Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by *SIR3* dosage

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In *Saccharomyces cerevisiae*, telomeres repress transcription of genes located nearby. This region-specific gene inactivation is thought to involve the packaging of telomeric domains into silent chromatin. To gain insight into the mechanism of telomeric silencing, a genetic assay to examine the spread of silencing along the distal right arm of chromosome V was developed. The frequency of silencing a telomere-adjacent *URA3* gene decreased with increasing distance of the gene's promoter from the telomere, irrespective of transcriptional orientation. The distance over which telomeric silencing of *URA3* was observed was extended by weakening the gene's promoter—specifically, by deleting PPR1, the *trans*-activator of *URA3*. The silent telomeric domain was extended even farther by increasing the gene dosage of *SIR3*. These results suggest that a gene's promoter is a key determinant in controlling silencing on that gene and that SIR3 is a crucial component of the silent chromatin domain that initiates at the telomere and is assembled inwardly along the yeast chromosome. Finally, silencing is not observed on the centromeric side of an actively transcribed telomeric gene, demonstrating that the repressed telomeric domain is propagated continuously along the DNA. Taken together, these data reflect the complex and dynamic organization of eukaryotic genomes into functionally distinct regions.

[*Key Words*: *Saccharomyces cerevisiae*; silencing; heterochromatin; gene expression]

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The eukaryotic genome is organized into regions distinct in their structure and function. Heterochromatin, which defines one such structural region, is condensed throughout the cell cycle while its counterpart, euchromatin, is more diffuse in appearance during interphase (Heitz 1928, as cited in Brown 1966). Chromosomal regions also differ functionally, as the expression of a eukaryotic gene can be profoundly affected by its chromosomal position. This phenomenon, chromosomal position effect, is observed in many eukaryotes (for review, see Lima-de-Faria 1983) and has been studied extensively in *Drosophila melanogaster* (for extensive reviews, see Lewis 1950; Baker 1968; Spofford 1976). When genetic rearrangements place euchromatic segments of the genome into or near heterochromatin, the expression of a translocated euchromatic gene is altered in a population of cells: Some cells express the gene, whereas others do not. Thus, a mosaic or variegated phenotypic pattern is produced.

Chromosomal position–effect phenomena can spread over great distances in the genome; for example, in *Drosophila*, genes located as far away as 80 chromosome polytene bands (~2000 kbp) are still subject to position–effect variegation (PEV) (Demerec 1940). This spreading effect is thought to reflect the dynamic nature of assembly of heterochromatin over a locus (for review, see Zuckerkandl 1974; Spofford 1976). When heterochromatin assembles far enough to include a locus, the gene within it is inactivated.

In *Saccharomyces cerevisiae*, chromosomal domains have been identified that exert position effect: the cryptic mating-type loci, *HML* and *HMR*, and telomeres (for review, see Laurenson and Rine 1992; Sandell and Zakian 1992). Genes located near or within these domains may be transcriptionally silenced and exhibit phenotypic variegation (Klar et al. 1981; Nasmyth et al. 1981; Schnell and Rine 1986; Mahoney and Broach 1989; Gottschling et al. 1990). At least six modifiers of position effect are shared between the *HM* loci and telomeres. A mutation in *SIR2*, *SIR3*, *SIR4*, *NAT1*, *ARD1*, or *HHF2* (which encodes histone H4) reduces or abolishes silencing at telomeres, *HML*, and *HMR* (Hopper and Hall 1975; Haber and George 1979; Klar et al. 1979, 1981; Ivy et al. 1986; Rine and Herskowitz 1987; Whiteway et al. 1987;

Kayne et al. 1988; Mullen et al. 1989; Megee et al. 1990; Park and Szostak 1990; Aparicio et al. 1991). The involvement of histone H4 and the observation that the *HM* loci and telomeres are refractory to DNA modifications in vivo in a SIR-dependent manner point to chromatin structure as being involved in silencing the *HM* loci and telomeres. Specifically, this chromatin structure is thought to hinder access of transcription factors to these loci (Nasmyth 1982; Kostriken et al. 1983; Klar et al. 1984; Gottschling 1992; Singh and Klar 1992).

The spreading of position effect also occurs in yeast (Abraham et al. 1984; Feldman et al. 1984). Genes located up to ~4.9 kbp from a telomere still are subject to position effect, whereas no silencing is detected at loci ~20 kbp from the chromosome end (Gottschling et al. 1990). Additionally, insertion of a 30-kbp Ty array between the E and I sites (*cis*-acting elements required for silencing) at *HMLα* relieves silencing at this locus. However silencing is re-established when this array is reduced to a single 7-kbp Ty (Mastrangelo et al. 1992). Thus, there is a limit to the size of silenced domains at both *HM* loci and telomeres.

Telomeric silencing in yeast provides an excellent opportunity to study the spread of position effect in a eukaryote, particularly because the initiation site of position effect is known to be the end of the chromosome (Gottschling et al. 1990). In this paper, a quantitative method to examine telomeric position effect was used to identify parameters that modulate spreading. The results provide a molecular and mechanistic insight into the propagation of silencing in yeast, as well as the functional organization of silent chromosomal domains.

Results

Silencing of URA3 *decreases with increased distance from the telomere*

In an earlier study, we detected telomeric position effect (TPE) in *S. cerevisiae* 4.9 kbp from the left end of a modified chromosome VII (VII-L) by measuring the level of transcriptional repression of a telomere–proximal *URA3* when various yeast genes were inserted between *URA3* and the telomere (Gottschling et al. 1990). However, the effect of each inserted sequence on *URA3* expression was not exclusively dependent on the size of the insert. To better characterize the spread of TPE in *S. cerevisiae*, we examined the expression of *URA3* as a function of its distance from a representative telomere, without introducing any new sequences between *URA3* and the end of the chromosome.

A set of isogenic strains was created with *URA3* placed at various distances from the right end of chromosome V (V-R) (Fig. 1); the normal chromosomal copy of *URA3* is nonfunctional in each strain. At each site of insertion, *URA3* was positioned in either transcriptional orientation. This set of strains may be divided into two groups: Those that maintained the original ~6.7-kbp telomere-associated Y′ element of V-R, and those in which the Y′ and some adjacent sequences were replaced with a new terminus of $(TG_{1-3})_n$. These Y′ elements are middle-repetitive DNA sequences found proximal to some, but not all, yeast telomeres; their function is unknown (for review, see Olson 1991).

Transcriptional repression as a function of distance from the chromosome end was analyzed by determining

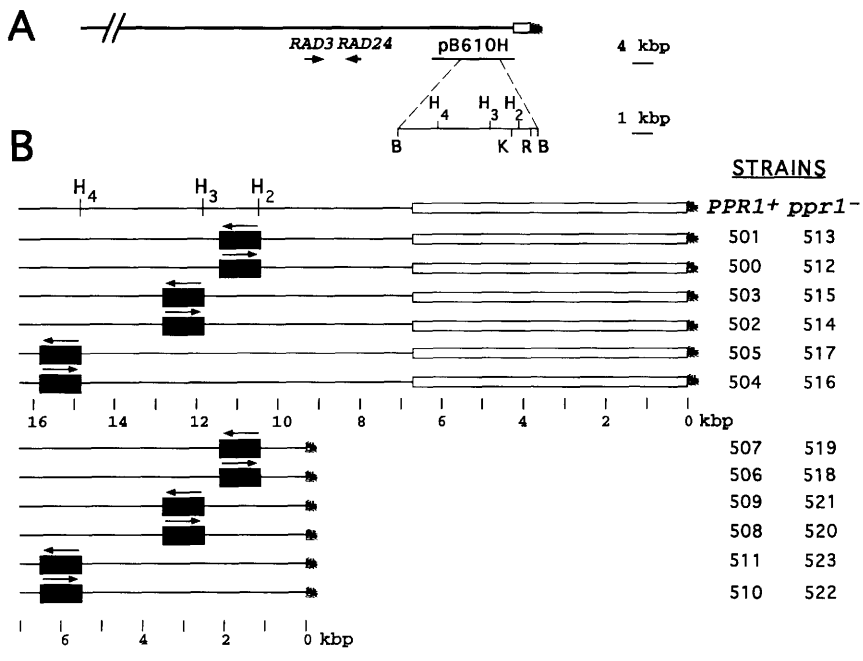

Figure 1. Chromosomal constructs used to study telomeric position–effect spreading along V-R. (*A*) A diagram of the chromosome V-R distal region in a wild-type strain is shown (adapted from Ferguson et al. 1991). The open box and the diffuse dark end represent the Y′ subtelomeric element and the telomeric $(TG_{1-3})_n$ repeat, respectively, and are not drawn to scale. Physical location and transcriptional orientation of the *RAD3* and *RAD24* genes are indicated by arrows. [The telomere-associated sequence X (Zakian 1989) was not detected by Southern analysis on V-R in the strains used in this study (data not shown). The X probe was made from an *Eco*RI–*Eco*RV fragment, which is presumably common to all X elements (Wright et al. 1992).] The region of V-R cloned into plasmid pB610H and used in plasmid constructs is underlined and shown in an expanded scale, with a partial restriction map (C. Newlon, pers. comm.). (B) *Bam*HI; (H) *Hin*dIII; (K) *Kpn*I, (R) *Eco*RI. *Hin*dIII sites are numbered with respect to the telomere, as indicated by their subscripts. (*B*) An expanded view of V-R along with a representation of each strain that contained *URA3* (solid box; arrows indicate transcriptional direction) along V-R. Strain designations for both *PPR1*$^+$ and *ppr1*$^-$ derivatives are indicated at *right*.

the level of *URA3* silencing in each strain. The level of silencing in a population of cells is quantified by determining the fraction of cells capable of forming colonies on 5-fluoro-orotic acid (5-FOA) medium; 5-FOA is lethal to cells expressing the *URA3* gene product (Boeke et al. 1987). In our analysis, the ability of a cell to give rise to a colony on 5-FOA (5-FOA^R) indicates that when it was plated onto the medium, the cell contained little or no *URA3* gene product. Thus, when *URA3* is telomeric, telomere-mediated transcriptional repression enables the cell to grow on 5-FOA (Gottschling et al. 1990).

Quantification of TPE spreading along V-R is summarized graphically in Figure 2 (PPR1$^+$), where the fraction of 5-FOA^R cells is plotted versus the distance of the *URA3* promoter from the telomere. A continuous gradient in frequency of silencing was observed, with the highest frequency occurring at the most telomere–proximal position. Repression was no longer detected when the *URA3* promoter was located 3.5 kbp away from the telomere. The steady decrease in frequency of repression with respect to promoter distance from the telomere suggested that the position of the *URA3* promoter was the key element in determining repression; transcriptional orientation with respect to the telomere did not appear to be significant in regulating *URA3* expression (Fig. 2). Finally, in strains with a Y′ element between the *URA3* gene and the V-R telomere (UCC500–505), no repression was detected at the tested distances of 10–16 kbp from the telomere (data not shown).

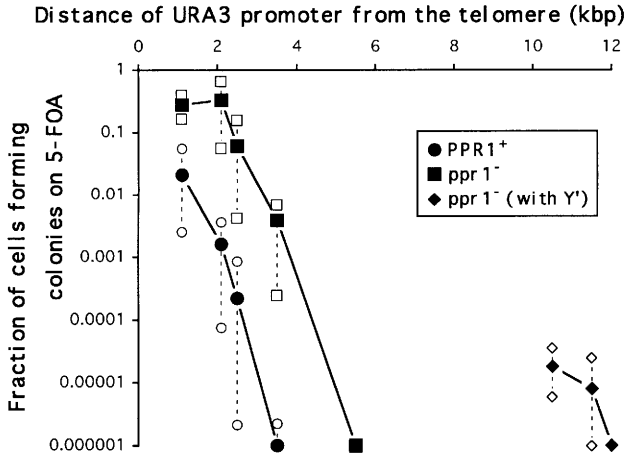

Figure 2. The effect of promoter location and strength on silencing of a telomeric *URA3*. Transcriptional repression of *URA3* in the strains described in Fig. 1B was determined by measuring resistance to 5-FOA. Data points from strains with V-R still bearing the original subtelomeric Y′ sequence are labeled "with Y′." PPR1 was either present (PPR1$^+$) or absent (*ppr1*$^-$) in the strains examined. Solid and open symbols are the mean and the extremes of the distribution, respectively, of the ratios of colonies formed on 5-FOA medium compared with colonies formed on nonselective medium, from a minimum of four independent trials. Values at or below 10^{-6} are beyond the sensitivity limit of the assay and are considered as zero.

Absence of a trans*-activator increases the extent of TPE spreading*

If promoter distance from the telomere is a primary determinant for governing TPE spreading, then weakening the promoter might result in an increase in spreading. To test this idea, *ppr1*$^-$ derivatives of the strains described above, with *URA3* at various distances from the telomere, were created. [PPR1 is a *trans*-activator protein that enhances expression of the *URA3* gene (Loison et al. 1980; Roy et al. 1990).] As shown in Figure 2, repression was more frequent at each location of *URA3* and detectable over a greater distance from the telomere in *ppr1*$^-$ than in *PPR1*$^+$ strains. Thus, the range over which TPE spreads seems to be inversely related to the promoter strength of the gene being assayed. Similarly, deleting *GCN4*, the *HIS3 trans*-activator (Hope and Struhl 1985; Hinnebusch 1988), reduced the ability of strains carrying a telomeric copy of *HIS3* to form colonies on medium lacking histidine (Fig. 5, below; data not shown), indicating that this effect is not specific to *URA3*.

In the *ppr1*$^-$ strains with the Y′ element present at V-R, a small fraction of 5-FOA^R cells were reproducibly observed in the two strains in which the *URA3* promoter is ~11 and 12 kbp from the telomere (Fig. 2; see Fig. 1, UCC513 and UCC512). Southern analysis revealed no change in chromosome structure between *URA3* and the telomere in these strains (data not shown). These results contrast with the data for strains lacking the Y′ element on V-R (UCC518-523), in which no repression was detected beyond ~6 kbp from the V-R telomere. Thus, it seems that 6.7 kbp of Y′ sequence has a greater ability to sustain telomere-dependent silencing than the same length of unique V-R sequence.

Overexpression of SIR3 *enhances TPE spreading*

The gene products of *SIR2*, *SIR3*, and *SIR4* are required for TPE, and it has been postulated that one or more of them is a structural component of silent yeast chromatin (Nasmyth 1982; Ivy et al. 1986; Marshall et al. 1987; Rine and Herskowitz 1987; Alberts and Sternglanz 1990; Johnson et al. 1990; Aparicio et al. 1991; Stone et al. 1991). To examine whether the normal cellular level of SIR2, SIR3, or SIR4 limits the range of silent telomeric domains, we tested whether introduction of multiple copies of the *SIR2*, *SIR3*, or *SIR4* genes would increase the spread of TPE. Only raising *SIR3* copy number enhanced position–effect spreading on telomere-adjacent genes (Figs. 3A and 6). No phenotype was observed in strains transformed with a multicopy plasmid carrying *SIR2* (data not shown). Increasing *SIR4* dosage relieved silencing on telomeric genes (data not shown); a similar effect has been observed previously at a weakened *HMR* (Sussel and Shore 1991).

The effect of *SIR3* overexpression was quantified in the sets of strains previously described (Fig. 1B, UCC500–523). Increased dosage of *SIR3* raised the frequency of *URA3* silencing in each strain (Fig. 3A). In *ppr1*$^-$ strains overexpressing *SIR3* on a high-copy plas-

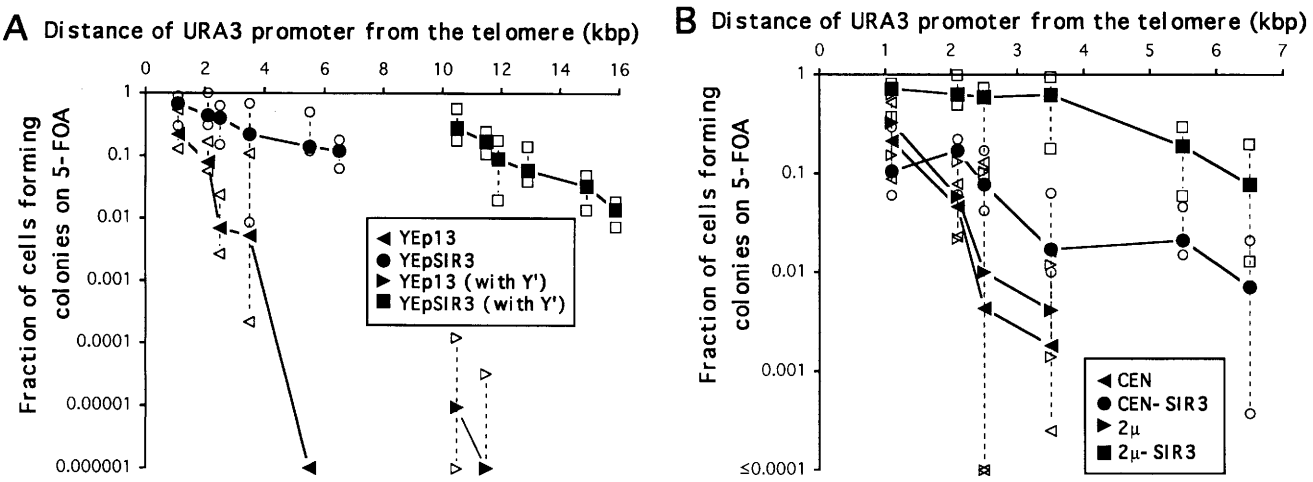

Figure 3. Increased *SIR3* gene dosage enhances telomeric position–effect spreading. (*A*) The *ppr1*$^-$ strains described in Fig. 1B were transformed with either YEp13 containing the *SIR3* gene (YEpSIR3) or the vector alone (YEp13). Data were collected as in Fig. 2, except that media lacked leucine to maintain the plasmids; the median (solid symbols) and the extremes (open symbols) of at least five independent trials are reported for each data point. (*B*) Strains UCC518–523 (*ppr1*$^-$) were transformed with *SIR3* carried on a centromeric plasmid (CEN–SIR3), a multicopy plasmid (2μ–SIR3), or with either vector alone (CEN or 2μ). To maintain the plasmids, all media lacked tryptophan. Plating efficiencies on 5-FOA medium were recorded and presented as in *A*. Resistance to 5-FOA for UCC523 transformed with either vector alone was at the limit of detection; this data point was not plotted.

mid (YEpSIR3), *URA3* was frequently silenced 16 kbp from the telomere (with a Y'), whereas in cells with vector alone (YEp13) no significant silencing was detectable beyond 4 kbp. Similar results were obtained in *PPR1*$^+$ strains transformed with YEp13 or YEpSIR3, although as expected from the data presented in the previous section, *URA3* transcription was somewhat less frequently repressed than in the *ppr1*$^-$ strains (data not shown). Again, the presence of a Y' element appeared to facilitate TPE spreading over longer distances than unique chromosomal sequences.

Extrapolation of the "YEpSIR3 with Y" curve (Fig. 3A) suggested that TPE spreading should extend inward ~25 kbp from the end of chromosome V-R in the *SIR3*-overexpressing strains. Consistent with this estimate, *URA3* was repressed at 22 kbp from the VII-L telomere when *SIR3* was overexpressed (Fig. 6B, below), but *URA3* expression was not affected at its normal locus (data not shown), ~120 kbp from telomere V-L (Mortimer et al. 1992). No increase in telomeric silencing was detected in strains transformed with plasmids carrying mutant alleles of *SIR3*, indicating that propagation of telomeric silencing is dependent on functional SIR3 (data not shown). These results are consistent with SIR3 being a limiting component required to assemble repressive telomeric chromatin.

If SIR3 is limiting, the spread of TPE should be very sensitive to *SIR3* gene dosage. This hypothesis was tested in *ppr1*$^-$ strains transformed with *SIR3* carried either on a centromeric (CEN–SIR3) or a multicopy plasmid (2μ–SIR3), or with the vectors alone (CEN and 2μ; Fig. 3B). With a single-copy plasmid (CEN–SIR3), the spreading effect was less enhanced than with a high-copy plasmid (2μ–SIR3) but greater than with either vector alone (Fig. 3B). Hence, the results indicate that *SIR3* dosage limits the spread of yeast telomeric position effect.

Increased SIR3 *dosage cannot suppress the requirements of SIR2, SIR4, NAT1, ARD1, and histone H4 for TPE*

In addition to SIR3, the gene products of *SIR2*, *SIR4*, *NAT1*, *ARD1*, and *HHF2* (histone H4) are required for transcriptional silencing at telomeres (Aparicio et al. 1991). We tested whether the increased dosage of *SIR3* could restore TPE in cells deficient for these other proteins. Strains containing *URA3* adjacent to the VII-L telomere and defective in each of the aforementioned genes were transformed with a high-copy *SIR3* plasmid. In no case did increased levels of *SIR3* restore telomeric silencing (Table 1).

Mutations in *SIR1* do not relieve silencing at telomeres, suggesting that SIR1 is not involved in controlling TPE (Aparicio et al. 1991). Consistent with this idea, *SIR3* overexpression in *sir1*$^-$ strains enhanced TPE spreading, as observed in wild-type strains (Table 1). Because the *SIR3* dosage-dependent enhancement of TPE cannot suppress the requirements for *SIR2*, *SIR4*, *NAT1*, *ARD1*, and histone H4, it appears that the *SIR3* effect operates through the normal mechanism of telomeric silencing, rather than introducing a novel mechanism of silencing.

Silenced chromosomal domains spread continuously from the telomere

The results presented above suggest that the silenced telomeric domain spreads inward along the chromosome

Table 1. *Increased* SIR3 *dosage cannot suppress the requirements of SIR2, SIR4, NAT1, ARD1, and histone H4*

Strain	Genotype	Fraction 5–FOA^R	
		2μ–SIR3	2μ
UCC1001	wild type	0.47 (0.29–0.60)	0.35 (0.20–0.45)
UCC3248	*sir2*	$<2 \times 10^{-6}$	$<2 \times 10^{-6}$
UCC3249	*sir3*	0.56 (0.39–0.73)	$<1 \times 10^{-6}$
UCC3250	*sir4*	$<4 \times 10^{-6}$	$<1 \times 10^{-6}$
UCC2031	wild type	0.42 (0.33–0.67)	0.55 (0.47–0.67)
UCC2032	*HHF2–gly16*	$<5 \times 10^{-6}$	$<4 \times 10^{-6}$
UCC2033	*HHF2–gln16*	$<7 \times 10^{-6}$	$<8 \times 10^{-6}$
UCC2034	*HHF2–gly17*	$<5 \times 10^{-6}$	$<7 \times 10^{-6}$
UCC18	wild type	0.55 (0.50–0.60)	0.43 (0.37–0.50)
UCC25	*ard1*	$<36 \times 10^{-6}$	$<32 \times 10^{-6}$
UCC16	*nat1*	$<50 \times 10^{-6}$	$<29 \times 10^{-6}$
UCC1003	wild type	0.0024 (0.0013–0.0036)	$<9 \times 10^{-6}$
UCC3243	*sir1*	0.0021 (0.0010–0.0047)	$<6 \times 10^{-6}$

Shown is resistance to 5-FOA of strains from various backgrounds carrying *URA3* at the *adh4* locus (UCC1003 and UCC3243) or adjacent to the VII-L telomere (all others). Strains were transformed with a 2μ-derived plasmid carrying no insert (2μ: YEp13 in UCC1001, UCC2031, and their derivatives; pHR59-33 in UCC18, UCC1003, and their derivatives) or *SIR3* (2μ–SIR3). The relevant genotype of the strains is indicated. Wild type: *ARD1, HHF2, NAT1, SIR1, SIR2, SIR3, SIR4.* Data were collected as in Fig. 3. The mean of at least three independent trials is presented; the range of values is given in parentheses. For transformants where no FOA resistance was detected, the actual limit of detection of the assay is reported.

in a continuous fashion. To test this idea further, two genes were placed adjacent to one another near the same telomere, and the transcriptional state of the centromere–proximal gene was examined when the telomere–proximal gene was transcriptionally active. If the silenced domain is spread continuously along the chromosome, then the centromere–proximal gene should always be derepressed when the telomere–proximal gene is active. However, if the repressed domain is discontinuous, then the centromere–proximal gene may be in a repressed state even when the telomere–proximal gene is active (Fig. 4).

Both the *URA3* and *HIS3* genes were inserted near the V-R telomere without a Y′ element present; each of the eight possible permutations of *URA3* and *HIS3* located near the V-R telomere was constructed (UCC2576–2583; Fig. 5). In addition, three strains were created in which *URA3* and *HIS3* were located on two different chromosomes (V-R and VII-L), with both genes adjacent to a telomere (UCC2590), with *URA3* at a telomere and *HIS3* nontelomeric (UCC2589), or the converse situation (UCC2591). To improve the sensitivity of the spreading assay, the promoters of *URA3* and *HIS3* were weakened by deleting *PPR1* and *GCN4*, the genes that encode their respective *trans*-activators, in each strain. All strains grew in the absence of histidine (Fig. 5; −his), indicating that *HIS3* was capable of being expressed at each chromosomal position, although expression was compromised at some telomeric locations (e.g., UCC2577; colony size was small and plating efficiency was reduced on

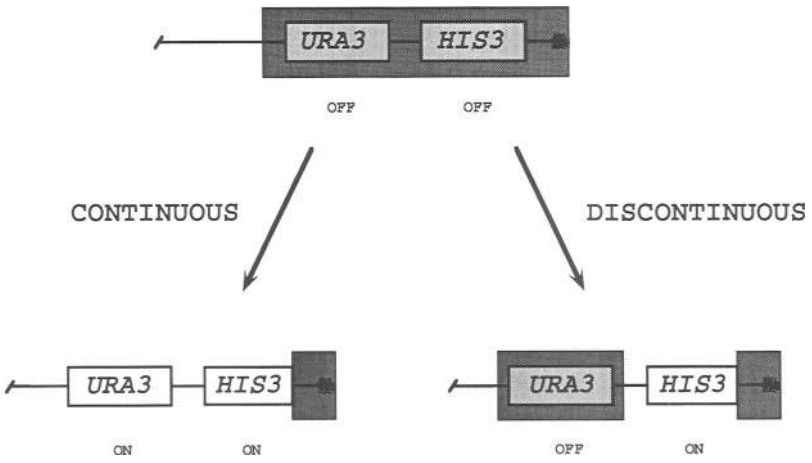

Figure 4. General models for the spreading of silent chromosomal domains. The scheme depicts the rationale used to test whether silencing spreads continuously from yeast telomeres. In this scheme the domain encompassing both genes has the potential to be repressed. If telomeric chromatin spreads continuously from the telomere and the *HIS3* gene is transcriptionally active (ON), *URA3* will not be silenced (it is ON). In the discontinuous model, silent chromatin may reinitiate beyond the active *HIS3* gene, repressing *URA3* (OFF). The diffuse dark end represents the telomeric $(TG_{1-3})_n$ repeat. The shaded box encompassing the chromosomal sequences depicts the silenced chromosomal domain.

−his). All strains carrying a telomeric *URA3* gave rise to colonies that grew on fully supplemented 5-FOA medium, reflecting transcriptional repression of *URA3* (Fig. 5; FOA).

The test for continuity of the silenced domain along the chromosome from the telomere is presented in the panel labeled FOA − his of Figure 5. In the four strains with both *URA3* and *HIS3* located near the V-R telomere and *HIS3* as the telomere–proximal marker (UCC2576–2579), no growth was detected on FOA − his medium. That is, when *HIS3* was nearer to the telomere and transcriptionally active, *URA3* was never transcriptionally repressed. In contrast, when *URA3* was telomere–proximal (UCC2580–2583) colonies were obtained on FOA − his, indicating that it was possible for *URA3* to be repressed while *HIS3* was active. Thus TPE spreads continuously inward from the telomere. These results also suggest that the spread of silencing can be blocked by transcription of an intervening gene.

Of the four strains with *URA3* in the telomere–proximal location, UCC2581 showed conspicuously poor growth on FOA − his. In this strain, the *URA3* and *HIS3* promoters are separated by only ~0.5 kbp. In such close proximity it might be difficult to open the *HIS3* chromatin structure without also disrupting the silencing apparatus over *URA3*. Another notable result was observed when *URA3* and *HIS3* were located at different telomeres (UCC2590); robust colonies grew on FOA − his medium, signifying repression at one telomeric locus while the other telomeric marker was expressed. This result indicates that telomeric silencing is locus specific.

We then examined whether the increased spread of silencing mediated by *SIR3* overexpression was also continuous. *TRP1* and *URA3* were inserted ~12.5 and 22 kbp, respectively, from the VII-L telomere (Fig. 6A). 5-FOA^R colonies were observed only when the cells were transformed with YEpSIR3 (Fig. 6B; FOA − leu); however, no 5-FOA^R was detected if *TRP1* was simultaneously expressed in these cells (Fig. 6B; FOA − trp − leu). *TRP1* expression by itself was only modestly impaired in YEpSIR3 transformants, as demonstrated by their high efficiency of plating on − trp − leu medium. Similar results were obtained when *ADE2* (inserted ~9 kbp from the same telomere) replaced *TRP1* in this experiment (data not shown). Taken together, these observations suggest that *SIR3* propagates silencing continuously from the telomere.

Discussion

We have carried out a systematic characterization of the spreading of TPE in *S. cerevisiae*. The telomeric position effect in yeast can be considered as a gradient of transcriptional silencing along the chromosome. We postulate that this gradient reflects the limited assembly of a silent chromatin (heterochromatic-like) structure that initiates at the telomere and proceeds continuously inward along the chromosome. In our analysis, the fraction of 5-FOA^R cells provided an estimate of the frequency at which a telomeric *URA3* was located within this repressive structure.

Transcriptional inactivation of a telomeric locus may be viewed as the final product of a reaction in which subunits of silent chromatin are assembled. In a simple model, silencing of a *URA3* gene 6 kbp from the telomere would require six times as many subunits as that needed to silence a *URA3* gene located 1 kbp away. If the assembly of telomeric repressive chromatin were a first-

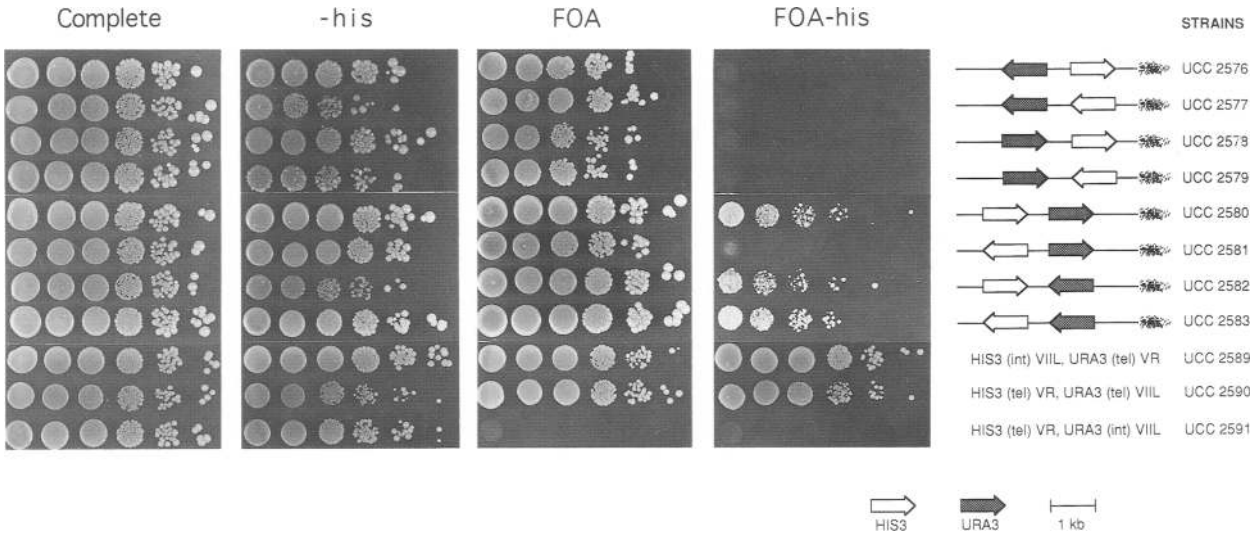

Figure 5. Silenced chromosomal domains spread continuously from the telomere. Cells were pregrown on rich medium, and 10-fold serial dilutions were plated onto nonselective medium (Complete), medium lacking histidine (−his), medium containing 5-FOA (FOA), and medium lacking histidine and containing 5-FOA (FOA − his). The arrows represent the genes and their transcriptional orientation. (tel and int) A telomeric and an internal location on the chromosome, respectively. The V-R telomeric repeat is not drawn to scale.

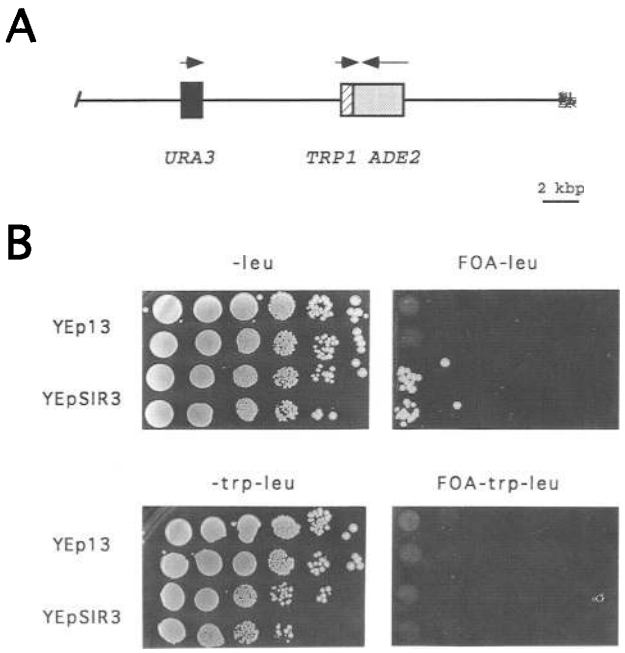

Figure 6. SIR3 propagates continuous silenced domains from the telomere. (*A*) A sketch of the VII-L arm in UCC1035 is shown. The shaded and solid boxes represent *ADE2* and *URA3*, respectively; the hatched box corresponds to the *TRP1* gene. The arrows indicate transcriptional orientation of the inserted genes. The diffuse dark end, which represents the telomeric $(TG_{1-3})_n$ repeat, is not drawn to scale. (*B*) Plating efficiency of two independent isolates of UCC1035 transformed by YEpSIR3 or YEp13. Cells were pregrown in −leu media to maintain the plasmids. Tenfold serial dilutions were plated onto medium lacking leucine (−leu). In addition, these plates either lacked tryptophan (−trp−leu), contained 5-FOA (FOA−leu), or lacked tryptophan and contained 5-FOA (FOA−trp−leu).

order reaction, then the occurrence of a repressed *URA3* gene at 1 kbp from the telomere would be expected six times as frequently as when *URA3* is 6 kbp away. From our results, this clearly is not the case. An exponential function more aptly describes the relationship between frequency of silencing and distance from the telomere (Fig. 2). Rather, the data suggest that telomeric silencing results from the cooperative assembly of subunits, and/or assembly of multiple components. Such a multimeric representation of silent chromatin is not surprising, as it is expected to involve the four core histones plus additional components (Eissenberg 1989; Henikoff 1990; Spradling and Karpen 1990; Grigliatti 1991). Nevertheless, this work provides the first in vivo quantitative examination of the silent chromatin assembly process.

It has been proposed that specific terminator sequences along the chromosome act as barriers to heterochromatic spreading (Tartof et al. 1984). No such regions were detected on the telomere–proximal 16 kbp of V-R, nor over the 20 kbp of a modified VII-L (Figs. 3 and 6), although our data do not rule out the existence of such sites in yeast.

Cells carrying *URA3* and *HIS3* located near the V-R telomere, with *HIS3* telomere–proximal, were unable to form colonies on FOA−his media (Fig. 5, UCC2576–2579). Because this medium selects for cells in which both *URA3* is repressed and *HIS3* is active, this result demonstrates that silent telomeric domains are continuously propagated from the end of the chromosome in yeast. Because a telomeric gene can be induced to become active (Gottschling et al. 1990; O.M. Aparicio and D.E. Gottschling, unpubl.), we suggest that transcription may actively block silent chromatin propagation. Alternatively, transcription may not act as a barrier to the spread of silencing per se, but, rather, reflect that the silent telomeric domain assembled only a short distance from the telomere, thus never encompassing the *HIS3* (or *URA3*) gene. The distinction between these two models should be considered regarding gene regulation within chromosomal domains.

The role of the promoter in TPE spreading

The presence of silent chromatin structures over a telomeric locus appears to impede the access of sequence-specific DNA-binding proteins to the DNA within, thereby generating a TPE (Gottschling et al. 1990; Aparicio et al. 1991; Gottschling 1992). The data plotted in Figure 2 show a steady decrease in the frequency of silencing when compared to the distance of the *URA3* promoter from the telomere. This result strongly suggests that a gene's promoter is a major determinant in *cis* for effective transcriptional repression near telomeres. Combined with the finding that the silencing of *URA3* does not appear to be dependent on the transcriptional orientation of *URA3* (Fig. 2), we propose that repression is primarily exerted on the gene's promoter and therefore blocks initiation rather than elongation.

Two important points about position effect are provided by the experiments in which *PPR1* was deleted. As with most *trans*-activator proteins, PPR1 appears to modulate transcription through the promoter (Roy et al. 1990). Hence, the increased frequency of telomeric silencing of *URA3* in *ppr1*$^-$ strains supports the result that promoter occlusion is critical in achieving position–effect repression (Fig. 2). These results also suggest that the spreading of position effect is a function of promoter strength of the gene being assayed.

A position effect on timing of replication has been detected at ~35 kbp from the V-R telomere (Ferguson et al. 1991; Ferguson and Fangman 1992), whereas position effect on *URA3* transcription is not detected beyond ~13 kbp from the same terminus (Fig. 2). At present, we cannot resolve whether this apparent discrepancy reflects differences between the two assays being used or inherent distinctions between the mechanisms of initiating replication and transcription.

Effect of Y′ elements on the spread of telomeric silencing

It has been suggested that Y′ elements overcome telomere position effect (Greider 1992), because genes em-

bedded into Y's are not transcriptionally repressed (Carlson et al. 1985; Louis and Haber 1990). However, our data argue that Y's do not block the spread of telomeric repression per se; we find that a 6.7-kbp Y′ element sustains a greater frequency of silencing than an equal length of unique chromosomal sequences (Figs. 2 and 3A). It is unclear whether Y's are involved in propagation or reinitiation of silencing or whether Y's simply lack elements present in unique chromosomal DNA that suppress the spreading of telomere-dependent transcriptional inactivation. Nevertheless, the presence of a Y′ element adjacent to a telomere results in a more extensive silent chromosomal domain. Perhaps this trait is important in maintaining the unique telomeric presence of Y′ elements.

SIR3 *enhances position effect in yeast*

Overexpression of *SIR3* enhances position–effect variegation of telomeric genes; this *SIR3* effect was also detected within and adjacent to the *HM* loci (H. Renauld and D.E. Gottschling, unpubl.). Thus, the modulation by *SIR3* of position–effect repression is likely to occur at other places in the genome where an initiation site for *SIR3*-dependent silencing resides.

The slope of the observed gradient in frequency of *URA3* silencing along V-R is altered by overexpressing *SIR3* in the cell (Fig. 3A), suggesting that in contrast to the effect of a *ppr1* mutation (Fig. 2), *SIR3* overexpression affects silent chromatin rather than an intrinsic property of *URA3*. In addition, the increase in telomeric silencing is sensitive to *SIR3* gene dosage (Fig. 3B), indicating that SIR3 is limiting in the cell. These data suggest that SIR3 may be a structural component of yeast repressive chromatin or a factor directly required for its assembly. Alternatively, SIR3 may act indirectly by regulating the level or activity of structural or assembly constituents of silent chromosomal domains.

SIR3 bears no significant similarity to any known enhancers of position effects, such as the *Drosophila* Su(var)2-5 (HP-1) or Su(var)3-7 proteins (Alberts and Sternglanz 1990) nor does it harbor a detectable chromodomain motif, which is thought to mediate the packaging of heterochromatin by the *Su(var)2-5* and *Polycomb* gene products (Paro and Hogness 1991; Messmer et al. 1992). Extragenic suppressor analysis of *HML* silencing indicates a physical interaction between SIR3 and histone H4 (Johnson et al. 1990). Thus, we favor the model that SIR3 interacts directly with yeast nucleosomes to facilitate the compaction of chromatin into a higher-order structure responsible for silenced regions of the yeast genome. In this light, SIR3 may be a functional equivalent of histone H1, mediating supranucleosomal organization of the genome (Weintraub 1984).

In addition to histone H4, telomeric silencing requires the products of *SIR2*, *SIR4*, *NAT1*, and *ARD1*. The roles of SIR2 and SIR4 in transcriptional repression are not yet clear. NAT1 and ARD1, which are subunits of an amino-terminal acetyltransferase (Park and Szostak 1992), presumably modify chromatin component(s) to facilitate assembly of repressed chromosomal states (Mullen et al. 1989; Park et al. 1992).

The ability of telomeric silencing to spread along the chromosome raises the question as to whether a cell can control the size of silenced domains. This issue is particularly critical for *S. cerevisiae*, in which inappropriate regional silencing might have immediate deleterious effects, owing to the high density of genes along the chromosome (Olson 1991). A *cis*-acting element can act as a chromosome-specific barrier against the spread of silent domains [e.g., active transcription units (this work) or homologs of the *Drosophila scs* sequences (Kellum and Schedl 1992)]. On a cellular scale, limiting the amount of *SIR3* in the cell could prevent excessive transcriptional inactivation of the entire genome. Because the *SIR3* gene itself is located near a telomere (Ivy et al. 1985) and no essential gene has been found between *SIR3* and the telomere (Basson et al. 1987; Brisco et al. 1987; Dietzel and Kurjan 1987; Mortimer et al. 1992), position–effect repression of the *SIR3* locus would provide a plausible negative feedback mechanism for control of position–effect spreading in yeast. If telomeric chromatin spread as far as the *SIR3* locus, transcription of *SIR3* would be repressed, thus limiting further spreading of the repressive chromatin. In apparent contrast to the yeast genome, larger eukaryotic genomes are extensively heterochromatic. This may be the result of the presence of more abundant functional homologs of SIR3. Extensive but carefully controlled heterochromatization of chromosomes may play a major role in control of cellular differentiation and development in complex eukaryotes.

In summary, the spread of telomeric position effect in *S. cerevisiae* is modulated by numerous factors, including promoter distance from the telomere, promoter strength, transcriptional status of telomere–proximal genes, presence of Y′ elements, and intracellular concentration of the *SIR3* gene product. These results highlight the complexity of mechanisms employed in regional gene expression and the plasticity of the eukaryotic genome.

Materials and methods

Construction of plasmids

Chromosomal location and a brief restriction map of the V-R sequences used in this study are diagramed in Figure 1A. The set of plasmids used to insert the *URA3* gene at various positions along V-R was constructed as follows, starting with plasmid pB610H (a gift from C. Newlon, New Jersey Medical School, Newark). Plasmid pHSS6TG carries a telomeric repeat sequence [derived from pYTCA-2 (Gottschling et al. 1990)] inserted between the *Eco*RI and *Bam*HI restriction sites of plasmid pHSS6 (Seifert et al. 1986). Orientation of the telomeric sequence is such that digestion of pHSS6TG with *Eco*RI will yield an end that is a substrate for telomere formation in yeast. A 7.3-kbp *Bam*HI fragment from plasmid pB610H (Fig. 1A) was ligated into the *Bam*HI site of pHSS6TG. A 7.4-kbp *Not*I fragment of this new plasmid, carrying unique V-R sequences adjacent to a telomeric $(TG_{1-3})_n$ repeat, was cloned into the *Not*I site of pVZ1 (Henikoff and Eghtedarzadeh 1987), generating pSC1. Plasmids pVURAH2(+) and pVRURAH2(−) were constructed by insert-

ing a 1.2-kbp *Hin*dIII fragment containing *URA3* into the H_2 site of pSC1 partially digested with *Hin*dIII (Fig. 1A). *URA3* transcriptional orientation is denoted plus (+) when transcription is toward the telomere and minus (−) when toward the centromere. *URA3* was cloned in a similar way into the H_3 and H_4 *Hin*dIII restriction sites, generating plasmids pVURAH3(+), pVURAH3(−), pVURAH4(+), and pVURAH4(−), respectively.

The *HIS3* gene was isolated from plasmid pHIS3 (Struhl 1985; Gottschling et al. 1990) by amplification using PCR (Innis et al. 1990), with the following primers: 5′ oligo, 5′- CCGGATCC-TGCCTCGGTAATGATTTTCATTTTTT-3′; 3′ oligo, 5′-CC-GGATCCTCTCGAGTTCAAGAGAAAAAAAAAAGAAA-3′. Restriction sites for *Bam*HI, which were placed at the ends of the oligonucleotides for convenient cloning, are underlined. Hence, we refer to this DNA segment as *HIS3 Bam*HI fragment.

Plasmids used to test for discontinuity of silenced chromosomal domains along V-R were created as follows. pH1.5HIS3(+) and pH1.5HIS3(−) were constructed in two steps. First, a 1.5 kbp *Hin*dIII fragment of V-R chromosomal DNA (between H_2 and H_3; Fig. 1A) was inserted into the *Hin*dIII site of pHSS6 to generate plasmid pHSS6(1.5). pHSS6(1.5) was then digested with *Kpn*I, blunt-ended, and ligated with the *HIS3 Bam*HI fragment, which had its ends filled in. A two-step process was also required to construct plasmids pVRUH2(−)HR1(+) and pVRUH2(−)HR1(−). Plasmid pVURAH2(−) was cut with *Xho*I and *Sal*I, and recircularized by ligation; a blunt-ended *HIS3 Bam*HI fragment was ligated into this plasmid that had been partially digested with *Eco*RI and blunted with T4 DNA polymerase. Plasmids pVRUH2(+)HR1(+) and pVRUH2(+)HR1(−) were constructed following the same procedure. Plasmids pYAHIS4-2(−) were made by cloning the *HIS3 Bam*HI fragment into the *Bam*HI site of pYA4-2 (Walton et al. 1986).

Plasmid pΔPPR1–HIS3 was constructed by replacing a 0.7-kbp *Bgl*II fragment containing the promoter region of *PPR1* (Kammerer et al. 1984), in plasmid pUC8–PPR1 (a gift from R. Losson, CNRS–IBMC, Strasbourg, France), with a 1.85-kbp *Bam*HI fragment from plasmid pHIS3. In plasmid pΔPPR1::LYS2 the same *Bgl*II fragment was replaced by a blunt-ended 4.8-kbp *Hin*dIII-*Xba*I fragment containing *LYS2*, isolated from pDP6 (Fleig et al. 1986).

Plasmid pVZ1ΔGCN4::TRP1 carries a deletion in the translation initiation region of *GCN4*. Plasmid pB238 [a derivative of plasmid p164 (Hinnebusch 1985)] was digested with *Bam*HI and *Bgl*II, and a 0.8-kbp *Bam*HI fragment containing *TRP1* from YDpW (Berben et al. 1991) was ligated into it. A *Sal*I–*Eco*RI 3.2-kbp fragment of the resulting plasmid was then ligated into pVZ1 digested previously with *Eco*RI and *Sal*I, to create pVZ1ΔGCN4::TRP1.

The plasmid pVZJL38TRP1(+)ADE2(-) was used to insert *TRP1* and *ADE2* between *ADH4* and telomere VII-L. Plasmid pUC19-JL3 contains a 0.4-kbp *Eco*RI–*Hin*dIII fragment including the JL3 region from VII-L (Walton et al. 1986). This plasmid was digested with *Eco*RI, its ends were made blunt, and the linearized plasmid was treated with *Hin*dIII. The JL3 region was ligated into plasmid pVZ1 digested previously with *Hin*cII and *Hin*dIII. Plasmid pVZJL38 was constructed by digesting the resulting plasmid, pVZJL3, with *Sma*I and *Eco*RI; an ~0.8-kbp *Eco*RI–*Hin*dIII fragment from plasmid pUC19-JL8 (Walton et al. 1986), with only its *Hin*dIII end made blunt, was ligated into the plasmid. A 1.45-kbp *Eco*RI fragment from plasmid YRp7 containing the *TRP1* gene (Struhl et al. 1979) was then inserted into this new plasmid, pVZJL38. The resulting plasmid, pVZJL38TRP1(+), was digested with *Bgl*II, and a 3.6-kbp *Bam*HI fragment containing *ADE2* was inserted (Gottschling et al. 1990). Plasmid pVZJL38TRP1(+)ADE2(−) has *ADE2* inserted in the opposite transcriptional orientation as *TRP1*.

YEpSIR3 (alias pKAN63; R.E. Esposito laboratory, The University of Chicago, IL) carries a ~7 kbp *Bam*HI genomic insert containing *SIR3* and flanking chromosomal sequences (Ivy et al. 1986) cloned into YEp13 (Broach et al. 1979). CEN–SIR3 (pHR62-16) contains a 3.7-kbp *Hpa*I fragment of plasmid pKAN63, encompassing *SIR3* and its putative transcriptional regulatory elements (Shore et al. 1984), inserted into the *Sma*I restriction site of plasmid pRS314 (Sikorski and Hieter 1989). Plasmid pHR67-23 (2μ–SIR3) carries the same *SIR3* fragment cloned into pHR59-33 (2μ), a derivative of pRS424 (Christianson et al. 1992) in which the *Cla*I site was deleted.

Plasmid pHR49-1 was constructed by inserting a 1.2-kbp *Bam*HI fragment containing *HIS3* from YDpH (Berben et al. 1991) into the *Bgl*II site of pRS316–SIR1 (a gift from Lorraine Pillus, University of Colorado, Boulder), which contains *SIR1* and flanking genomic sequences. All other plasmids used for strain construction have been described previously (Ivy et al. 1986; Kimmerly and Rine 1987; Gottschling et al. 1990; Aparicio et al. 1991).

DNA manipulations were performed as reported previously (Sambrook et al. 1989; Gottschling et al. 1990). *Escherichia coli* strains MC1066 (*r⁻ m⁻ trpC9830 leuB600 pyrF::Tn5 lacΔX74 strA galU galK*) (Casadaban et al. 1983), JF1754 (*r⁻ m⁻ leuB metB hisB*) (Himmelfarb et al. 1987), and TG1 [*supE hsdΔ5 thiΔ(lac–proAB) F′(traD36 proAB⁺ lacI^q lacZΔM15)*] (Sambrook et al. 1989) were used as plasmid hosts. Media for bacterial strains were prepared as described (Sambrook et al. 1989). Complementation of bacterial mutations by homologous yeast genes was used when applicable.

Yeast strains and methods

Media used for the growth of *S. cerevisiae* were described previously (Gottschling et al. 1990); all cultures were grown at 30°C. Yeast transformation was performed by electroporation in the presence of sorbitol (Becker and Guarente 1991) or the lithium acetate procedure (Schiestl and Gietz 1989). 5-FOA resistance (5-FOA^R) was determined as described previously (Aparicio et al. 1991). Yeast strain manipulations were carried out as described (Rose et al. 1990).

Strains UCC500–505 were constructed by transformation of YPH250 (Sikorski and Hieter 1989) with *Bam*HI-digested plasmids pVURAH2(+), pVURAH2(−), pVURAH3(+), pVURAH3(−), pVURAH4(+), and pVURAH4(−), respectively. Strains UCC506–511 were constructed by transformation of strain YPH250 with the same plasmids digested with *Not*I. In both cases, Ura$^+$ colonies were selected. *ppr1⁻* derivatives of these strains were constructed by transformation with *Eco*RI-digested pΔPPR1–HIS3 and selection for His$^+$ transformants.

URA3 was inserted into the *ADH4* locus (~20 kbp from telomere VII-L) of YPH250 to yield UCC1003, as described (Gottschling et al. 1990). UCC3248, UCC3249, and UCC3250 are derivatives of UCC1001 (Gottschling 1992) that are *sir2::HIS3*, *sir3::HIS3*, and *sir4::HIS3*, respectively, and were created by transformation as described (Kimmerly and Rine 1987; Aparicio et al. 1991). A *sir1::HIS3* derivative of UCC1003 (UCC3243) was constructed by transforming UCC1003 with *Cla*I- and *Sma*I-digested pHR49-1.

Plasmid pH1.5HIS3(+) was digested with *Not*I and transformed into UCC506 and UCC507 to make UCC2515 and UCC2517, respectively. pH1.5HIS3(−) was transformed in the same way into UCC506 and UCC507, to generate UCC2516 and UCC2518, respectively. Strains UCC2524–2527 were derived from YPH250 after transformation with the various pVRUH2(+/−)HR1(+/−) constructions digested with *Sph*I and *Not*I. UCC1005 is derived from YPH250 (Sikorski and Hie-

Table 2. *Yeast strains*

Strain	Relevant genotype	Origin
LJY153	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 hhfl::HIS3*	L. Johnson/M.Grunstein
W303-1a	*MAT***a** *ade2 can1-100 his3 leu2 trp1 ura3-1*	R. Rothstein
YPH250	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1, ura3-52*	Sikorski and Hieter (1989)
UCC16	W303-1a *nat1::LEU2 adh4::URA3*–TEL	Aparicio et al. (1991)
UCC18	W303-1a *adh4::URA3*–TEL	Aparicio et al. (1991)
UCC25	W303-1a *ard1::LEU2 adh4::URA3*–TEL	Aparicio et al. (1991)
UCC(500–511)	YPH250 *DIU5-(2–13)*	this study
UCC(512–523)	UCC(500–511) *ppr1::HIS3*	this study
UCC1001	YPH250 *adh4::URA3*–TEL	Gottschling (1992)
UCC1003	YPH250 *adh4::URA3*	this study
UCC1005	YPH250 *DIU5-1*	this study
UCC1035	UCC1003 *DIA7-1 DIT7-1*	this study
UCC2031	LJY153 *adh4::URA3*–TEL	Aparicio et al. (1991)
UCC2032	LJY153 *HHF2(gly-16) TRP1 adh4::URA3*–TEL	Aparicio et al. (1991)
UCC2033	LJY153 *HHF2(gln-16) TRP1 adh4::URA3*–TEL	Aparicio et al. (1991)
UCC2034	LJY153 *HHF2(gly-17) TRP1 adh4::URA3*–TEL	Aparicio et al. (1991)
UCC2576	UCC507 *gcn4::TRP1 ppr1::LYS2 DIH5-1*	this study
UCC2577	UCC507 *gcn4::TRP1 ppr1::LYS2 DIH5-2*	this study
UCC2578	UCC506 *gcn4::TRP1 ppr1::LYS2 DIH5-1*	this study
UCC2579	UCC506 *gcn4::TRP1 ppr1::LYS2 DIH5-2*	this study
UCC2580	UCC506 *gcn4::TRP1 ppr1::LYS2 DIH5-3*	this study
UCC2581	UCC506 *gcn4::TRP1 ppr1::LYS2 DIH5-4*	this study
UCC2582	UCC507 *gcn4::TRP1 ppr1::LYS2 DIH5-3*	this study
UCC2583	UCC507 *gcn4::TRP1 ppr1::LYS2 DIH5-4*	this study
UCC2589	UCC1005 *gcn4::TRP1 ppr1::LYS2 adh4::HIS3*	this study
UCC2590	YPH250 *gcn4::TRP1 ppr1::LYS2 adh4::URA3*–TEL *DIH5-5*	this study
UCC2591	YPH250 *gcn4::TRP1 ppr1::LYS2 adh4::URA3 DIH5-5*	this study
UCC3243	UCC1003 *sir1::HIS3*	this study
UCC3248	UCC1001 *sir2::HIS3*	this study
UCC3249	UCC1001 *sir3::HIS3*	this study
UCC3250	UCC1001 *sir4::HIS3*	this study

The following convention was adopted to describe insertions of genes along a chromosome where no open reading frame or genetic locus has been mapped. Dominant mutant alleles of *URA3* on chromosome V were designated *DIU5* [directed integration of *URA3* on chromosome V (in Arabic numerals)]. This convention was extended for insertion of *ADE2*, *TRP1*, or *HIS3*. The precise chromosomal structure of the relevant alleles is illustrated in Figs. 1B, 5, and 6A.

ter 1989) by transformation with pVR-URA3-TEL, as described (Gottschling et al. 1990). UCC1005 was transformed with pYA-HIS4-2(−) that had been digested with *Eco*RI and *Sal*I, yielding strain UCC2509. Strain UCC2528 carries a telomeric *URA3* at the VII-L telomere; it was created by transformation of YPH500 (Sikorski and Hieter 1989) with pVII-L URA3-TEL as described (Gottschling et al. 1990). The UCC2535 strain was created by transforming YPH250 with pVRUH2(−)HR1(+), selecting for His^+ transformants, and then screening for Ura^- cells. *URA3* was integrated at the *ADH4* locus of UCC2535 by transformation with padh4::URA3, as described (Gottschling et al. 1990), generating strain UCC2585. UCC2536, a meiotic segregant of a cross between UCC2528 and UCC2535, carries *HIS3* on V-R and *URA3* on VII-L. *ppr1*$^-$ *gcn4*$^-$ derivatives of strains UCC2515–2518, UCC2524–2527, UCC2509, UCC2536, and UCC2585 were constructed by transformation with *Eco*RI-digested pΔPPR1::LYS2 and selection for Lys^+ colonies; the *GCN4* locus was then disrupted by transformation with pVZ1ΔGCN4::TRP1 digested with *Not*I and *Sal*I, yielding UCC2580–2583, UCC2576–2579, and UCC2589–2591. Chromosomal structures of these strains are diagramed in Figure 5.

The gamma-deletion method (Sikorski and Hieter 1989) was used to introduce *TRP1* and *ADE2* between the JL3 and JL8 regions on VII-L (Walton et al. 1986). Plasmid pVZJL38TRP1(+)ADE2(−) was digested with *Bam*HI and transformed into UCC1003 to yield strain UCC1035.

The expected structures of the various chromosomal constructs were confirmed by Southern analysis as described previously (data not shown; Gottschling et al. 1990; Aparicio et al. 1991). All other strains have been described previously (Aparicio et al. 1991). Table 2 summarizes the relevant genotypes and origins of the strains used in this study.

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Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage.

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