirm the location of the insertion and the lack of truncation (data not shown). We assume that the insertion of the TG_{1-3} sequence acts through the binding of Rap1, which has been shown by both molecular and immunological techniques to bind yeast telomeric repeats (for review, see Gilson and Gasser 1995). These observations suggest that a critical element provided by proximity to a telomere is an abundance of Rap1 molecules and perhaps Rap1 ligands.

Overexpression of *Sir1*, *Sir3*, and *Sir4* enables silencer-mediated repression at *LYS2*

Why are internal chromosomal sites less conducive to silencer-mediated repression? Based on the unequal dis-

Figure 4. Silencing is not propagated continuously from the telomere. The expression of the *LEU2''lacZ* silencing cassette (E>I) is monitored either at the *HML* site of integration (EG93 and EG47) or at *LYS2* (EG86). In all three strains the only wild-type *URA3* allele is under the influence of TPE as follows: In EG93, the *URA3* gene is integrated at a fragmented VIII telomere and the silencing cassette is at *HML* on chromosome III. In EG47, the *URA3* gene is located in between the telomere and *HML::LEU2''lacZ* on chromosome III, and similarly in EG86, the promoter of *URA3* is at 1 kb from the telomeric repeat and 12 kb from the *lys2::LEU2''lacZ* reporter on chromosome II. As indicated at left, cells were grown in the absence or presence of uracil and in the absence or presence of 1 mg/ml of 5-FOA, to select for cells that either express (– uracil, ON) or repress (+ 5-FOA, OFF) *URA3*. Cells grown with uracil but without 5-FOA are subject to variegated expression, which is indicated as ON/OFF. Cultures grew at least 10 generations before being assayed for β -galactosidase activity. The standard deviation of the mean was calculated on at least four independent trials and is presented as described in Fig. 1.

Strain	Diagram	Uracil	5-FOA	<i>URA3</i> expression	relative β -galactosidase activity
EG93		-	-	ON	1 ± 0.2
		+	-	ON/OFF	1.2 ± 0.2
		+	+	OFF	0.7 ± 0.2
EG47		-	-	ON	1.2 ± 0.2
		+	-	ON/OFF	1 ± 0.1
		+	+	OFF	0.8 ± 0.2
EG86		-	-	ON	1 ± 0.2
		+	-	ON/OFF	1 ± 0.2
		+	+	OFF	0.7 ± 0.2

tribution of Rap1, Sir3, and Sir4 proteins in the nucleus (Klein et al. 1992; Palladino et al. 1993; Cockell et al. 1995), we speculated that internal chromosomal sites, like *LYS2*, may not have ready access to the high concentrations of Sir proteins found near telomeres. Consistent with this idea that Sir proteins are limiting, we see that a small degree of repression can be conferred on the reporter construct at *LYS2*, by deleting the *HML* locus in the control strain and thereby lowering the number of sites competing for Sir factors (Fig. 2B, column 1). To determine which Sir proteins might be limiting for silencer-mediated repression at *LYS2*, we transformed the strain carrying *lys2::HML::LEU2''lacZ* with plasmids expressing *SIR* gene products or with control vectors carrying only the selectable marker. In all cases the effects of *SIR* overexpression were also monitored in a fully isogenic strain carrying the *LEU2''lacZ* reporter at *HML* instead of at *LYS2*. The levels and localization of the proteins were checked by immunoblotting (Fig. 5) and immunofluorescence (Fig. 6).

As described above, the β -galactosidase activity produced from the *LEU2''lacZ* construct integrated at *LYS2* is ~40-fold higher than that detected from the same construct at *HML* (Fig. 2A,B, column 1). However, if Sir1 (Fig. 2B, column 5) or Sir3 (Fig. 2B, columns 6–8) or Sir4 (Fig. 2B, columns 9,10) are overproduced, a drop in the level of β -galactosidase is observed, reflecting a partial restoration of silencing at an internal location. These results suggest that these Sir proteins are limiting for repression at *LYS2* or else that their overexpression alters the availability and/or the expression of other factors that are of limited supply. This last explanation was examined by Western blotting with affinity-purified monospecific antibodies, to determine the protein levels conferred by various *SIR*-expressing plasmids (Fig. 5). Within a twofold margin of error, Rap1 levels remain constant in all cells tested (data not shown), whereas Sir3

and Sir4 levels increase between 8- and 50-fold upon transformation with plasmids overexpressing the appropriate gene (see legend to Fig. 5 for quantitation). The amount of Sir3 in the cell is largely unchanged by Sir4 overexpression and vice versa, indicating independent regulation of the two polypeptides. We are unable to monitor Sir1 by Western blot but can nonetheless confirm that the introduction of YEpSIR1 does not significantly alter levels of Rap1 (data not shown), Sir3, or Sir4 (Fig. 5).

The Sir3 expression from a low-copy-number plasmid (pRS6.3) is sixfold lower than that from a multicopy plasmid carrying *SIR3* under control of the *ADC1* promoter (p2 μ -ASir3; see Fig. 5), yet repression of the *lys2::LEU2''lacZ* reporter is approximately equal, even in the presence of both plasmids (Fig. 2B, column 8). Similarly, the partial repression brought about by roughly 30-fold higher levels of Sir4 (pC-ASir4; Fig. 5) is not improved by further increasing *SIR4* gene expression (data not shown). This suggests that Sir3 and Sir4 become saturating for internal silencing at sufficiently high dosage, further supporting the hypothesis that more than one silencing factor is limiting for internal silencing.

This hypothesis was tested directly by the pairwise introduction of constructs such that both Sir1 and Sir3, both Sir1 and Sir4, or both Sir3 and Sir4 or all three could be concomitantly expressed (Fig. 2B, columns 11–15). Under these conditions the enhanced repression conferred by the plasmid-borne genes is additive. Maximal repression is achieved in the presence of all three plasmids and corresponds to a drop in the *LEU2''lacZ* expression level of 83% (Fig. 2B, column 15). The fact that overexpression of three Sir proteins does not repress 100% may reflect either the limiting dosage of another silencing factor or cell-to-cell variability in expression levels from the plasmid-borne genes. It is noteworthy

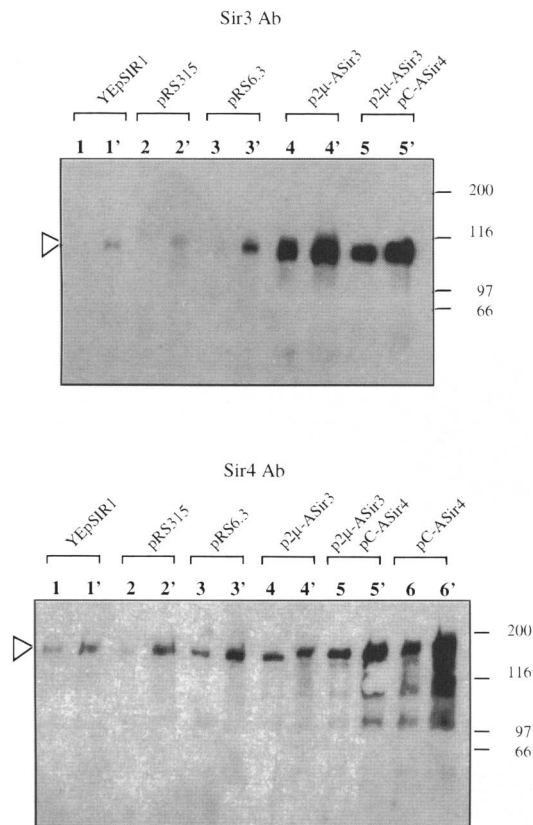


Figure 5. Levels of Sir3 and Sir4 overexpression as determined by Western blot with affinity-purified antibodies. Crude nuclear fractions from EG37 carrying the indicated plasmids and grown in selective media (see Fig. 2; Materials and methods) were analyzed on SDS gels and Western blots by standard techniques. Identical filters were probed with affinity-purified rabbit antibodies against Sir3 (labeled Sir3 Ab), Sir4 (labeled Sir4 Ab), Rap1, and tubulin (data not shown). EG37 carrying the indicated plasmids bear labels of 1–5, and for each strain the pair of lanes corresponds to proteins from 1 and 2 OD₂₆₀ units of nuclei, respectively. Lanes 6 and 6' contain the double of this. The open triangles indicate the expected migration of Sir3 and Sir4, and the molecular weight markers are given at the right. Quantitation was performed as described in Materials and methods and shows Sir3 levels to be constant in strains carrying YEpSIR1 (1) and pRS315 (2), 7-fold higher in the presence of pRS6.3 (3), and between 40- and 50-fold higher in strains with p2 μ -ASir3 (4) or p2 μ -ASir3 and pC-ASir4 (5). The Sir4 protein levels are equivalent in strains 1, 3, and 4 and are slightly lower in the presence of the control plasmid pRS315 (lane 2). In the presence of p2 μ -ASir3 and pC-ASir4 together (5), the level is between 30- and 60-fold elevated, as it is in the presence of pSIR4-2 μ alone (lane 6). Compounded error rates gives a 50% error range for these values.

that the introduction of pC-ASir4 and either YEpSIR1 or pKAN63 (a high-copy-number plasmid with the *SIR3* gene under its own promoter) at the same time also compensates for the derepression of *HML* conferred by Sir4 overexpression alone (Fig. 2A, cf. columns 12–14). One explanation of this is that Sir4 forms complexes, probably with Sir3, in which the stoichiometry is carefully

balanced. Because Sir protein dosage does not affect the *LEU2* promoter in the absence of silencers, we conclude that Sir overexpression reinforces silencer-mediated repression at internal chromosomal locations.

A dispersed localization of Sir3 and Sir4 proteins correlates with silencing at lys2::HML::LEU2''lacZ

Using monospecific affinity-purified antibodies for indirect immunofluorescence, we show in Figure 6 that Sir3, Sir4, and Rap1 are highly concentrated in a limited number of foci in the haploid reporter strain EG37 carrying a *LEU2* vector with no insert (Fig. 6a,d,g). To demonstrate the specificity of the anti-Sir immunofluorescence, we tested affinity-purified anti-Sir3 on a *sir3::LEU2* strain and anti-Sir4 on a *sir4::HIS3* strain, and neither produced staining above background (Fig. 6l,m). Elsewhere, we have shown that these immunoreactive foci coincide in a statistically significant manner with foci detected by in situ hybridization with a highly conserved subtelomeric sequence (Y'; Gotta et al. 1996; see also Fig. 6k). In the control strain, Rap1, Sir3, Sir4, and the Y' telomeric sequences all produce a very similar pattern of staining: between 4 and 9 discrete foci (green) superimposed on the ethidium bromide stain of genomic DNA [red; Fig. 6a,d,g,k]. The specificity of anti-Sir3 and anti-Sir4 for single yeast polypeptides of the appropriate size is demonstrated by Western blot (Fig. 5) and was demonstrated previously for anti-Rap1 (Klein et al. 1992). We then asked the question whether the establishment of silencing at *LYS2* owing to overexpression of *SIR1*, *SIR3*, and/or *SIR4* reflects the “release” of Sir proteins from this focal localization pattern.

The distribution of Rap1, Sir3, and Sir4 was determined under the conditions of overexpression that allow silencing of the internal *lys2::HML::LEU2''lacZ* reporter. Overexpression of Sir3 results in a diffuse staining pattern of Sir3 throughout the nucleoplasm (Fig. 6e), consistent with an excess of Sir3 in the foci. Interestingly, in the same cells, the Rap1 and Sir4 foci are visible but again are more diffuse (Fig. 6b,h). This is shown for a strain carrying pRS6.3, but similar patterns were observed in the presence of p2 μ -ASir3 (data not shown). The overexpression of Sir4 also leads to a diffuse staining of Sir4 (Fig. 6i), a slightly more dispersed Rap1 focal staining (Fig. 6c), and a diffuse Sir3 staining superimposed on foci (Fig. 6f). These results clearly show that when either Sir3 or Sir4 are overproduced, Sir3, Sir4, and, to a lesser extent, Rap1 are delocalized in a coupled manner. Thus, the restoration of an efficient silencing at internal sites correlates with an increase of available Sir3 and Sir4 proteins throughout the nucleus. On the other hand, in strains overexpressing Sir1, the focal staining of Rap1, Sir3, and Sir4 is preserved (data not shown). Intriguingly, when both Sir3 and Sir4 are overexpressed, the focal staining pattern for Rap1, Sir3, and Sir4 was partially restored (shown for anti-Sir4; Fig. 6n), indicating that a balanced expression of Sir3 and Sir4 is critical for their localization at telomeric foci. This is consistent

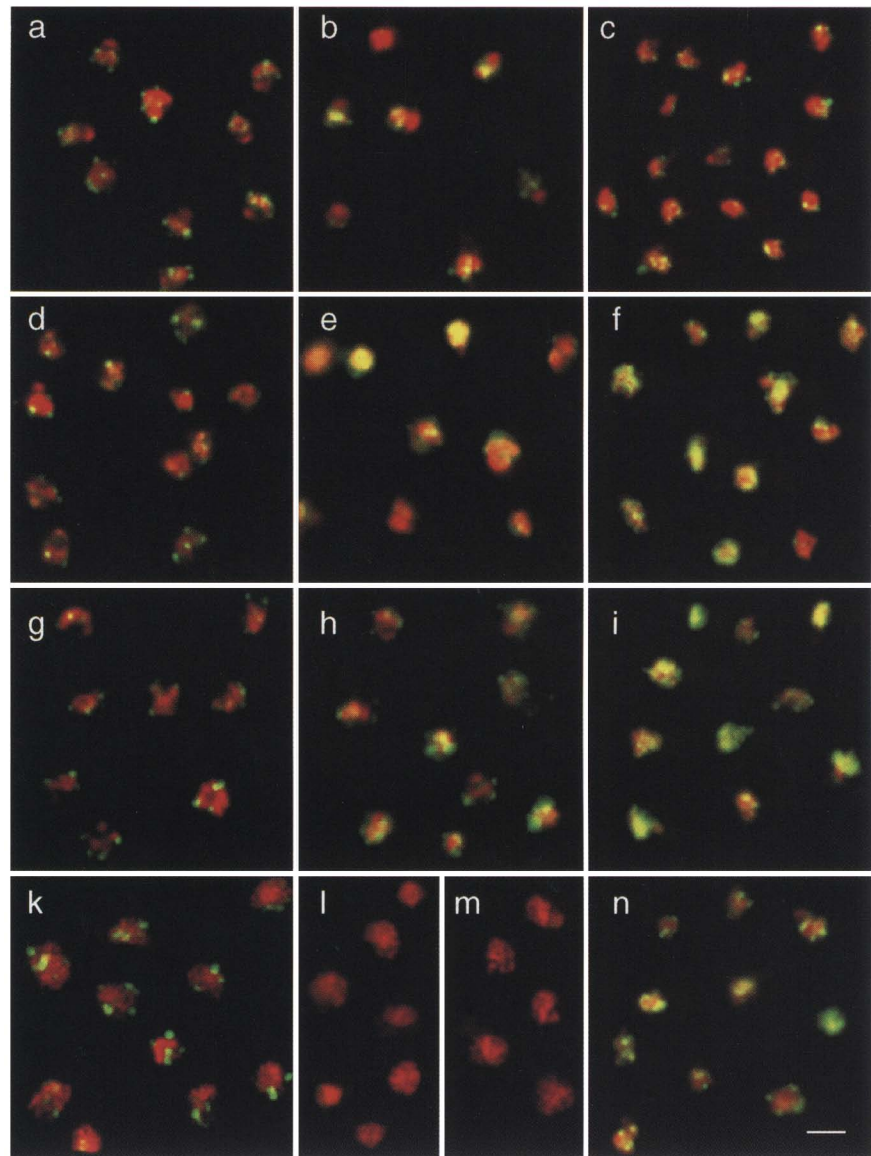


Figure 6. Sir3 and Sir4 overexpression correlates with a dispersed staining of both Sir proteins. Immunofluorescence was performed as described in Materials and methods with the anti-Sir3 (*d–f*) and anti-Sir4 (*g–i,n*) used on the Western blots in Fig. 5 and with affinity-purified Rap1 (panels *a–c*). These were visualized by a DTAF-coupled secondary antibody (green signal), whereas the nuclear DNA is counterstained with ethidium bromide (red). Superposition of the two signals is yellow. (*a,d,g*) EG37 carrying pRS315; (*b,e,h*) EG37 carrying pRS6.3; (*c,f,i*) EG37 with pC-ASir4; (*n*) EG37 with both pC-ASir4 and p2 μ -ASir3. (*l*) Anti-Sir3 staining on S150-2B carrying a *sir3::LEU2* disruption; (*m*) anti-Sir4 staining on a *sir4::HIS3* disruption (JRY3411; see Palladino et al. 1993). (*k*) In situ hybridization with a 4.8-kb fragment of the short Y' repeat of *S. cerevisiae* (Louis et al. 1994) visualized by an FITC-coupled anti-digoxigenin Fab fragment (Boehringer Mannheim). A diploid wild type for all known loci affecting silencing (GA225; see Palladino et al. 1993) was used for the hybridization. All images are taken as described in Materials and methods. Bar, 1.5 μ m.

with the hypothesis that Sir3 and Sir4 form complexes of defined stoichiometry important for repression (Marshall et al. 1987; Chien et al. 1991; Moretti et al. 1994; Cockell et al. 1995).

Discussion

Silencer function depends on chromosomal context

Using a quantitative reporter system for yeast gene repression in which there is no selection for or against expression of the reporter gene, we have shown that the Sir-dependent repression conferred by *HML* silencers depends on chromosomal context. That is, a *LEU2''lacZ* reporter construct flanked by *HM* silencers is repressed when integrated at *HML*, at a subtelomeric domain, or if carried on a plasmid. It is not repressed, however, when integrated at *LYS2*, *HIS3*, *KEX2*, and *SIN4* that are 342

kb, 250 kb, 200 kb, and 205 kb, respectively, from the nearest telomeres of chromosomes II (*LYS2*), XV (*HIS3*), and XIV (*KEX2* and *SIN4*). Silencing can be restored at the *LYS2* internal site by either truncation of the chromosome 13 kb from the reporter construct or by the insertion of 350 bp of telomeric tract, which is not sufficient to repress transcription on its own (Stavenhagen and Zakian 1994). This requirement for proximity to a telomeric repeat is not promoter nor strain specific, nor is the Sir-dependent repression of this construct simply an extension of telomere repeat-mediated repression.

Sir protein concentration affects internal silencing

We have demonstrated that Rap1, Sir3, and Sir4 proteins, which play essential roles in telomeric and *HM* silencing, are not randomly distributed throughout the wild-

type yeast cell nucleus but localize in four to nine bright foci of staining, as does subtelomeric DNA, detected by in situ hybridization (Palladino et al. 1993; Cockell et al. 1995; Fig. 6). Because we have demonstrated that the majority of the Rap1, Sir3, and Sir4 foci coincide with the FISH signals of the Y' subtelomeric probe (Gotta et al. 1996), we can conclude that in wild-type cells Sir3 and Sir4 are present in high concentrations at their major sites of action, that is, near telomeric repeats.

We argue that this unequal distribution of Sir proteins in the yeast nucleus is responsible for the inability of *HML* silencers to repress at *LYS2* and other internal loci for the following reasons: First, the elevated expression of silent information regulators Sir1, Sir3, or Sir4, confers repression to a limited degree on the "internal" *LEU2''lacZ* reporter construct. Concomitant overexpression of both Sir3 and Sir4 or of either in combination with Sir1 further improves repression, suggesting that all three proteins are limiting for silencer-mediated repression at internal sites. Coincident with the elevated expression of Sir3 or Sir4, we observe a diffuse staining of both proteins throughout the yeast nucleus, which is consistent with the model that the overexpressed proteins are free to diffuse to the internal *lys2::HML::LEU2''lacZ* reporter. There may be other yet uncharacterized proteins that are limiting for silencing also released by overexpression of Sir3 or Sir4. Second, we show that the insertion of 350 bp of telomeric tract will allow full repression of the *HML::LEU2''lacZ* reporter at *LYS2*, facilitating the ability of the silencer to function, but not creating a continuum of repression from the telomere. It was shown previously that TG_{1-3} tracts of a similar length do not alone confer silencing at *LYS2*, although insertion of an internal tract of >800 bp does (Stavenhagen and Zakian 1994). It is assumed that the TG_{1-3} tracts function by binding Rap1, which in turn, targets Sir3 and Sir4 to the nearby promoter (Moretti et al. 1994). Finally, it has been demonstrated that the artificial targeting of Sir3 or Sir4 constructs near a reporter gene at internal positions in the chromosome repress the reporter only if the strains carry carboxy-terminally truncated forms of Rap1 (Lustig et al. 1996; Marcand et al. 1996). These mutations result in the delocalization of both Sir3 and Sir4 from the telomeric foci (Cockell et al. 1995), which resembles the distribution observed in strains overexpressing *SIR3* or *SIR4* (Fig. 6). Thus, to repress at internal loci, it appears necessary to achieve a critical local concentration of Sir proteins. This can be achieved either by their release from the telomeric foci, by their overexpression, or through interaction with a sufficient amount of Rap1 (or other Sir-binding equivalents) targeted to the reporter gene. The ability to reach this "Sir threshold" is also facilitated by proximity to a chromosomal end. This is depicted schematically in Figure 7.

We show that Sir1 is also limiting for silencer-mediated repression and that its overexpression with Sir3 and/or Sir4 enhances repression of *lys2::HML::LEU2''lacZ* in an additive manner. Intriguingly, the overexpression of Sir1 or Sir4 affect differentially the same reporter construct at *LYS2* and at *HML*, decreasing repression at

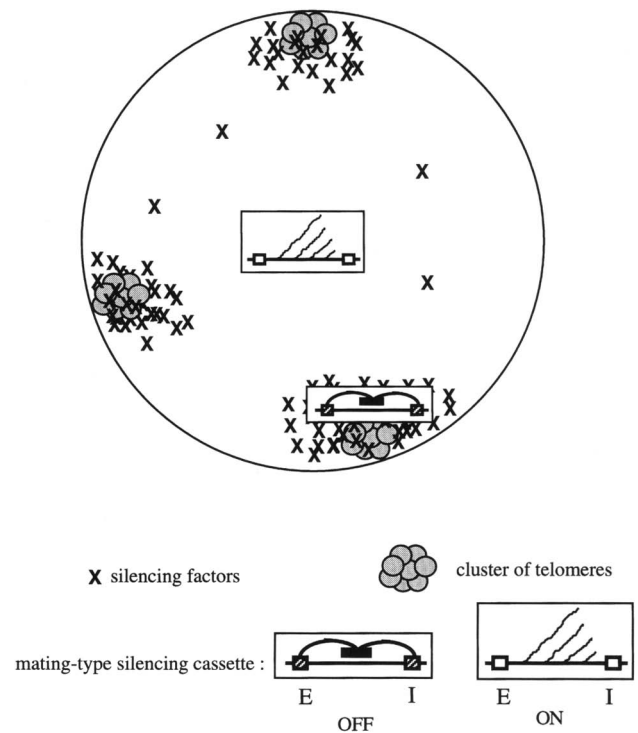


Figure 7. Nuclear subdomains and concentration gradients can affect gene expression. In this model we depict a nucleus with three zones in which silencing factors (e.g., Sir3 and Sir4) are highly concentrated, coinciding with clusters of telomeric repeats. Lower concentrations of Sirs are found throughout the nucleoplasm. We suggest that *HM* silencer-flanked genes have a better chance to be repressed if they have access to the pool of Sirs at telomeres. To repress at weak or potential silencers that are not subtelomeric, higher concentrations or delocalization of Sir proteins may be required. The "zoning" or indexing of the nucleus is dynamic and should be thought of as concentration gradients in flux, that nonetheless can influence transcription. Indexing may be particularly important for the establishment of inherited patterns of gene expression.

HML but improving repression at *LYS2* (Fig. 2). This may indicate either that an alternative mechanism for silencing functions at internal loci or that the concentration of the factors is different at the two loci, and, therefore, elevated expression provokes opposite effects. The latter explanation is consistent with the available data on Sir4 localization. The subnuclear localization of Sir1 is unknown, but it seems unlikely that it will be concentrated at telomeres, because *sir1* mutants derepress only at *HM* loci (Aparicio et al. 1991) and Sir1 appears not to bind Rap1 (Chien et al. 1993). Because slight variations in the number of silencers within the nucleus affects *HM* silencing (Boscheron et al. 1996) and deletion of *HML* slightly improves silencing at *LYS2*, we reason that much of the available Sir1 pool may be sequestered at *HM* loci. Thus, overexpression of Sir1 may "free" it, not from telomeric clusters but from association with factors at *HM* silencers. Additionally, Sir1 could help re-

cruit the limiting concentrations of Sir3 and Sir4 to the *LYS2* reporter construct, through interaction with ORC1 that binds both silencer elements (Triolo and Sternglanz 1996).

Physiological implications of unequal Sir distribution

The immediate physiological consequence of such a compartmentation of silencing factors is to enable bifunctional proteins like Rap1 and Abf1, which can mediate both activation and repression of transcription, to have both functions in the same nucleus, depending on their localization with respect to the pools of Sir factors. Because Sir3 and Sir4 are more concentrated near telomeric sequences, a Rap1 molecule in this compartment will be less likely to bind a coactivator like Gcr1 (Tornow et al. 1993), which may compete with Sir proteins for the Rap1 carboxyl terminus. The opposite is likely to be true at an internal Rap1 site. Indication that Sir proteins can compete for the *trans*-activating potential of the Rap1 carboxyl terminus was demonstrated in two-hybrid studies using a Rap1–GBD fusion (Moretti et al. 1994).

The bifunctional nature of Rap1-binding sites is particularly important when we consider that our *HML::LEU2''lacZ* construct is not silent at internal loci, whereas the *HML α* cassette can be repressed when integrated far from a telomere (Thompson et al. 1994). Similarly, the introduction of even a single silencer confers partial repression on the *MAT α* locus (Shei and Broach 1995). The fact that *HML α* is less dependent on telomere proximity than our silencer-flanked reporter appears to be attributable to the Rap1 site in the $\alpha 2$ promoter, which stimulates $\alpha 2$ transcription in other contexts. We have demonstrated that the insertion of a promoter-proximal Rap1 site in our *HML::LEU2''lacZ* reporter (Fig. 1B; Boscheron et al. 1996) also improves repression by reinforcing the silencer. Thus, it appears as if *HM* silencing has evolved a means to reduce dependency on telomere proximity and to improve the stability of repression, through mechanisms like Sir1 and additional repressor binding sites.

Are chromosomal domains constrained within the nucleus?

If local Sir concentrations are too low at *LYS2*, *HIS3*, *KEX2*, and *SIN4* to confer silencing, are internal sequences constrained from interacting with this pool of highly concentrated Sir protein near telomeres? In situ hybridization studies with probes specific for *LYS2* indicate that the distribution of this region of the chromosome is random with respect to either the nuclear periphery or to Rap1 (Gotta et al. 1996). If positioning of sequences in the nucleus is stochastic, then the low level of silencer-mediated repression at *LYS2* suggests that the coincidence of *LYS2* and telomeres occurs relatively infrequently, at least as compared with *HML*. On the other hand, the plasmid-borne *HML::LEU2''lacZ* reporter shows full Sir-dependent repression (Fig. 1B). One expla-

nation for this may be that this small extrachromosomal element is able to move more freely within the nucleoplasm than a chromosomal segment and thus might be more efficiently associated with telomere foci and high concentrations of Sir proteins. This implies that although an internal yeast chromosomal segment might not have a strictly defined subnuclear localization, it could nonetheless be constrained from moving freely in the nucleoplasm. Further in situ studies to localize repressed and derepressed domains within the nucleus should shed light on this question.

Nuclear "indexing" and transcription

In summary, we present evidence that the clustering of telomeric sequences and their attraction for large numbers of Rap1, Sir3, and Sir4 molecules create a subcompartment that favors repression. Unequal distribution of Sir proteins in the nucleus also ensures that Sir complexes are not repressing promiscuously, that is, binding nucleosomes near promoters of genes that use Rap1 or Abf1 for *trans*-activation. This compartmentation of the nucleus into different transcriptional states was suggested previously for *Drosophila*, where genes normally located in either euchromatin or heterochromatin were shown to require their normal chromosomal environment for proper regulation (for review, see Karpen 1994). A clustering of sequences was proposed previously to provide a basis for nuclear indexing, originally based on the idea that AT-rich scaffold attached regions might be brought together in a limited space within the nucleus, providing for a functional nuclear organization by creating zones where polymerases and *trans*-acting factors are highly concentrated (Gasser and Laemmli 1987). This variation in local concentrations of factors conferred by their cooperative binding to clustered motifs may indeed be a major function for other repeated sequences, such as the centromeric alphoid DNA (Zuckerandl and Villet 1988).

A disturbance of this distribution phenomenon may result in a modification of the normal transcription pattern, as reported in yeast for a truncated allele of *SIR4* (*SIR4-42*, Kennedy et al. 1995), which was shown to act in a dominant manner to prolong the cell's life span. Because the effect was Sir dependent, a putative "aging" locus was postulated to be repressed by silencing complexes that were mislocalized from the telomeric compartment. The focal staining of Sir3 and Sir4 is disrupted in the *SIR4-42* mutant (T. Laroche, M. Gotta, and S.M. Gasser, unpubl.). We predict that the behavior of our internally inserted *HML* silencing cassettes mimics cryptic silencers like those postulated for the aging gene.

A further prediction from this model of compartments is that the sequestering of silencing factors is telomere length dependent. In support of this, silencing at *HMR* is impaired in yeast cells carrying long telomeres (Buck and Shore 1995). Such a mechanism might also be relevant for mammalian cells where telomere shortening appears to act as an antiproliferation signal leading to cellular

senescence (for review, see Wright and Shay 1995). In this case, decreasing the size of telomeric repeats might liberate factors that are normally telomere bound, to modulate gene expression at other chromatin sites. Two recently described human telomeric proteins (Chong et al. 1995; Bilaud et al. 1996) may play a role similar to that of Rap1, sequestering chromatin-modifying ligands at telomeres. Thus, one can imagine that cellular proliferation control and senescence might indeed reflect the misprogramming of transcription through disturbance of nuclear indices.

Materials and methods

Plasmids

Standard molecular biology techniques were performed as described in Sambrook et al. (1989). The *LEU2''lacZ* silencing cassettes used in this study are derived from plasmids described in Boscheron et al. (1996) that contain a *LEU2''lacZ* reporter gene flanked by various combinations of *HML-E* and *HML-I* alleles. **E** indicates the wild-type *HML-E* sequence, **e₁** indicates a 43-nucleotide deletion of **E**, **I** indicates the wild-type sequence of *HML-I*, and **i** indicates a complete deletion of **I**. The different cassettes are named according to the allele combination and with the symbol > or < indicating the orientation of the reporter gene; for example, **E>I** corresponds to the reporter gene flanked by the two wild-type silencers with the *LEU2* promoter

adjacent to *HML-E*, and so on. **Eε<I** carries wild-type silencers with an additional Rap1-binding site (**ε** sequence) adjacent to *HML-E* and downstream of the *LEU2''lacZ* reporter. To facilitate usage in the text, insertion of the *LEU2''lacZ* reporter flanked by functional silencers at *HML* will be indicated as *HML::LEU2''lacZ* and nonfunctional silencers will be indicated as *hml::LEU2''lacZ*.

pLYS2URA is a derivative of pD6 (Fleig et al. 1986) in which a 2.3-kb *HindIII* fragment from p1URA3 I^{AB}, containing *URA3* flanked by sequences derived from *HML-E* and *HML-I* (**e₁** and **I^{AB}** alleles, respectively; Boscheron et al. 1996), was inserted at *XhoI*. To integrate a *LEU2''lacZ* reporter gene flanked by *HML-E* and *HML-I* at the *HIS3* locus, a 5.2-kb *HindIII* fragment from pE>I was inserted into the *EcoRV*–*NotI* sites of the integrative plasmid pRS303, leading to pHIS3E>I. To integrate a *LEU2''lacZ* reporter gene flanked by *HML-E* and *HML-I* at the *KEX2* locus (respectively *SIN4*), a 1.8-kb *XhoI* DNA fragment spanning the *KEX2* stop codon (respectively *SIN4*) was inserted at the *XhoI* site of pHIS3E>I, such that the *KEX2* stop codon (respectively *SIN4*) is located 450 nucleotides (respectively 1.4 kb) from the *HML-E* insert. The resulting plasmid was named pKEX2E>I (respectively pSIN4E>I). The primers used to amplify by PCR the *KEX2* 1.8-kb fragment are 5'-TATTCACCTC-GAGTGATATCAACGGCAGATGC and 5'-GCCCCATATCA-TGCTCGAGATCACCAGAC; those used to amplify the *SIN4* 1.8-kb fragment are 5'-TCCATCGCTCGAGTGGATGAGGTTTACC and 5'-TTTCACTTCTCGAGGAATGATCATGAGAGCC. An *XhoI* restriction site was placed in each primer for convenient cloning (underlined). pC–E>I, a *LEU2*–

Table 1. Yeast strains used in this study

Strain	Genotype	Source
S150-2B	<i>MATa leu2-3,112 ura3-52 trp1-289 his3 gal2</i>	a gift from J. Broach (Princeton University, NJ)
W303-1B	<i>Matα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15</i>	a gift from R. Rothstein (Columbia University, New York, NY)
EG5	S150-2B <i>HML::E > I</i> (pRS315)	Boscheron et al. (1996)
EG28	EG5 <i>sir3::TRP1₁</i>	Boscheron et al. (1996)
EG35	S150-2B <i>lys2::e1-URA3-I^{AB}</i>	this study
EG36	S150-2B <i>lys2::e1 > I</i> (pRS315)	this study
EG37	S150-2B <i>HML::E > I</i>	this study
EG47	EG5 P78:: <i>URA3</i>	this study
EG59	S150-2B <i>lys2::E > I</i> (pRS315)	this study
EG70	S150-2B <i>lys2::E > I</i>	this study
EG82	S150-2B <i>lys2::Eε < I</i> (pRS315)	this study
EG84	EG5 X W303-1B	this study
EG85	EG59 X W303-1B	this study
EG86	EG85 <i>LYS2-GRS1/lys2::E > I-GRS1::URA3-TEL</i>	this study
EG87	EG85 <i>LYS2-GRS1::URA3-TEL/lys2::E > I-GRS1</i>	this study
EG90	EG59 <i>hml::URA3</i>	this study
EG93	EG5 <i>adh4::URA3-TEL</i>	this study
EG108	EG59 <i>sir3::TRP1</i>	this study
EG109	EG37 <i>sir1::LEU2</i>	Boscheron et al. (1996)
EG111	EG70 <i>sir1::LEU2</i>	this study
EG112	S150-2B <i>HIS3::E > I</i> (pRS315)	this study
EG139	EG37 <i>sir4::HIS3</i> (pAAH5)	this study
EG162	EG70 <i>sir4::HIS3</i> (pAAH5)	this study
GA210	S150-2B <i>hml::e1-URA3-I^{AB}</i>	Boscheron et al. (1996)
LM1	EG59 <i>GRS1::URA3-Tel350</i>	this study
LM2	S150-2B <i>KEX2::E > I</i> (pRS315)	this study
LM3	S150-2B <i>SIN4::E > I</i> (pRS315)	this study
Z2	S150-2B (pC–E > I)	this study
Z11	S150-2B (pC–e > i)	this study

CEN-ARS plasmid, carries the 5.4-kb *XbaI*-*HindIII* fragment from pE>I inserted between the *XbaI* and *HindIII* sites of pRS315 (Sikorski and Hieter 1989). pC-e>i is similar to pC-E>I except that it carries the 5-kb *XbaI*-*HindIII* fragment from pe₁>i.

For disrupting the *HML* locus, we constructed pe1URAI that contains a *KpnI* *URA3* fragment inserted at the *KpnI* site of a plasmid DNA carrying null alleles of both *HML* silencers (e₁ and i) with the *URA3* promoter located adjacent to i. Plasmid pURATel80LYS derived from pVII-L *URA3*-TEL (Gottschling et al. 1990) by replacing the *ADH4* fragment by a 1-kb *SalI*-*HindIII* *GRS1* fragment, such that the *SalI* site was positioned away from *URA3*. The primers used to amplify by PCR the *GRS1* fragment are 5'-TTGATGACATTGTCGACGGAAACC-TCTAACTTGCTTGG and 5'-TGATGGCAGAGATTGAACA-TTTCGTTGACCC. A *SalI* restriction site was placed at the end of one primer for convenient cloning (underlined). The *HindIII* cloning site was provided by an internal site present in the PCR-generated fragment that is positioned 11404 nucleotides away from the *XhoI* site within *LYS2* that was used for inserting the reporter silencing cassettes. Plasmid pURATel350LYS derived from pURATel80LYS by replacing the 1.1-kb *BamHI*-*HindIII* fragment by a *BamHI*-*HindIII* fragment of pYTel, carrying the *URA3* gene and 270 nucleotides of yeast telomeric repeats in pUC19.

To integrate the *URA3* gene into the left subtelomeric region of chromosome III, we replaced an internal 2408-nucleotide *KpnI* fragment from p78 (kindly provided by C. Newlon, New Jersey Medical School, Newark), a derivative of Yip5 that contains a 4530-nucleotide *EcoRI* fragment located 2 kb from the left telomere of chromosome III, by a 1-kb *KpnI* *URA3* fragment. The resulting plasmid is named p78URA.

p2 μ -ASir3 was obtained by cloning a 3.7-kb *HpaI* fragment carrying *SIR3* excised from pRS6.3 (*SIR3* in pSEYC58, a gift from J. Rine, University of California, Berkeley) into the *HindIII* site of pAAH5, a 2 μ plasmid carrying the *ADC1* promoter upstream of a cloning *HindIII* site and the 3' *ADC1* region downstream of this site. pC-ASir4 is a *URA3*-CEN-ARS plasmid containing the *XbaI*-*SalI* *SIR4*-containing fragment from pADH-SIR4 (Cockell et al. 1995) cloned between the *XbaI* and *SalI* sites of pRS316 (Sikorski and Hieter 1989).

Yeast strain constructions

Yeast media and methods were as described in Rose et al. (1990). The strains used in this study are described in Table 1. β -galactosidase assays on permeabilized yeast cells were performed as described in Boscheron et al. (1996). The targeted integration of the *LEU2''lacZ* silencing reporter cassette at the *LYS2* locus was done in two steps (Boscheron et al. 1996). First, pLYS2URA cut with *BamHI* and *NruI*, generating terminal sequences homologous to the *LYS2* locus, was transformed into S150-2B producing EG35. Insertions were checked by a Southern blot analysis. Second, an *XbaI*-*NotI* fragment from the plasmids carrying the *LEU2''lacZ* reporter gene was used to replace the *URA3* gene by cotransformation with pRS315. The Leu⁺ transformants were replica-plated to SD-uracil, SD+uracil, and SD+uracil+5-FOA. The Ura⁻, 5-FOA^R colonies were analyzed both by Southern blot analysis and by PCR. The mutant or wild-type E and I silencer alleles were checked by the presence of a *BglIII* site in the case of e₁ (or absence for E) and by an *EcoRV* site in the case of I^{AB} (or its absence at I). Insertion of the additional Rap1 site (the ϵ oligonucleotide) is described in Boscheron et al. (1996).

The integration of pHIS3E>I into the *his3* locus was performed by transforming *PstI*-linearized plasmid DNA into S150-

2B, selecting His⁺ cells and screening the correct integration events by a Southern restriction site analysis, producing EG112. The integration of pURATel350LYS into the *GRS1* locus was performed by transforming *BglIII*-linearized plasmid DNA into EG59, selecting Ura⁺ cells, and screening the correct integration events by PCR and Southern restriction site analysis. The resulting strain is called LM1. The integration of pKEX2E>I into the 3' region of the *KEX2* locus was performed by transforming *BsrGI*-linearized plasmid DNA into S150-2B, selecting His⁺ cells, and screening the correct integration events by Southern restriction analysis. The resulting strain is LM2. An identical process was performed for integration of pSIN4E>I, linearized with *Tth111I*, at *SIN4*, producing LM3.

The fragmentation of the *ADH4*-distal part of chromosome VII in strain EG5 with pVII-L *URA3*-TEL was performed as described in Gottschling et al. (1990), leading to EG93. For the fragmentation of the *GRS1*-distal part of chromosome II, pURATel80LYS DNA cut with *EcoRI* and *SalI* was transfected into the diploid strain EG85, selecting Ura⁺ cells. The fragmentation of either homolog was analyzed by a Southern restriction site analysis by running the agarose gel on a FIGE mapper apparatus (Bio-Rad). *MluI* digestion of the fragmented chromosome II carrying the *LEU2''lacZ* silencing cassette inserted at *LYS2* released a terminal 15.3-kb fragment, visualized by Southern hybridization and sensitive to a BAL 31 digestion (data not shown). The resulting strain was named EG86. The fragmented chromosome II with a wild-type *LYS2* region produces a 18.4-kb fragment, sensitive to BAL 31 digestion but not detectable with a *lacZ* probe, in a strain named EG87.

The *sir1* and *sir3* disruption cassette is described in Boscheron et al. (1996), the *sir4* gene disruption used pJR276 (Kimmerly and Rine 1987), and disruption of *HML* was done with a linear fragment from pe1URAI (see Materials and methods, Plasmids) containing the *URA3* gene flanked by *HML* silencer null alleles using one-step gene replacement.

For overexpressing Sir1, Sir3, and/or Sir4, we transformed the appropriate strains with the high-copy plasmid YEpSIR1 (Stone et al. 1991), with the CEN-ARS plasmid pRS6.3 (see above), with the high-copy plasmid p2 μ -ASir3 expressing Sir3 from an *ADC1* promoter (see above), with the high-copy plasmid pKAN63 (Ivy et al. 1986), and with either pC-ASir4, which is a CEN-ARS plasmid expressing Sir4 from an *ADC1* promoter, or from the high-copy plasmid pFP320, which carries a *BglIII*-*EcoRI* fragment containing *SIR4* cloned in pRS424, a *TRP1*-2 μ vector (a gift from F. Palladino, ISREC, Lausanne, Switzerland).

Immunofluorescence, in situ hybridization, and Western blots

Haploid strains carrying the indicated plasmids were precultured and grown overnight in selective media. Cells were treated for 10 min at 30°C with 1000 U/ml of lyticase (Verdier et al. 1990) in growth media containing 1.2 M Sorbitol to partially digest the cell wall, after which cells were washed and fixed with formaldehyde (Palladino et al. 1993) and reacted with affinity-purified anti-Rap1 (Klein et al. 1992), anti-Sir3, or anti-Sir4 antibodies (Gotta et al. 1996). Secondary antibodies conjugated to DTAF were preadsorbed against fixed spheroplasts prior to use. In situ hybridization was performed on cells prepared identically to those for immunofluorescence, using a short Y' probe described in Louis et al. (1994). The probe was nick-translated using digoxigenin-derivatized dUTP (Boehringer Mannheim) and detected with FITC-coupled sheep anti-digoxigenin Fab fragments (Boehringer Mannheim) as described (Gotta et al. 1996). DNA was detected by EtBr staining (1 μ g/ml) in the mounting solution [1 \times PBS, 50% glycerol, 24 μ g/ml 1,4

diazabicyclo-2,2,2,octane [DABCO]). Confocal microscopy was performed on a Zeiss Axiovert 100 microscope (Zeiss Laser Scanning Microscope 410 system) with a 63× Plan-Apochromat objective (1.4 oil). Standardized conditions were used for image scanning, averaging, and processing, such that all treatments of images were done in parallel and identically for all images shown.

Western blots were performed on proteins from crude nuclear preparation [Verdier et al. 1990] from the indicated strains. In sequential lanes 1 and 2, OD₂₆₀ units of each nuclear preparation denatured in 2% SDS and sonicated to fragment DNA were loaded per slot, as indicated, and transferred to nitrocellulose by standard procedures. The same affinity-purified antibodies used in the immunofluorescence were used to probe the filter, and the primary antibodies were detected with a peroxidase-coupled secondary antibody. The peroxidase signal was detected using ECL chemiluminescence on preflashed film (Amersham, UK). Quantitation was done by scanning three exposures of the autoradiograph with a CCD camera and quantifying the images with the PhosphorImager, and correction for loading equality was achieved by standardization to the anti-tubulin and total Coomassie blue signals. For every sample at least one of the two lanes was consistently in the linear response curve of the luminescence reaction.

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Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression.

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