Overcoming telomeric silencing: a *trans*-activator competes to establish gene expression in a cell cycle-dependent way

Oscar M. Aparicio and Daniel E. Gottschling

Department of Molecular Genetics & Cell Biology, University of Chicago, Chicago, Illinois 60637 USA

Genes located near telomeres in yeast are subject to position-effect variegation. To better understand the mechanism of this variegation, we investigated how a telomeric *URA3* gene switches from a silent to an expressed state. We found that silencing of a telomeric *URA3* gene was attributable to the elimination of its basal transcription. The reversal of that silencing was dependent on the presence of PPR1, the *trans*-activator protein of *URA3*. Maximum expression of *URA3* required a higher concentration of PPR1 when *URA3* was telomeric compared with when it was at a nontelomeric location. The ability of PPR1 to overcome silencing varied at different points in the cell cycle. In cells arrested in G₂/metaphase, PPR1 was able to activate transcription of a telomeric *URA3*, but in cells arrested in G₀, G₁, or early S phase it was not. In comparison, a nontelomeric *URA3* could be activated by PPR1 at all times. We conclude that once established, telomeric silent chromatin is a relatively stable structure, making a gene recalcitrant to activation. Following the disassembly of silent chromatin during DNA replication, competition of assembly ensues between components of telomeric chromatin, to establish a silent state, and the *trans*-activator, to establish gene expression. These results help explain the stochastic nature of phenotypic switching in variegated gene expression.

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In multicellular eukaryotes, chromosomal position effects generally involve the repression of a euchromatic, wild-type gene when it has been placed in or near heterochromatin as the result of a chromosomal rearrangement (for review, see Lima-de-Faria 1983). In a population of cells with such a rearrangement, the gene may escape repression; consequently, the resulting phenotype is variegated, exhibiting patches of normal and mutant tissue. A classic example of this phenomenon is the mosaic red-and-white eye of *Drosophila* in which the *white* gene has been translocated within centromeric heterochromatin (for review, see Eissenberg 1989; Henikoff 1990; Spradling and Karpen 1990).

When a wild-type gene is located near a telomere in the budding yeast *Saccharomyces cerevisiae*, it too is subject to position-effect variegation (Gottschling et al. 1990). For instance, when yeast cells with the *ADE2* gene placed near a telomere form a colony on solid medium, the colony is composed of subpopulations in which the *ADE2* gene is either expressed (white sectors) or repressed (red sectors). The different phenotypes of the sectors in a colony reflect the ability of genetically identical cells to switch between phenotypic states. The fact

that large sectors are phenotypically uniform reflects the ability of each state to be heritably propagated for multiple generations. Similarly, yeast cells with a telomeric *URA3* gene can form colonies on medium containing 5-fluoro-orotic acid (5-FOA), a drug lethal to cells expressing *URA3* (Boeke et al. 1987), indicating that the cells are phenotypically *ura3*⁻. However, these 5-FOA-resistant cells can form colonies when placed on medium lacking uracil; thus, the cells are able to switch their phenotypic status by activating expression of the telomeric *URA3* gene (Gottschling et al. 1990).

Silencing of telomeric genes in *S. cerevisiae* is likely attributable to a structurally distinct chromatin domain that initiates at the telomere. Evidence for this specialized chromatin structure includes identification of mutations in the histone H3 and H4 genes that relieve telomeric silencing (Aparicio et al. 1991; M. Grunstein, pers. comm.), the finding that telomere-adjacent chromatin contains histone H4 in a hypoacetylated state compared with H4 in actively transcribed chromatin regions of the genome (Braunstein et al. 1993), and the relative inaccessibility of telomere-proximal DNA to *in vivo* modification by the *Escherichia coli* dam methyl-

transferase protein (Gottschling 1992). In addition, the frequency with which a gene is silenced decreases with increasing distance from the telomere, suggesting that the structure nucleates at the telomere and the extent of its inward assembly along the chromosome varies between cells (Renaud et al. 1993). The extent of this assembly is proportional to the cellular concentration of SIR3, a gene product required for silencing at telomeres and the silent mating loci, *HML* and *HMR* (Aparicio et al. 1991; Laurenson and Rine 1992; Renaud et al. 1993). These results suggest that SIR3 is rate-limiting for assembly of the silent chromatin structure and implicate SIR3 as a component of the silent structure.

Two intriguing questions that arise in the study of position–effect variegation are how does a gene switch between phenotypic states; and once a state is determined, how is it heritably propagated? Both questions have been considered in fundamental issues of developmental biology. In particular, a great deal of interest has been focused on the role of transcriptional regulation in addressing these questions (for review, see Brown 1984; Weintraub 1985). With respect to position–effect variegation and the first question, two models of regulation that involve a role for chromatin structure have evolved (for review, see Felsenfeld 1992). Both models propose that transcription of a gene is inhibited by assembly of its DNA into chromatin. Furthermore, one or more transcriptional activator proteins (*trans*-activators) bind in a sequence-specific manner to DNA located in proximity to the gene and facilitate transcription of that gene, thus overcoming the repressive nature of the chromatin. Where the models differ is that in one case chromatin prevents the *trans*-activator from gaining access to the DNA, in essence keeping the gene “irreversibly” repressed. However, during DNA replication the chromatin structure of the gene is perturbed and the *trans*-activator has the opportunity to gain access and establish transcription before reassembly of the chromatin is completed. In the second case, the *trans*-activator can induce gene transcription at anytime in a replication-independent manner, effectively disrupting the repressive nature of the chromatin.

At its normal locus, *URA3*, like many biosynthetic pathway genes, is constitutively expressed at a basal level but can be induced to higher levels of expression (Lacroute 1968). *URA3* induction is contingent upon binding of an activated form of the *trans*-activator PPR1 to the upstream activating sequence (UAS) of the gene (Losson and Lacroute 1981; Roy et al. 1990). Interestingly, when *URA3* is located adjacent to a telomere, its basal level of expression may be repressed because the cells are phenotypically *ura3*[−] (Gottschling et al. 1990).

In our attempt to comprehend position–effect variegation, we have focused on how a gene switches between phenotypic states. We report on our efforts to understand how a gene located near a telomere overcomes silencing. Specifically, we examined the role of PPR1 in the expression of a telomeric *URA3* gene. Our results lead us to conclude that silent telomeric chromatin inhibits basal expression of *URA3* and prevents the transcriptional ac-

tivation of the telomeric *URA3* gene by PPR1 during the G₁ and early S phases of the cell cycle, in addition to when cells are arrested in G₀. Furthermore, our results suggest that upon replication of the telomeric DNA, a competition takes place between assembly of a silent chromatin structure and assembly of a PPR1-mediated transcriptionally active gene. These findings provide a peek into the mechanism of transcriptional control by silent chromatin and the establishment and inheritance of transcriptional states.

Results

The URA3 trans-activator PPR1 is required for overcoming telomeric silencing of URA3

To test the idea that the *trans*-activator PPR1 plays a role in overcoming silencing of a telomere-linked *URA3* gene, the *PPR1* gene was deleted from a strain in which *URA3* was located adjacent to telomere VII-L (UCC1001). To determine whether deletion of *PPR1* had a specific effect on *URA3* expression at a telomere, *PPR1* was also deleted in a strain with *URA3* inserted at an internal chromosomal position, the *ADH4* locus, which is ~20 kb from telomere VII-L (UCC1003). *PPR1* was also deleted in a strain lacking *URA3* (*ura3-52*; YPH250). *URA3* expression was measured by two methods: plating viability assays on medium containing 5-FOA and on medium lacking uracil (−URA), and RNA blot hybridization analysis. 5-FOA is converted into a toxic metabolite by the *URA3* gene product, such that cells expressing normal levels of the *URA3* gene product are sensitive to 5-FOA, whereas cells that lack it are resistant to 5-FOA (Boeke et al. 1984). For the RNA analysis, transcript levels were analyzed from *URA3*, *URA1*, and *ura3-52* [(Rose and Winston 1984) in this allele the *URA3* transcript is truncated], each of which is regulated by the PPR1 protein (Losson and Lacroute 1981). Thus, *URA1* and *ura3-52* RNA levels reflect the *in vivo* level of PPR1 activity as a transcriptional activator in each experimental sample.

Figure 1 shows that *PPR1* is required for overcoming silencing of the telomeric *URA3* gene. Wild-type (*PPR1*⁺) cells with *URA3* near a telomere formed colonies on 5-FOA medium and medium lacking uracil. This reflects the ability of the telomeric *URA3* gene to switch between transcriptionally repressed and active states. Deletion of *PPR1* abolished the ability of cells with a telomeric *URA3* gene to grow in the absence of uracil (Fig. 1A). Deletion of the PPR1-binding site within the *URA3* gene promoter had the same effect as deletion of *PPR1*, indicating that specific binding of PPR1 at the *URA3* UAS was required for overcoming silencing (Fig. 3, below; *UAS_{GAL}–URA3–TEL*, GAL4 plasmid “−”, the strain is *PPR1*⁺). Thus, in this telomeric context, PPR1 is required for the transcriptional activation of the *URA3* gene. The very small colonies that arose on −URA medium from the *ppr1*[−] strain with a telomeric *URA3* gene had acquired *trans*-acting mutations or local chromosomal rearrangements that permitted expression of

Figure 1. Expression of a telomere-linked *URA3* gene requires *PPR1*. (A) Plating efficiency of strains with *URA3* present at a telomeric (TEL) (UCC1001, UCC111) or internal (INT) (UCC1003, UCC113) chromosomal locus or absent (YPH250, UCC115), having *PPR1* present (+) or deleted (-). Cells were grown for 3 days on nonselective medium, suspended in H₂O, and equivalent aliquots of tenfold serial dilutions were placed onto complete synthetic medium (Complete), medium lacking uracil (- URA), and medium containing 5-FOA (FOA). (B) RNA blot hybridization analysis of strains in A. Total RNA isolated from mid-log-phase cells grown in medium containing uracil (+) or lacking uracil (-), was electrophoretically separated and blotted to nylon. The blot was sequentially hybridized with ³²P-labeled *URA3* and *ACT1* antisense RNA probes, and a *URA1* DNA probe.

URA3 [M.S. Singer and D.E. Gottschling, unpubl.]. Therefore, essentially no *URA3* gene product was produced from this telomeric site when *PPR1* was absent from the cell. In contrast, deletion of *PPR1* had no effect on 5-FOA or -URA viability when *URA3* was located at an internal chromosomal locus, (there is a slight decrease of colony size on -URA; Fig. 1A). This result suggests that at an internal location transcription of *URA3* still occurs, independently of *PPR1*, and is consistent with *URA3* regulation at its normal chromosomal locus [Losson et al. 1985]. As expected, *PPR1* deletion had no effect on the plating viability of cells lacking a functional *URA3* gene (Fig. 1A).

Telomeric *URA3* mRNA was undetectable when *PPR1* was deleted (Fig. 1B). However, *PPR1*⁺ cells with a telomeric *URA3* maintained the ability to activate *URA3* transcription. Deletion of *PPR1* had little or no effect on expression of an internal copy of *URA3*, or on expression of *URA1*.

Both the plating viability on -URA medium and the RNA analysis indicate that the constitutive or basal (*PPR1*-independent) expression of *URA3* at telomere VII-L is repressed by the telomeric silencing machinery. However, the *trans*-activator *PPR1* is able to circumvent the telomeric repression, thus facilitating *URA3* expression.

Increased *PPR1* dosage prevents silencing of a telomeric *URA3*

Because a telomeric *URA3* could exist in either an active or repressed state, and because *PPR1* was required for the active state, we postulated that *PPR1* might compete against the assembly of a repressed state. If this hypoth-

esis were true, then increasing the dosage of *PPR1* should increase the frequency with which an active state is established. To test this hypothesis, *PPR1* was expressed from a multicopy plasmid (FAT-*PPR1*, FAT is the vector alone) in strains with *URA3* absent, *URA3* at a telomeric, or *URA3* at an internal chromosomal locus. Cell viability of the resulting strains was quantified on 5-FOA medium and medium lacking uracil (Fig. 2). Increase of *PPR1* protein concentration from FAT-*PPR1* [verified by *ura3-52* and *URA1* RNA levels and quantitative electrophoretic mobility shift analyses (EMSAs; data not shown)] resulted in complete 5-FOA sensitivity of cells with *URA3* at the telomeric locus, along with improved growth on -URA. As expected, viability was not affected by overproduction of *PPR1* when *URA3* was at the internal locus or absent. Thus, high levels of *PPR1* compete against telomeric silencing to perpetually re-establish the *URA3* gene in an active state. These results also suggest that in a wild-type cell, the concentration of *PPR1* is limiting for telomeric *URA3* expression.

GAL4 can overcome telomeric silencing

To determine whether the ability of *PPR1* to overcome telomeric silencing on *URA3* transcription was a general characteristic of transcriptional activator proteins, the *PPR1* binding-site upstream of the *URA3* gene was replaced with a binding site for the *GAL4* *trans*-activator protein [Verdier 1990]. This modified *URA3* gene [*UAS-GAL-URA3*] was placed next to telomere VII-L (UCC420) or within the *ADH4* locus (UCC419) in strain YM725 (*gal4*⁻, *gal80*⁻, *ura3*⁻). The *gal80* mutation relieves negative regulation of the *GAL4* protein so that activity of *GAL4* is proportional to its concentration [Johnston

Figure 2. Silencing of a telomere-linked *URA3* gene can be reversed by overexpression of *PPR1*. Plating efficiency, as described in Fig. 1A, of strains with *URA3* present at a telomeric (TEL) (UCC1001) or internal (INT) (UCC1003) chromosomal locus or absent (YPH250), which contained the *PPR1*-overproducing plasmid (FAT-*PPR1*) or the vector alone (FAT) on complete synthetic medium (Complete), medium lacking uracil (–URA), and medium containing 5-FOA (FOA). All media lacked tryptophan to maintain selection for the plasmids.

1987). *UAS_{GAL}-URA3* was silenced when placed at telomere VII-L, as the cells were 5-FOA resistant and *Ura*[–], but *UAS_{GAL}-URA3* was not repressed when internally located on the chromosome as cells were 5-FOA-sensitive and *URA*⁺ (Fig. 3, *GAL4* plasmid –).

The wild-type *GAL4* protein or a series of carboxy-terminal truncations of the *GAL4* protein were expressed in the strains with *UAS_{GAL}-URA3* located at the telomere or at the internal locus. The carboxy-terminal truncation derivatives of *GAL4* maintain the amino-terminal DNA-binding domain and bind to *UAS_{GAL}* in vitro but are defective in transcriptional activation in vivo (Johnston and Dover 1988). Expression of wild-type *GAL4*, from a single copy centromeric plasmid, completely reversed silencing of the telomeric *UAS_{GAL}-URA3*, as indicated by the sensitivity of this strain to 5-FOA and robust growth on –URA medium (Fig. 3). None of the truncated *GAL4* derivatives were able to activate *UAS_{GAL}-URA3* adjacent to the telomere. Expression of *GAL4* or its derivatives had no effect on 5-FOA-sensitivity, or –URA viability, of strains with *UAS_{GAL}-URA3* located internal on the chromosome. It appears that the activation domain of *GAL4* is required

to compete for telomeric gene expression. These results suggest that the ability to overcome telomeric silencing is a general function of *trans*-activators.

Modulating the dosage of PPR1^c reveals that its accessibility to the telomeric URA3 gene is limited

The finding that *PPR1* dosage has a demonstrable effect on telomeric *URA3* expression, but not for internal *URA3* expression (Figs. 1 and 2), suggested that the telomeric *URA3* gene is relatively resistant to transcriptional activation by *PPR1* compared with when *URA3* gene is nontelomeric. To investigate this, a chimeric gene, *GALPPR1-1*, was constructed with the coding sequence of the *PPR1-1* allele under control of the *GAL1,10* promoter (Johnston and Davis 1984). The *PPR1-1* allele encodes a constitutively active protein, *PPR1^c*; thus, the level of *PPR1^c* activity as a *trans*-activator is directly proportional to its total cellular concentration (Losson and Lacroute 1983). The *GAL1,10* promoter permitted precise regulation of *PPR1^c* protein concentration within the cell (Durrin et al. 1991), because the intracellular level of *PPR1^c* was proportional to the level

Figure 3. Expression of a telomere-linked *UAS_{GAL}-URA3* gene requires the activation domain of the *GAL4* *trans*-activator protein. Plating efficiency, as in Fig. 2, of *gal4 gal80* strains that had *UAS_{GAL}-URA3* [*URA3* gene with the *PPR1*-binding site (*UAS_{URA}*) replaced by the *GAL4*-binding site (*UAS_{GAL}*)] at a telomeric (TEL) (UCC420) or internal (INT) (UCC419) chromosomal locus, and contained a plasmid expressing the wild-type *GAL4* protein (WT-881) or a plasmid expressing a carboxy-terminal truncation of *GAL4* at amino acids 292, 684, 174, or 383 (the numbers also denote the respective plasmids), or no plasmid (–). All media contained 3% raffinose as the sole carbon source and lacked tryptophan to maintain selection for the plasmids, except the media used for the strains harboring no plasmid that did contain tryptophan.

of galactose in the medium [based on *ura3-52* RNA levels (Fig. 4) and quantitative EMSAs (data not shown)]. As a control, a nonfunctional version of the gene fusion (*GALppr1-1*), which contains an inversion within the *PPR1-1*-coding sequence, was also created. These gene fusions were inserted at the *leu2* locus in isogenic *ppr1*[−] strains containing *URA3* at a telomeric (UCC2016) or internal chromosomal locus (UCC431) or in which *URA3* was absent (UCC432).

The resulting strains were tested for viability on 5-FOA and −URA medium that also contain galactose. Expression of the *GALPPR1-1* fusion, but not the mutated *GALppr1-1* fusion, effectively overcame silencing of the telomeric *URA3* in all cells of the population; the cells were URA⁺ and 5-FOA sensitive (data not shown). Expression of *GALPPR1-1* or *GALppr1-1* had no effect on the 5-FOA sensitivity or the −URA viability of strains with *URA3* either at the internal locus or absent (data not shown).

Levels of mRNA were analyzed from these strains grown in rich medium containing 3% raffinose and 0.25% galactose, which induced expression of *GALPPR1-1* or *GALppr1-1*. Expression of *GALPPR1-1* strongly activated transcription from *URA3*, *URA1*, and *ura3-52*, although compared with expression of the internal *URA3* gene, expression of the telomeric *URA3* was reduced (Fig. 4A). Equivalent levels of PPR1^c activity [based on *URA1* and *ura3-52* mRNA levels (Fig. 4A), and EMSAs (data not shown)] were present in the *GALPPR1-1* strains. This result supports the idea that

compared with the internal *URA3*, the telomeric *URA3* gene is relatively resistant to transcriptional activation at this concentration of PPR1^c.

We compared the relative expression levels of the telomeric *URA3* gene and the internal *URA3* gene when different concentrations of PPR1^c protein were expressed. The level of *ura3-52* RNA was used as a standard for PPR1^c concentration in vivo in comparing the two *URA3* loci; *ura3-52* has the same upstream sequences as *URA3* and is responsive over a wide range of PPR1^c concentrations (Fig. 4). We varied the level of *GALPPR1-1* expression by growing cells with different concentrations of galactose in the medium; levels of *ura3-52* RNA confirmed that higher concentrations of galactose resulted in higher intracellular PPR1^c protein concentrations (Fig. 4B). The results show that *URA3* at the telomeric locus was less responsive to low levels of the *trans*-activator than *URA3* at an internal locus (Fig. 4B,C). In addition, although both loci can achieve the same maximum level of expression, a higher PPR1^c concentration was required for the telomeric *URA3* compared with the internal *URA3* (Fig. 4B,C). These results suggest that there is a competition for binding at the telomeric *URA3* promoter between PPR1^c and silent chromatin.

PPR1^c activation of a telomeric URA3 gene is cell cycle regulated

The experiments described above were performed on ac-

Figure 4. Modulating the dosage of PPR1^c reveals a competition for accessibility of the *trans*-activator to the telomeric *URA3* gene. (A) RNA blot hybridization analysis of strains with *URA3* at a telomeric (TEL) (UCC409, UCC410) or internal (INT) (UCC435, UCC436) chromosomal locus or absent (UCC433, UCC434), which had a *GALPPR1-1* (*PPR1-1*: “+”) or *GALppr1-1* (*PPR1-1*: “−”) gene fusion integrated at the *leu2* locus. The normal *PPR1* gene locus was deleted in all strains. Total RNA was isolated from cells grown in YEP–3% raffinose/0.3% galactose to induce expression of the *GALPPR1-1* gene fusions. The RNA was electrophoresed, blotted, and sequentially hybridized with the probes described in Fig. 1B, except that a *URA3* DNA probe was used in this and subsequent figures. (B) RNA blot hybridization analysis of cells with the integrated *GALPPR1-1* gene fusion and either a telomeric *URA3* (UCC409) or an internal *URA3* (UCC435), grown in YEP–3% raffinose plus the indicated galactose concentration (%GAL). The blot was hybridized with the probes described in A. (C) The amount of probe hybridizing the RNA samples in B was quantified. The *ura3-52* and *URA3* message levels were calculated relative to *ACT1*, and the *ura3-52* and *URA3* values at 0% galactose (no PPR1^c) were set as a background to zero. The level of *ura3-52* message above the zero value is directly proportional to the in vivo level of active PPR1 in each sample [ppr1].

tively dividing cells. Hence, the cells were transiting through the cell cycle during the analysis. Keeping this in mind, two simple models can be set forth to explain the competition between PPR1 and telomeric chromatin for expression of the *URA3* gene. In the first model, the competition only occurs within specific periods of the cell cycle. During part of the cell cycle the telomeric *URA3* gene is resistant to activation by PPR1 if the silent chromatin state has been established. Only when the silent chromatin is weakened or disassembled, which might occur during DNA replication of the telomeric region, does PPR1 have the opportunity to activate the gene. In the second model, PPR1 competes with equal efficacy throughout the cell cycle.

To test and distinguish between these models, cells were grown in rich medium containing 3% raffinose and no galactose. Thus, PPR1^c was not present and the telomeric *URA3* gene was maintained in a silent state. The cells were then synchronously arrested by treatment with either α -factor pheromone, to arrest them late in G₁ (Pringle and Hartwell 1981), or nocodazole, an inhibitor of microtubule assembly (Pillus and Solomon 1986). In many eukaryotes, nocodazole produces a synchronous arrest at metaphase. Nocodazole also produces a very synchronous arrest in yeast; however it is unclear whether the arrest occurs late in G₂ or at metaphase. By the criterion of spindle pole body separation, the cells appear to be in G₂ (Jacobs et al. 1988); however, recent studies suggest that the chromosomes may be condensed as expected for a metaphase arrest (Guacci et al. 1994). In light of this uncertainty, we will refer to the arrest as G₂/metaphase. Once arrested, galactose was added to induce expression of *PPR1-1*, and half of the culture was released from the arrest while arrest was maintained in the other half. Expression of the telomeric *URA3* gene and the internal *URA1* and *ura3-52* genes was compared. The transcript levels of *CLN2* and *SWI5* were also analyzed to monitor the progress of cells through the cell cycle. *CLN2* is transiently expressed in late G₁ near the time of Start (Wittenberg et al. 1990), and *SWI5* is transiently expressed beginning sometime in S, through G₂, and on into M (Nasmyth et al. 1987).

The telomeric *URA3* was not activated by PPR1^c during α -factor arrest (Fig. 5). The analysis clearly shows that while cells were arrested with α -factor, the telomeric *URA3* gene remained repressed. The increase in *URA1* and *ura3-52* mRNA levels indicates that PPR1^c was active in these cells. Following release from the α -factor arrest, PPR1^c was able to activate the telomeric *URA3* gene. The analysis of the *SWI5* transcript (Fig. 5) and microscopic analysis of cell morphology (data not shown) were consistent with the cell-cycle arrest imposed by α -factor and release thereafter. The low level of telomeric *URA3* transcript seen late during the continued α -factor arrest correlated with the small fraction of cells (~5%) that escaped from the arrest.

In striking contrast to the repressed state of telomeric *URA3* during α -factor arrest, the telomeric *URA3* gene in G₂/metaphase, nocodazole-arrested cells was effectively activated by PPR1^c (Fig. 6). In the absence of func-

Figure 5. Silencing of a telomere-linked *URA3* gene is not reversible while cells are arrested in late G₁ with α -factor. Cells with a telomeric *URA3* and the integrated *GALPPR1-1* gene fusion (UCC409) were grown in YEP-3% raffinose and arrested with α -factor. When >95% of the cells were arrested as unbudded cells, the culture was pelleted by centrifugation, and half of the culture (α -factor: +) was resuspended in medium containing 0.3% galactose and α -factor, whereas the other half of the culture (α -factor: -) was resuspended in medium containing 0.3% galactose but lacking α -factor; $t=0$ hr. Aliquots of each culture were harvested to prepare RNA at the indicated times. The blot was hybridized with the probes described in Fig. 4A and with a *SWI5* DNA probe.

tional PPR1^c (Fig. 6A, *GALppr1-1*), no activation of the telomeric *URA3* or the internal *URA1* and *ura3-52* genes occurred. Not even basal expression of the telomeric *URA3* was seen in the absence of PPR1^c. Analyses of *CLN2* and *SWI5* expression (Fig. 6), as well as microscopic analyses of cell morphology (data not shown), confirmed the successful arrest with nocodazole and the release that followed.

To determine whether the effects of the α -factor and nocodazole treatments were attributable to the specific cell cycle arrests and not to other physiological effects of the treatments, we tested the effect of α -factor on telomeric gene expression in cells arrested in G₂ with nocodazole and, conversely, the effect of nocodazole on telomeric gene expression in cells arrested in G₁ with α -factor. The α -factor treatment did not prevent the expression of the telomeric *URA3* gene in cells arrested previously with nocodazole, and nocodazole treatment did not result in expression of the telomeric gene in cells arrested previously with α -factor (Fig. 6B). Thus, it appears that the effects on telomeric gene transcription by α -factor and nocodazole were attributable to the specific cell cycle arrests. These results suggest that the ability of a *trans*-activator (PPR1^c) to function in a telomeric domain is cell cycle regulated. We propose that a *trans*-activator is inaccessible to the telomeric domain in G₁ phase and becomes accessible by the time the cells are in G₂/metaphase.

To more accurately determine the period of the cell cycle in which PPR1^c activation of a telomeric *URA3*

Figure 6. *PPR1-1* can induce telomeric *URA3* gene expression while cells are arrested with nocodazole. (A) RNA blot hybridization analysis of telomeric *URA3* expression in cells during and after arrest with nocodazole. Cells with *URA3* adjacent to telomere VII-L, and the *GALPPR1-1* (UCC409) or *GALppr1-1* (UCC410) gene fusion integrated at the *leu2* locus were grown in YEP-3% raffinose and arrested with α -factor. These presynchronized G_1 cells were released from the α -factor arrest into medium containing nocodazole. Cultures were incubated with nocodazole until >85% of the cells arrested as large budded cells. The cells were pelleted by centrifugation, and half of each culture was resuspended in medium containing galactose and nocodazole (Nocodazole: +), whereas the other half of each culture was resuspended in medium containing galactose but lacking nocodazole (Nocodazole: -); this is $t = 0$ hr. Aliquots of each culture were harvested for RNA preparation at the indicated times. The blot was sequentially hybridized with the probes described in Fig. 5 and with a *CLN2* DNA probe. (B) Cells with a telomeric *URA3* were arrested in G_1 with α -factor (α -factor arrest) or in G_2 /metaphase with nocodazole (Nocodazole arrest). Next, hydroxyurea (+HU), nocodazole (+Noc), α -factor (+ α F), or nothing (-) was added to the cultures while α -factor arrest or nocodazole arrest was maintained. After 1 hr, galactose was added to all cultures to 0.3%, incubation of the arrested cells was continued for 1 hr, and the cultures were harvested for RNA preparation. RNA blot hybridization analysis of the samples was carried out with the probes described in Fig. 5 and quantified. The ratio of telomeric *URA3* to *ura3-52* RNA is plotted as telomeric *URA3* expression. The ratio of *SWI5* to *ACT1* mRNA is plotted below as *SWI5* expression.

could occur, cells were arrested in S phase with hydroxyurea (HU), an inhibitor of DNA replication (Slater 1973). Yeast cells with a telomeric *URA3* and the integrated *GALPPR1-1* fusion were pregrown in medium lacking galactose, to maintain repression of the telomeric *URA3* gene, and arrested with α -factor. Galactose was added to the α -factor-arrested cells to induce expression of *PPR1-1*, and the cells were released from the α -factor arrest; half of the culture was released into medium containing HU. Cells treated with this α -factor/HU protocol arrest very early in S phase, significantly before telomeric regions replicate (Hartwell 1976; McCarroll and Fangman 1988). HU prevented the activation of the telomeric *URA3* (Fig. 7) but did not affect

transcriptional activation of the internal *URA1* and *ura3-52* genes (data not shown). Telomeric *URA3* and *SWI5* expression following release from the HU arrest indicated that the arrest was reversible (Fig. 7, HU wash-out). Additionally, HU did not prevent activation of the telomeric *URA3* gene in cells that were arrested previously in G_2 /metaphase with nocodazole, indicating that the presence of HU itself does not prevent telomeric *URA3* expression (Fig. 6B). These results indicate that early in S phase the *trans*-activator cannot gain access to the telomeric *URA3* and, taken together with the results above, suggest that progression through S phase is required for the establishment of the transcriptionally active state in the telomeric domain.

Figure 7. Silencing of a telomere-linked *URA3* gene is not reversible while cells are arrested in early S phase with HU. Cells with *URA3* adjacent to telomere VII-L and the *GALPPR1-1* gene fusion integrated at the *leu2* locus (UCC409) were grown in YEP-3% raffinose and arrested with α -factor. Galactose was added to these presynchronized G_1 -arrested cells; following a 45-min incubation with galactose, the cells were released from the α -factor arrest into medium containing HU and galactose (+HU), or into medium containing galactose but no HU (-HU) at $t=0$ min. The HU-treated culture was released from the arrest ($t=180$ min) HU washout) and resuspended in medium containing galactose but no HU. Cells were collected for RNA isolation at the times indicated and subjected to RNA blot hybridization analysis with the probes described in Fig. 5. Hybridization was quantified, and the ratio of *URA3* to *ura3-52* RNA levels was plotted graphically as telomeric *URA3* expression. Expression of *SWI5* was standardized to the level of *ACT1* expression and plotted graphically as *SWI5* expression.

Temperature-sensitive alleles of *CDC* (cell division control) genes represent another method commonly used to arrest yeast cells at a specific point in the cell cycle (Pringle and Hartwell 1981). Cells are typically shifted from a permissive growth temperature ($\sim 23^\circ\text{C}$) to a non-permissive temperature (37°C) to cause arrest. We began to use temperature-sensitive alleles of *CDC* genes to define the cell cycle period in which PPR1 activation occurred. However, we discovered that PPR1^c-induced expression of a telomeric *URA3* was compromised severely at 37°C in wild-type (*CDC*⁺) cells (Aparicio 1993). This finding precluded our use of temperature-sensitive al-

leles in dissecting the period of activation in the cell cycle. The effect appeared to be telomere specific, because the *ura3-52* locus was activated. It is not clear whether the effect of temperature on telomeric *URA3* activation was specific to PPR1^c (e.g., a reduction in the effective concentration of PPR1^c) or whether it reflects a general strengthening of telomeric repression (e.g., the silent state was assembled more rapidly at 37°C ; see Discussion).

Telomeric silencing is irreversible when cells are in stationary phase (G_0)

An additional means to synchronously arrest a population of yeast cells is to maintain a culture in stationary phase (for review, see Werner-Washburne et al. 1993). Stationary-phase cells of *S. cerevisiae* arrest in a state referred to as G_0 ; the cells are unbudded and their genomes are unreplicated. Cells enter G_0 by exiting from G_1 phase, and general transcriptional repression occurs upon entry to stationary phase (Choder 1991). Strains with *URA3* at a telomeric or a nontelomeric locus and an integrated *GALPPR1-1* were grown to stationary phase in rich medium containing 3% raffinose, so that PPR1^c was absent and, hence, the telomeric *URA3* gene was silenced. Cells were determined to be in stationary phase when the optical density of the culture had not increased during the previous 24-hr period, and >98% of cells were unbudded. Expression of *GALPPR1-1* was induced in the stationary cells by adding 0.3% galactose to the cultures. Incubation was continued as aliquots were collected for RNA analysis. Although the internal *URA3* gene, as well as the *URA1* and *ura3-52* genes, was transcriptionally activated by PPR1^c in the stationary cells, the telomeric *URA3* gene was not activated (Fig. 8). Only after 48 hr of induction was a telomeric *URA3* transcript observed, just slightly above limits of detection (Fig. 8). Thus, silencing of a telomeric gene in stationary-phase cells is essentially irreversible. As expected, basal levels of transcription decreased in the stationary cells (Fig. 8, cf. uninduced *URA1* levels in the stationary and log cells). Moreover, the *SWI5* transcript was not detected in G_0 cells, confirming that cells were not progressing through the mitotic cell cycle. In the experiment shown here, galactose was added to cultures ~ 48 hr after mid-log phase; equivalent results were obtained when the experiment was performed with 7-day-old cultures (data not shown).

Discussion

In this study we examined the ability of *trans*-activator proteins to overcome silencing of a telomere-adjacent gene in *S. cerevisiae*. We found that the *trans*-activator protein PPR1 is absolutely required for expression of a *URA3* gene located immediately adjacent to the left telomere of chromosome VII (Fig. 1). In contrast, when *URA3* is at a nontelomeric location, PPR1 merely pro-

Figure 8. Silencing of a telomere-linked *URA3* gene is not reversible in stationary-phase cells. Strains with a telomeric (UCC409) or an internal (UCC435) *URA3* gene and the integrated *GALPPR1-1* gene fusion were grown in YEP-3% raffinose into stationary phase. Galactose was added to both cultures to 0.3% ($t=0$ hr), and aliquots were harvested for RNA preparation at the indicated times. The percentage of unbudded cells in the cultures remained above 98% at each time point. Ethidium bromide staining of the 18S rRNA in the gel prior to transfer is shown. RNA isolated from uninduced cells of the same strains during logarithmic growth in YEP-3% raffinose (no galactose) were included at the far right as controls for *SWI5* expression. The blot was analyzed with the same probes described in Fig. 5.

vides a modest increase in expression (Roy et al. 1990, Fig. 1). Two conclusions may be drawn from these results: (1) Telomeres inhibit basal transcription; and (2) *trans*-activators have a mechanism to circumvent this inhibition.

It is likely that the basal transcription apparatus of *URA3* is prevented from accessing the gene's promoter because of steric occlusion by silent telomeric chromatin. This notion is supported by the observation that other DNA-binding proteins, such as *E. coli* dam methylase, are excluded from telomere-proximal DNA regions in vivo (Gottschling 1992). Note that basal expression of *URA3*, as with most housekeeping genes in yeast, requires not only a TATA element but additional sequences upstream that bind PPR1-independent factors (Roy et al. 1990).

Although it is unclear how PPR1 or any other *trans*-activator protein activates transcription, several of our results address the mechanism of overcoming silencing. First, PPR1 cannot activate transcription of the telomeric *URA3* gene in G_1 , early S, or G_0 cells. Only in a G_2 /metaphase arrest is activation observed (Figs. 5–8). Second, the cellular concentration of PPR1 dramatically affects the frequency with which telomeric *URA3* expression is established (Figs. 1, 2, and 4). Third, the complete activation domain of a *trans*-activator is essential for its efficacy (Fig. 3). Although a telomeric gene with a

GAL4 UAS can be activated in the presence of wild-type *GAL4*, the gene remains silenced when the wild-type *GAL4* is replaced by derivatives that remove the *GAL4* transcriptional activation domain.

On the basis of our results, we propose a replication-dependent model (see introductory section) to explain how a telomeric gene can overcome silencing to become transcriptionally active. In G_1 of the cell cycle, a silenced telomeric gene is packaged in a repressive chromatin structure that is relatively "static" and prevents interactions of the DNA with other DNA-binding proteins such as basal transcription factors and *trans*-activators. However, the telomeric chromatin loses its static structure as a result of the DNA replication process or some other coordinate cellular event. Alternatively, one of the two newly replicated sister chromatids retains the silent chromatin, whereas the other is essentially "naked" DNA and awaits assembly into chromatin. Regardless of which pathway occurs, upon completion of replication, two distinct assembly processes compete to establish the transcriptional state of a telomeric gene. Assembly of silent chromatin initiates at the telomere and propagates inward along the DNA. This process not only requires the histones but a number of additional factors, such as RAP1, SIR2, SIR3, and SIR4 (Aparicio et al. 1991; Kyrion et al. 1993). The competing process involves the binding of the *trans*-activator protein to the telomeric gene and assembly of an active transcription complex. The competition ends when one of the two processes is fully established at the promoter region of the telomeric gene. In the absence of competition from the *trans*-activator, the silent chromatin eventually assembles into its static structure. The moment that this silent structure forms defines the end of the cell cycle period in which the *trans*-activator has an opportunity to compete. Although our results identify the existence of a cell cycle "window" for silent chromatin assembly, definition of its precise beginning and end awaits a more detailed analysis.

Having a limited period in the cell cycle during which a transcriptional state is established has several ramifications. Environmental or genetic changes that alter the duration of the silent chromatin assembly process could dramatically affect the frequency of establishing a state. Such changes may be direct. For instance, the SIR3 gene product appears to be a component of silent chromatin that is rate limiting in its assembly (Johnson et al. 1990; Renaud et al. 1993). Thus, increasing SIR3 concentration increases the frequency of establishing repression (Renaud et al. 1993). Alternatively, changes that extend periods of the cell cycle in which silent chromatin assembly occurs, such as G_2 , provide a *trans*-activator greater opportunity to establish an active state. Conversely, a shorter G_2 would favor establishment of a silent state. In essence, such changes can dictate the amount of phenotypic variegation within a population of cells.

The assembly of silent telomeric chromatin may consist of several distinct, sequential steps rather than an "all-or-none", concerted process. Evidence for this suggestion comes from Figure 6A. In nocodazole-arrested

cells, telomeric *URA3* expression was rapid when PPR1 was present (*GALPPR1*). However, basal or PPR1-independent (*GALppr1*) expression of the telomeric *URA3* was not detected, even after a lengthy arrest (~5 hr), whereas basal expression at internal loci was normal. These results suggest that at the nocodazole-arrest point silent chromatin is assembled up to a stage that precludes basal expression yet does not prevent PPR1-induced expression. This postulated intermediate of silent chromatin assembly may not be locked into a fully static structure, yet it is still more recalcitrant to gene expression than other areas of the genome. The static chromatin structure likely requires several contributions: binding of the core histones by accessory proteins such as SIR3 (Renauld et al. 1993); modifications of telomeric histones such as hypoacetylation (Braunstein et al. 1993); and localization of the structure to the nuclear periphery (Palladino et al. 1993). Any of these contributions may be absent at an intermediate stage. Thus, we propose that the system defined here will be very useful in examining the assembly steps of silent chromatin in vivo.

Our results both complement and extend observations made at the yeast silent mating type loci *HML* and *HMRI* (Miller and Nasmyth 1984). Telomeres and the *HM* loci share a number of silencing factors (e.g., SIR2, SIR3, and SIR4) (Aparicio et al. 1991). Using temperature-sensitive alleles of *SIR3* and *SIR4*, Miller and Nasmyth determined that establishment of silencing at the *HM* loci requires passage through S phase and, thus, presumably DNA replication. Their conclusion is consistent with the model we propose, that the competition for assembly occurs after replication. Furthermore, we show that at least in the case of the VII-L telomeric locus, assembly of silencing is not completed until sometime after G_2 /metaphase (nocodazole arrest). In their experiments, Miller and Nasmyth also found that inactivating the *SIR3* or *SIR4* gene product at any time in the cell cycle resulted in gene expression at the *HM* loci. Here, we show that passage through S phase is required for activation of a telomeric gene. Thus, dismantling of the repressive chromatin, either by artificially compromising it with a defective *SIR3* or *SIR4* allele, or in every cell cycle during passage through S phase, allows a renewal of the competition between establishment of active and silent states.

As the result of a telomeric location, *URA3* can be much more highly regulated than at its normal locus. When *URA3* is at a nontelomeric location, the presence of PPR1⁺ produces a three- to sevenfold induction over basal expression (Liljelund et al. 1984; Fig. 4B). However, with *URA3* near a telomere, an equivalent amount of PPR1 induces expression ~100-fold (Fig. 4B). We suggest that eukaryotic genomes may have evolved to take advantage of this type of telomeric regulation. For example, trypanosomes depend on the highly regulated expression of the telomeric VSG (variable surface glycoprotein) genes (for review, see Borst 1991; Cross 1990).

When cells were in G_0 , essentially no amount of *trans*-activator protein was sufficient to overcome telomeric silencing, whereas at an internal nonsilenced position

the *trans*-activator readily induced expression (Fig. 8). Interestingly, general transcriptional repression, apparently mediated by chromatin changes, occurs upon entry to stationary phase (Choder 1991). Stationary-phase chromosomes display different sedimentation properties than G_1 -phase chromosomes, suggesting that chromosomes assume a distinct compact structure in G_0 cells (Piñon 1978). It is possible that the same machinery and mechanism of telomeric silencing in G_1 extends to other regions of the genome in G_0 , thus facilitating the more global compaction and transcriptional repression.

Whatever the nature of the silent telomeric chromatin, it contrasts with the chromatin structure of the *PHO5* gene in yeast. Although this locus is transcriptionally repressed by nucleosomes upstream of the transcription initiation site, it can be induced rapidly at any time in the cell cycle or in G_0 -arrested cells (Schmid et al. 1992). The induction involves the displacement of a nucleosome by the gene's transcriptional activator protein. In contrast, overcoming telomeric silencing requires that the nucleosomes be modified or removed by passage through S phase before the *trans*-activator protein can have its effect. This emphasizes that telomeric chromatin is inherently different than chromatin at *PHO5* or most other regions of the yeast genome.

In an attempt to understand telomere position-effect variegation, we have examined one aspect of this phenomenon: the ability of a telomeric gene to switch from a silent to an expressed state. Our findings have provided insights into the cell cycle-regulated nature of the silent state, the establishment of an active state, and the realization that the establishment process must recur each time a cell replicates its genome. We expect that the study of telomere position effect as presented here will serve as a model system for understanding the process of chromatin assembly in vivo. In addition, it will assist us in understanding the role of chromatin in propagating a phenotypic state through successive cell generations.

Materials and methods

Plasmid constructions

Plasmid FAT-PPR1 was constructed by ligating a 4.4-kb *EcoRI* fragment containing the *PPR1* gene (from pUC8-PPR1, a gift from R. Losson, CNRS, Strasbourg, France) into plasmid YEP-FAT10 (referred to as "FAT" in the legend to Fig. 2 and in text), [2 μ *ARS*, *TRP1*, *leu2-d*, a gift from K. Runge (Runge and Zakian 1989)]. A 3.7-kb *HindIII*-*SphI* fragment containing the entire *PPR1-1* allele [from plasmid pFL11 (Losson and Lacroute 1983)] was inserted into plasmid pVZ1 (Henikoff and Eghtedarzadeh 1987). The resulting plasmid (pVZPPR1-1) provided a 3.7-kb *HindIII*-*BamHI* fragment containing *PPR1-1* that was ligated into pRS425 (Sikorski and Hieter 1989) to yield plasmid pRS4-PPR1-1. Plasmids pRS305-GALPPR1-1 and pRS305-GALppr1-1 were constructed in a series of steps. A 685-bp *EcoRI*-*BamHI* fragment containing the *GAL1,10* promoter [from pBM150, (Johnston and Davis 1984)] was ligated into *EcoRI*-*BamHI*-digested pRS314 (Sikorski and Hieter 1989); the resulting plasmid (pRS314GAL) was digested with *ApaI*-*EcoRI*, and a 2.8-kb *ApaI*-*EcoRI* fragment containing the 3' portion of *PPR1-1* from plasmid pRS4-PPR1-1 was inserted yielding pRS3GAL3'PPR1-1. Next, a 500-bp fragment containing the 5'

portion of the *PPR1-1* allele was produced by PCR amplification (Innis et al. 1990). The primers were designed to introduce an *EcoRI* site 28 bp upstream of the *PPR1* ATG initiation codon and to include the *EcoRI* site within the *PPR1-1*-coding sequence (PPR1-ATG oligonucleotide, 5'-CCGGAATTCATACGAAGATGATGATTAAATC-3', the new *EcoRI* site is underlined; PPR1-n650 oligonucleotide, 5'-GGCTTGCCATAGACTTGCTCG-3'). The fragment was digested with *EcoRI* and inserted between the *GAL1,10* promoter and the 3' *PPR1-1* sequence in pRS3GAL3'PPR1-1; one orientation of the insert yielded pGALPPR1-1, which has the *GAL1,10* promoter fused to the entire *PPR1-1*-coding sequence (*GALPPR1-1*), whereas the other orientation of the insert yielded pGALppr1-1, which has the 5' portion of the *PPR1-1* allele inverted resulting in a mutated gene fusion (*GALppr1-1*). The 3.5-kb *ApaI*–*BamHI* fragments containing *GALPPR1-1* and *GALppr1-1* from pGALPPR1-1 and pGALppr1-1, respectively, were inserted into pRS305 (Sikorski and Hieter 1989) yielding pRS305-GALPPR1-1 and pRS305-GALppr1-1.

Plasmid pVZADH4 contains the *ADH4* locus on a 3.1-kb *EcoRI*–*SalI* fragment (Gottschling et al. 1990). A 4.8-kb *HindIII*–*XbaI* fragment containing the *LYS2* gene from plasmid pDP6 (Fleig et al. 1986) was inserted into *XbaI*–*HindIII*-digested pVZADH4 creating pVZadh4::LYS2. The *UAS_{GAL}*–*URA3* allele was produced by sequential PCR amplification steps (Ausubel et al. 1989). The primers were designed to replace the PPR1 binding-site [*UAS_{URA}*, 5'-TTCGGTAATCTCCGAA-3' (Roy et al. 1990)] with a *GAL4*-binding site [*URA3*–*GAL*–5' oligonucleotide, 5'-CGGACGACTGTCTCCGTCAGAAAAAATTTCAGGAAACCG, *URA3*–*GAL*–3' oligonucleotide, 5'-CGGACGACAGTCGTCGCGCAGAGAAGAAGCAAGGAA; the *GAL4*-binding sequence is underlined (Verdier 1990)]. The *UAS_{GAL}*–*URA3* PCR product was digested with *SalI* and *BamHI* and inserted into pRS315(–*PstI*) producing pRS315(–*PstI*)–*GALURA3*; the *PstI* site in pRS315 was deleted previously by digestion of pRS315 (Sikorski and Hieter 1989) with *PstI*, making the ends blunt with T4 DNA polymerase, and religating the plasmid. Plasmid pRS315(–*PstI*)–*GALURA3* was digested with *HindIII* and *SmaI* and religated, resulting in the *UAS_{GAL}*–*URA3* fragment being inverted in the vector to yield pRS315(–*PstI*)–*GALURA3*-flip. This plasmid provided a 1.1-kb *HindIII*–*BamHI* fragment containing *UAS_{GAL}*–*URA3*, which was inserted into pVII-L *URA3*–TEL (Gottschling et al. 1990) to produce pADH4GALURA3TEL; the same 1.1-kb *HindIII*–*BamHI* *UAS_{GAL}*–*URA3* fragment was inserted into *HindIII*–*BamHI*-digested pVZADH4 resulting in plasmid pAdh4::GALURA3. A 1.2-kb *HindIII*–*NotI* fragment (made blunt-ended with T4 DNA polymerase, from pVII-L *URA3*–TEL) was ligated into *HindIII* (made blunt-ended with T4 DNA polymerase) digested pVZadh4::LYS2 producing pURA3–TEL–LYS2. A 1.5-kbp *PstI* fragment (from pADH4GALURA3TEL) containing the *UAS_{GAL}*–*URA3* promoter was inserted into *PstI*-digested pURA3–TEL–LYS2 to replace the wild-type *URA3* promoter; the resulting plasmid was pGALURA3–TEL–LYS2.

A 1.35-kbp *BamHI* fragment containing the entire *URA1* gene (Roy 1992) produced by PCR amplification of genomic DNA (5'*URA1* oligonucleotide, 5'-CGAACGGATCCCCCTTCAGCCACTACAGCCTACTT-3'; 3'*URA1* oligonucleotide, 5'-CGAAGGGATCCGCCAATTGCGAATGCACTCACCG-3', the *BamHI* sites are underlined) was inserted into pVZ1 to yield plasmid pVZURA1. A 1.1-kb *HindIII*–*BamHI* *URA3* fragment was ligated into *HindIII*–*BamHI*-digested plasmid YDpK (Berben et al. 1991), yielding plasmid YDpK–*URA3*. Plasmid p5'*URA3* contains a 415-bp *HindIII*–*EcoRV* 5' *URA3* fragment ligated into *HindIII*–*EcoRV*-digested pVZ1. Plasmid CY807 + TRP1 (*bar1::TRP1*) was constructed by inserting a 723-

bp *BamHI* fragment containing *TRP1*, from YDp-W (Berben et al. 1991), into the *BglII* site in the *BAR1* sequence in plasmid CY807 (from S. Honigberg, Syracuse University, NY). Plasmids pBM292 (*GAL4*–wild type, 881 amino acids), pBM430 (*GAL4*, carboxy-terminal amino acid 292), pBM433 (*GAL4*, carboxy-terminal amino acid 684), pBM789 (*GAL4*, carboxy-terminal amino acid 174), and pBM1268 (*GAL4*, carboxy terminal amino acid 383) are *CEN*, *TRP1* plasmids described previously and provided by M. Johnston and Dover (1988). Plasmids pBD57 and pJM206 were provided by F. Cross (Rockefeller University, New York), and plasmid pPL9 was provided by R. Surosky (Surosky and Esposito 1992).

Methods for DNA manipulation were described previously (Renaud et al. 1993).

Yeast methods and strains

S. cerevisiae were grown at 30°C; liquid cultures were agitated during incubation at 180 RPM. All experiments in liquid culture were carried out with mid-log-phase cells unless otherwise indicated. Plating efficiency analysis and synthetic media have been described previously (Gottschling et al. 1990), except for α -amino adipate-containing medium, which was prepared as described in (Sikorski and Boeke 1991). Experiments involving galactose control employed YEP-3% raffinose, and 0.3% galactose for induction unless otherwise indicated. For experiments involving drug or α -factor washout, cells were pelleted by centrifugation for 3 min at 1500g and washed and/or resuspended in prewarmed medium (30°C). Cells were arrested with 20 nM α -factor for 3 hr, and 50 mM phthalic acid (pH 5.5) was included in the medium. For release from α -factor arrest, 1 mg/ml of pronase E was included in the fresh resuspension media, except for the experiment described in Figure 6, where one water wash of the pellet was carried out and pronase E was not included in the resuspension medium. Cells were arrested with 10 μ g/ml of nocodazole (from a 1000 $\times$ stock solution in dimethylsulfoxide) for 3 hr. HU was dissolved directly in medium immediately before use to a final concentration of 400 mM, except in the experiments described in Figure 6B, where it was dissolved directly in the cultures. Cells were fixed and stored in 10 mM Tris-HCl, 100 mM EDTA, (pH 8.0), and 3.7% formaldehyde and sonicated before microscopy to assess cell morphology.

S. cerevisiae were transformed using the lithium acetate procedure (Ito et al. 1983). The *URA3* gene was placed adjacent to the telomere sequence (TG₁₋₃)_n on the left end of chromosome VII (UCC2013) or inserted at the *ADH4* locus about 20 kb from the telomere on VII-L (UCC432), as described previously (Gottschling et al. 1990). UCC2013 was derived from YPH499 (see Table 1), and UCC432 was derived from UCC431 (described below).

Strains UCC111, UCC113, UCC115, UCC412, and UCC2014 were constructed by transformation of strains UCC1001, UCC1003, YPH250 (see Table 1), UCC411 (described below), and UCC2013 respectively, with plasmid pAPPRI::HIS3 and selection for HIS⁺ transformants; this plasmid has been described previously (Renaud et al. 1993). Strains UCC116, UCC117, and UCC151 were derived from strains UCC1001, UCC1003, and YPH250, respectively, by transformation with plasmid pFAT–PPR1 and selection for TRP⁺ cells; strains UCC238, UCC152, and UCC153 were derived from strains UCC1001, UCC1003, and YPH250, respectively, by transformation with plasmid YEpFAT10 (FAT) and selection for TRP⁺.

Strain UCC411 was derived from YPH499 by transformation with *HpaI*-digested YDpK–*URA3* and selection for LYS⁺ cells. UCC413 and UCC2016 were derived from UCC412 and UCC2014, respectively, by transformation with plasmid CY807 + TRP1-digested with *ClaI*. Strain UCC431 was a

Table 1. Genotypes and sources of strains

Strain	Genotype	Source
YPH250	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1</i>	(Sikorski and Hieter 1989)
YPH499	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>	(Sikorski and Hieter 1989)
YM725	<i>MATa ura3-52 lys2-801 ade2-101 trp1-901 his3-200 tyr1-501 met⁻ can1 gal4-542 gal80-538</i>	(Johnston and Dover 1988)
UCC1001	YPH250 <i>URA3-TEL-VII-L</i>	(Gottschling 1992)
UCC1003	YPH250 <i>adh4::URA3</i>	(Renauld et al. 1993)
UCC111	UCC1001 <i>ppr1Δ::HIS3</i>	this study
UCC113	UCC1003 <i>ppr1Δ::HIS3</i>	this study
UCC115	YPH250 <i>ppr1Δ::HIS3</i>	this study
UCC419	YM725 <i>adh4::UAS_{GAL}-URA3</i>	this study
UCC420	YM725 <i>UAS_{GAL}-URA3-TEL-VII-L</i>	this study
UCC409	YPH499 <i>ppr1Δ::HIS3 bar1::TRP1 URA3-TEL-VII-L leu2::GALPPR1-1::LEU2</i>	this study
UCC410	YPH499 <i>ppr1Δ::HIS3 bar1::TRP1 URA3-TEL-VII-L leu2::GALPPR1-1::LEU2</i>	this study
UCC433	YPH499 <i>ppr1Δ::HIS3 bar1::TRP1 leu2::GALPPR1-1::LEU2</i>	this study
UCC434	YPH499 <i>ppr1Δ::HIS3 bar1::TRP1 leu2::GALPPR1-1::LEU2</i>	this study
UCC435	YPH499 <i>ppr1Δ::HIS3 bar1::TRP1 adh4::URA3 leu2::GALPPR1-1::LEU2</i>	this study
UCC436	YPH499 <i>ppr1Δ::HIS3 bar1::TRP1 adh4::URA3 leu2::GALPPR1-1::LEU2</i>	this study

5-FOA^R (Ura⁻, Lys⁻) derivative of UCC413. Strains UCC409, UCC433, and UCC435 were derived from strains UCC2016, UCC431, and UCC432 respectively, by transformation with *Hpa*I-digested pRS305-GALPPR1-1; strains UCC410, UCC434, and UCC436 were derived from strains UCC2016, UCC431, and UCC432, respectively, by transformation with *Hpa*I digested pRS305-GALppr1-1.

To place *UAS_{GAL}-URA3* (or another nonselectable marker) adjacent to telomere VII-L, a method was developed based on the phenomenon of new telomere formation at internal telomeric sequences (Gottschling et al. 1990). Plasmid pGALURA3-TEL-LYS2 was used to integrate within the *ADH4* locus: *UAS_{GAL}-URA3* adjacent to 81 bp of telomere repeat sequence followed by *LYS2* as the selectable marker (centromere-proximal to centromere-distal). At a frequency of $\sim 10^{-6}$, loss of chromosomal sequences distal to the 81-bp internal telomeric sequence (including *LYS2*) resulted in formation of a new and stable telomere having the *UAS_{GAL}-URA3* gene adjacent to it (data not shown). Cells that were transformed with pGALURA3-TEL-LYS2, and were LYS⁺ and had the correct sequences inserted within the *ADH4* locus (verified by DNA blot hybridization analysis), were grown nonselectively for ~ 25 generations. Cells that had lost *LYS2* were selected for survival on medium containing α -aminoadipate; the expected structure of telomere VII-L in the resulting Lys⁻ strain was verified by DNA hybridization analysis. UCC418 was derived from YM725 (see Table 1) by transformation with *Not*I-*Sal*I-digested plasmid pGALURA3-TEL-LYS2 and selection for LYS⁺ transformants; UCC420 was an α -aminoadipate resistant (Lys⁻) derivative of UCC418 that has *UAS_{GAL}-URA3* adjacent to telomere VII-L. UCC419 was derived from YM725 by transformation with *Eco*RI-*Sal*I-digested plasmid pAdh4::GALURA3 and selection for URA⁺ transformants. Strains UCC419 and UCC420 were transformed with plasmids pBM292, pBM430, pBM433, pBM789, and pBM1268, to yield strains UCC421-UCC425, respectively, for the UCC419 parent, and strains UCC426-UCC430, respectively, for the UCC420 parent. The expected structures of the various chromosomal constructs were confirmed by DNA blot hybridization analysis (data not shown).

Analysis of nucleic acids

Methods for DNA preparation and analysis have been described previously (Gottschling et al. 1990).

RNA was isolated from mid-log-phase cells, unless otherwise

indicated, as described previously (Gottschling et al. 1990). RNA hybridization analyses were performed as described previously (Gottschling et al. 1990), except that 15 or 20 μ g of total RNA was denatured in the presence of 20 μ g/ml of ethidium bromide and separated by electrophoresis on a 1.2% agarose–5% formaldehyde (37% stock)–MOPS gel. Immediately following electrophoresis the gel was photographed and washed twice for 15 min in H₂O, 15 min in 10 $\times$ SSC and transferred to nylon (Micron Separations, Inc., Westboro, MA). Photography of the gel following transfer verified that complete transfer of the rRNA had occurred. RNA was immobilized on the nylon membrane by UV irradiation (120 mJ) of the damp membrane, followed by prehybridization of the membrane. Prehybridization and hybridization solutions contained 5 $\times$ SSC, 50% formamide, 5 $\times$ Denhardt's solution, 0.2 mg/ml of denatured and degraded herring sperm DNA, 0.2% SDS; hybridization solution also contained 10% dextran sulfate, and was filtered through a 45- μ m membrane to remove particulates. Prehybridization [1–6 hr] and hybridization (18–30 hr) were carried out at 42°C for DNA probes and 53°C for RNA probes. Blots were washed 5 min at 23°C in 2 $\times$ SSC, 0.1% SDS, followed by two 15-min washes at 55°C in 0.1 $\times$ SSC, 0.1% SDS for DNA probes, or three 20 min washes at 60°C in 0.1 $\times$ SSC, 0.1% SDS for RNA probes, and exposed to film. The relative levels of *URA3* and *URA1* RNAs were quantified on a Radioanalytic Imaging System (Ambis, San Diego, CA). For rehybridization experiments, probes were removed from the blots with three 20-min washes with boiling 0.2% SDS.

RNA antisense probes were labeled with [³²P]CTP or [³²P]UTP (3000 Ci/mmol) by in vitro transcription of linearized plasmids with T7 RNA polymerase or SP6 RNA polymerase (Sambrook et al. 1989). DNA probes were labeled with [³²P]dCTP (3000 Ci/mmol) by random oligonucleotide priming as described (Sambrook et al. 1989). Plasmid p5'URA3 (T7) was the template for the *URA3* RNA probe. Plasmid pPL9 (SP6) was the template for the *ACT1* RNA probe. The *URA3* DNA probe was a 1.1-kb *Hind*III fragment containing the entire coding sequence, the *URA1* probe was a 1.3-kb *Bam*HI fragment containing the entire *URA1* gene in plasmid pVZURA1, the *SWI5* probe was a 3.3-kb *Hind*III fragment from pBD57, and the *CLN2* probe was a 640-bp *Hind*III–*Spe*I fragment in pJM206.

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