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To cite this version:
Laurent Maillet, Cécile Boscheron, Monica Gotta, Stéphane Marcand, Eric Gilson, et al.. Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression. Genes and Development, Cold Spring Harbor Laboratory Press, 1996, 10 (14), pp.1796-1811. 10.1101/gad.10.14.1796. hal-02448182

HAL Id: hal-02448182
https://hal.archives-ouvertes.fr/hal-02448182
Submitted on 22 Jan 2020

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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]

Received April 11, 1996; revised version accepted May 29, 1996.

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 envelope 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 Laureson and Rine 1992). In a similar fashion, polymerase II genes positioned adjacent to the poly[TG]_n 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 TG_13 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; Buchanan 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-1 or HML-1; 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 TG_13 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 hp [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 Laureson 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], although 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 TLE1 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...
essentialy 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 α1 and α2 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 quantitively 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 HMLα and HMRα [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 HML.E and, like natural HML 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 HMLα and HMRα [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.

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<1 includes a Rap1-binding site in between E and the 3’ end of the reporter gene. (X) XbaI, (H) HindIII. [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 strain tested. All strains are isogenic except for the integration or plasmid indicated in the strain tested. 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served for the construct at \textit{HML} in cells mutant for \textit{sir1}, \textit{sir3}, or \textit{sir4} (Fig. 2A, columns 2–4). The expression of the reporter gene at \textit{LYS2} is only slightly increased by mutations in the \textit{SIR} genes (Fig. 2B, columns 2–4) or by overexpression of the Sir4 carboxy-terminal domain (data not shown), which fully derepresses the \textit{E>I} construct integrated at \textit{HML} (Boscheron et al. 1996). These results suggest that \textit{HML} silencer function depends at

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Silencer-mediated repression at the \textit{LYS2} locus requires elevated concentrations of Sir proteins. The dependence of the \textit{LEU2"lacZ} expression on silencing factors is illustrated in \textit{EG37} (\textit{hml::E>I}; \textit{A}) and \textit{EG70} (\textit{lys2::E>I}; \textit{B}) by the disruption of \textit{sir1} by \textit{LEU2}, \textit{sir3} by \textit{TRP1}, and \textit{sir4} by \textit{HIS3} (columns 2, 3, and 4, respectively) or by transforming with plasmids overexpressing the genes encoding Sir1 (\textit{YEpSIR1}) or Sir3 (pkAN63, p2μ−ASir3, pRS6.3) or Sir4 (pC−ASir4, pFP320). All the cells contain a functional \textit{LEU2} gene either in the chromosome (\textit{sir1::LEU2}) or on a plasmid (pRS315, \textit{YEpSIR1}, p2μ−ASir3, or pkAN63) to allow growth in media selective for leucine. The \textit{sir} disruptions and plasmids are listed below the columns and are applicable to both \textit{A} and \textit{B}. Plasmids in the top row all carry \textit{LEU2} and in the middle carry \textit{URA3}, and pFP320 carries \textit{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. \textit{EG37} and \textit{EG70} carrying any combination of one, two, or three of the following parental plasmids without \textit{SIR} gene inserts produced similar amounts of \textit{β-galactosidase}: pRS315 (\textit{CEN−ARS LEU2}), pAAH5 (2μ−LEU2), pRS314 (\textit{CEN−ARS TRP1}), pRS316 (\textit{CEN−ARS URA3}). The comparison of \textit{β-galactosidase} activities from \textit{LEU2"lacZ} reporter at \textit{LYS2} (\textit{EG59}) in the presence and absence of an intact \textit{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 \textit{EG5} carrying pRS315 (\textit{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 \textit{HML} are indicated above the bars.}
\end{figure}
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 III). 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 silenceds. To test this we inserted the URA3 gene 3.1 kb away from the TG13 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 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-
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**Figure 3.** Insertion of yeast telomeric repeats 13 kb from LYS2 restores repression. (A) The integration of yeast telomeric repeats at the GR5I locus on chromosome II is depicted on the genetic map of the GR5I–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 GR5I of either (a) a linear DNA fragment derived from pURATelSOlyS exposing at one end 80 nucleotides of $(TG_1)_3$ DNA or (b) the plasmid pURATel350LYS cut at a unique site within the GR5I region, which inserts 350 nucleotides of $(TG_1)_3$ 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 LMl. (B) The diploids EG84 and EG85 are described in Materials and methods, and EG87, EG86, and LMl are described in A. β-Galactosidase activity and calculation of relative activity is as described in Fig. 1.

**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 α2 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 Ee'<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-
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Figure 4. Silencing is not propagated continuously from the telomere. The expression of the LYS2::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 VII 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::HML::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.

Table: Silencing is not propagated continuously from the telomere.

| Strain | Ura8 | 5-FOA | URA3 expression | relative β-galactosidase activity |
|--------|------|-------|----------------|-------------------------------|
| EG93   | -    | -     | ON             | 1 ± 0.2                       |
|        | +    | -     | ON/Off         | 1.2 ± 0.2                     |
|        | +    | +     | OFF            | 0.7 ± 0.2                     |
| EG47   | -    | -     | ON             | 1 ± 0.2                       |
|        | +    | -     | ON/Off         | 1.2 ± 0.2                     |
|        | +    | +     | 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 detected 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 [p2μ−-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
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, and 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 p2μ–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
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-
Telomeres as subnuclear silencing domains

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 telomeric, 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 HML 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-

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 TG1-3 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 TG1-3 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 telomic foci, or 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
cruit the limiting concentrations of Sir3 and Sir4 to the LYS2 reporter construct, through interaction with ORC1 that binds both silencer elements [Triolo and Stern glanz 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–Geb fusion [Moretti et al. 1994].

The bifunctional nature of Rap1-binding sites is particularly important when our HML::LEU2 'lacZ' construct is not silent at internal loci, whereas the HMLα 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 MA Tα locus [Shei and Broach 1995]. The fact that HMLα is less dependent on telomere proximity than our silencer-flanked reporter appears to be attributable to the Rap1 site in the α2 promoter, which stimulates α2 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 SIR4 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 explana
tion 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 [Zucker kandl and Villett 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
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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, e^ 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 by primer for convenient cloning (underlined). pC-E>I, a 1.8-kb Xhol DNA fragment spanning the KEX2 stop codon was inserted into the EcoRV-NotI sites of the integrative plasmid pRS303, leading to pHIS3-E>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 Xhol fragment from pE>I was inserted into the EcoRV-NotI sites of the integrative plasmid pRS303, leading to pHIS3-E>I. In this case, the KEX2 stop codon (respectively SIN4) was inserted at the Xhol site of pHIS3-E>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 pKEX2-E>I (respectively pSIN4-E>I). The primers used to amplify the KEX2-E>I (respectively pSIN4-E>I). The primers used to amplify the

Table 1. Yeast strains used in this study

| Strain     | Genotype | Source                                           |
|------------|----------|--------------------------------------------------|
| S150-2B    | MATa leu2-3,112 ura3-52 trp1-289 his3 gal2      | a gift from J. Broach [Princeton University, NJ] |
| W303-1B    |                      | a gift from R. Rothstein [Columbia University, New York, NY] |
| EG5        | S150-2B HML::E > I [pRS315]                      | Boscheron et al. [1996]                          |
| EG28       | EG5 str3::TRP1                                   | Boscheron et al. [1996]                          |
| EG35       | S150-2B lys2::e1-URA3-Ipa                        | this study                                       |
| EG36       | S150-2B lys2::e > I [pRS315]                      | this study                                       |
| EG37       | S150-2B HML::E > I                               | this study                                       |
| EG47       | EG5 P78::URA3                                    | this study                                       |
| EG59       | S150-2B lys2::E > I [pRS315]                      | this study                                       |
| EG70       | S150-2B lys2::E > I                              | this study                                       |
| EG82       | S150-2B lys2::E < I [pRS315]                      | this study                                       |
| EG84       | EG5 X W303-1B                                   | this study                                       |
| EG85       | EG59 X W303-1B                                  | this study                                       |
| EG86       | EG85 lys2-GRSL/lys2::E > I-GRSL::URA3-TEL        | this study                                       |
| EG87       | EG85 lys2-GRSL::URA3-TEL/lys2::E > I-GRSL        | this study                                       |
| EG90       | EG59 hml::URA3                                   | this study                                       |
| EG93       | EG5 adh4::URA3-TEL                               | this study                                       |
| EG108      | EG59 sir3::URA3-TEL                              | this study                                       |
| EG109      | EG37 sir1::LEU2                                  | Boscheron et al. [1996]                          |
| EG111      | EG70 sir1::LEU2                                  | this study                                       |
| EG112      | S150-2B HIS3::E > I [pRS315]                      | this study                                       |
| EG139      | EG37 sir2::HIS3 [pAAHS]                          | this study                                       |
| EG162      | EG70 sir4::HIS3 [pAAHS]                          | this study                                       |
| GA210      | S150-2B hml::e-URA3-Ipa                          | Boscheron et al. [1996]                          |
| LM1        | EG59 GRSL::URA3-Tel50                            | this study                                       |
| LM2        | S150-2B KEX2::E > I [pRS315]                      | this study                                       |
| LM3        | S150-2B SIN4::E > I [pRS315]                      | this study                                       |
| Z2         | S150-2B [pC-E > I]                               | this study                                       |
| Z11        | S150-2B [pC-e > i]                               | this study                                       |
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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 is similar to pC->E except that it carries the 5-kb XbaI–HindIII fragment from pE->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 i.e., and it 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 Sall–HindIII GRS1 fragment, such that the Sall site was positioned away from URA3. The primers used to amplify by PCR the GRS1 fragment are 5'-TTGATGATTGACCC-3' and 5'-TGATGGCAGAGATTGAACA-GTCTAACTTGGCTTGG and 5'-TGATGGCAGATTGACCTTCCGTTGACCC. A Sail 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 the 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-base KpnI fragmnet from p78 (kindly provided by C. Newlon, New Jersey Medical School, Newark), a derivative of Ylp5 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.

p2μ–ASir3 was obtained by cloning a 3.7-kb Pml fragment carrying Sir3 excised from pRS63.3 (SIR3 in pSEyC58, a gift from J. Rine, University of California, Berkeley) into the HindIII site of pAAHS, a 2μ plasmid carrying the ADCl promoter upstream of a cloning HindIII site and the 3' ADCl region downstream of this site. pC–ASir4 is a URA3–CEN–ARS plasmid containing the XbaI–Sall Sir4-containing fragment from pADH–SIR4 [Cockell et al. 1995] cloned between the XbaI and Sail 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 Nrul generating terminal sequences homologous to the LYS2 locus, was transformed into S150-2B producing EG5. Insertions were checked by a Southern blot analysis, producing EG11. The integration of pKEX2E>I into the 3' region of the KEX2 locus was performed by transforming BglII-linearized plasmid DNA into S150-2B, selecting His+ cells and screening the correct integration events by Southern blot site analysis, producing EG112. The integration of pURATel350LYS into the GRS1 locus was performed by transforming BglII-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 BglII-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>l, linearized with Tth1111, 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 Sall was transfected into the diploid strain EG85, selecting Ura+ cells. The fragmentation of either homolog was analyzed by a Southern site restriction site analysis by running the agarose gel on a FIGE mapper apparatus (Bio-Rad). MII 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 pRF276 (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 pRS63.3 [see above], with the high-copy plasmid p2μ–ASir3 expressing Sir3 from an ADCl promoter [see above], with the high-copy plasmid pKAN63 [by et al. 1986] and with either pC–ASir4, which is a CEN–ARS plasmid expressing Sir4 from an ADCl promoter, or from the high-copy plasmid pFK320, which carries a BglII–EcoRI fragment containing Sir4 cloned in pRS424, a TRPI–2μ vector [a gift from F. Palladino, ISREC, Lausanne, Switzerland].

Immunofluorescence, in situ hybridization, and Western blots

Haploid strains carrying the indicated plasmids were pre cultured 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-Rapl [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 μg/ml] in the mounting solution [1× PBS, 50% glycerol, 24 μg/ml, 1.4
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We are grateful to J. Broach, D. Gottschling, F. Palladino, L. Pillus, J. Rine, and D. Shore for gifts of plasmids, A. Formenton and M. Roberge for antibodies prepared in the Gasser laboratory, E. Revardel for strain constructions (EG84 and EG85), and T. Laroche for excellent technical help. M.G. thanks ISREC for a Ph.D. fellowship; E.G. thanks the European Molecular Biology Organization for his long-term fellowship at ISREC where this work was initiated. This work was supported by grants from the Swiss National Science Foundation and the Human Frontiers Research Program (to S.M.G.) and by Association pour la Recherche sur le Cancer (ARC), Groupement de Recherches et d’Études sur les Génomes [GREG], Ligue Nationale contre le Cancer, Région Rhône-Alpes, and Association Française de lutte contre la Mucoviscidose [AFLM] (to E.G.).

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*Genes Dev.* 1996, 10:
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