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Effect of Replicative Age on Transcriptional Silencing Near Telomeres in *Saccharomyces cerevisiae*

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Individual yeasts have a finite replicative life span in similarity to normal human fibroblasts. Telomere loss is a hallmark of replicative senescence in normal human fibroblasts and has been proposed to play a role in cellular senescence, perhaps by affecting subtelomeric genes. While telomere loss does not occur with replicative age in yeast, subtelomeric genes are subject to transcriptional silencing. It is possible that components of the silencing machinery other than telomeres change with replicative age and that these changes then lead to alterations in gene expression that contribute to aging. In an initial test of this possibility, we have examined the silencing of the *URA3* gene at two different telomeres as a function of yeast replicative age. Silencing declined rapidly and significantly at one telomere consistent with the involvement of silencing in aging, but it remained in comparison nearly constant at the other. These changes in silencing raise the possibility that the transcriptional status of genes in the subtelomeric region may be important for the senescence of both dividing cells and postmitotic cells, in which telomeres remain constant in length.

A yeast cell can divide only a limited number of times before it dies, but the daughters that it produces at each division have a full replicative life span (1,2). Progress through this life span is accompanied by many morphological and physiological changes (3,4). Thus, it is possible to characterize yeast longevity as an aging process. Among these age changes are an increase in size and the accumulation of bud scars, but these do not impose a limit to the life span encountered by yeasts (3,4). We initiated the genetic analysis of yeast aging several years ago. We found that the activity of certain genes is altered as a function of replicative age (5), and some of these genes play a role in determining yeast longevity (6,7). Normal human fibroblasts in tissue culture are also limited in their proliferative capacity, but in contrast to yeast, in which there is asymmetry between a cell and its progeny in terms of replicative potential, the entire fibroblast culture senesces and stops growing (reviewed in 8). Studies using human diploid fibroblasts suggest that replicative senescence in mammalian cells, and the lack of asymmetry mentioned above, may be causally related to the gradual loss of telomere sequences (9–11). A genetic mutation which results in such a progressive erosion of telomeres in yeast leads to the demise of the entire culture (12), highlighting the importance of telomere maintenance in an immortal or stem cell population. In the yeast *Saccharomyces cerevisiae*, the normal aging process is not accompanied by telomere shortening (13). In this organism however, telomeric regions elicit a position effect so that genes inserted in or near telomere sequences are subject to transcriptional silencing (14,15). Transcriptional silencing of subtelomeric genes is particularly interesting because the epigenetic inheritance of different regulatory states of chromatin may serve as a molecular mechanism of aging that can explain both finite replicative life span and its asymmetric inheritance (3,4). To explore the possibility that telomeric silencing may play a role in yeast aging, we have initially examined whether there is any age-dependent change in the degree of transcriptional silencing of a subtelomeric gene.

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Abbreviations: FOA, 5-fluoroorotic acid; kb, kilobase(s).

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MATERIALS AND METHODS

Strains. UCC81, UCC513, UCC519, and UCC521 were previously constructed to study telomeric silencing in yeast and were provided by D. Gottschling (14,15). UCC513, UCC519, and UCC521 are *MATα ade2 his3 leu2 lys2 trp1 ura3-52 ppr1*. UCC81 is *MATα lys2 his4 trp1 ade2 leu2 ura3-52*. UCC81 has the *URA3* gene immediately adjacent to the telomere sequence of the left arm of chromosome VII. UCC513, UCC519, and UCC521 have the *URA3* gene about 10.5 kb, 1 kb, and 2.5 kb, respectively, away from the telomere sequence of the right arm of chromosome V. The original *ura3* locus in all these strains is mutated by a Ty transposon insertion.

Cell sorting. 1 ml of stationary phase cell cultures was stained with 0.1 mg/ml of the fluorescent dye, Cellufluor (Polysciences, Warrington, PA). Cellufluor intercalates into the chitin of the bud scar. After 20 min staining, cells were sonicated at 60W for 30 sec, washed twice in water, and loaded into the FACS Vantage (Becton-Dickinson). The UV source was a Coherent Enterprise Laser with 251–364 nm emission and 6.0 mW output.

Age determination. Cells were stained with 0.1 mg/ml Cellufluor for 20 min, washed twice with water, and mounted on a Nikon Microphot FX epifluorescence microscope with the Nikon UV-2A filter cube (16). Fluorescence readings were performed on individual cells with 0 to 5 bud scars, using the Nikon P1 photometer. At least 10 different cells of the same age were measured. To estimate the number of bud scars on sorted cells, a linear regression equation relating fluorescence and the number of bud scars was obtained from unsorted stationary phase cells, as shown in the inset of Fig. 2A. Then, fluorescence intensity of individual sorted cells was measured as shown in Fig. 2A. These measurements were converted to the estimated bud scar numbers using the linear regression equation. The average replicative age of cells in each age group, estimated in this way, appears to match closely that obtained by measuring remaining life spans (Table 1), except for R2 and R3. The discrepancy between these two methods for these two groups is ascribed to the technical limitations associated with the fluorescence measurements (16). Remaining life spans were determined microscopically on 35 sorted cells from each age group by counting the number of daughters individual cells produced, as described previously (16). Daughters were removed consecutively by micromanipulation. The replicative age, expressed in generations, was obtained by subtracting the median life spans of the three older age groups from that of the virgin cells.

RESULTS AND DISCUSSION

Three yeast strains, in which a 1.1-kb DNA fragment containing the *URA3* gene with its promoter is inserted in the vicinity of the yeast 5′-C_{1-3}A-3′ telomeric repeats (Fig. 1A), were used in this experiment. It has been shown previously that the subtelomeric *URA3* gene in yeast strains UCC519, UCC 521, and UCC81 is subject to telomeric transcriptional silencing (14,15). It has also been shown that size and the number of bud scars, which incrementally mark the mother cell at each division, increase as yeast cells progress through the replicative life span (16). Therefore, cells from stationary cultures were stained with Cellufluor, and sorted according to size and fluorescence, using a fluorescence-activated cell sorter, into four groups of increasing age, R1 to R4 (Fig. 1C). The average age of sorted cells in each group was estimated by restaining them with Cellufluor and examining them under a fluorescence microscope to count the number of bud scars on individual cells. This method applied well to the virgin and young cells, because these cells contain fewer than 5 bud scars, and it showed that more than 99% of the R1 cells were in fact virgin cells (never budded). However, the accuracy of counting bud scars on cells decreases as the number of bud scars increases. Therefore, a linear regression equation relating the number of bud scars and the intensity of fluorescence was derived for each strain using unsorted stained cells with fewer than 6 bud scars, and the bud scar numbers were estimated from this equation for the R4 cells (Fig. 2A, Table 1). This showed that this fraction consisted of cells containing 14, 7, and 14 bud scars on average for strains UCC519, UCC81, and UCC521, respectively. In order to confirm the average age of sorted cells, their remaining life spans were determined (Fig. 2B, Table 1). The replicative ages of the oldest cells obtained using this method were in close agreement with those determined with the first procedure (Table 1), as shown previously (16).

To determine the frequency of transcriptionally silent Ura^- cells in each age group, equal numbers of sorted cells were simultaneously plated on a complete agar medium and a complete agar medium containing FOA, and the number of colonies growing on each medium was counted. FOA is used to select for Ura^- cells because of its toxicity to Ura^+ cells (17). Therefore, the relative frequency of FOA-resistant cells is a measure of the degree of silencing exerted on the subtelomeric *URA3* gene. Our data show that the frequency of FOA-resistant cells decreased with age in

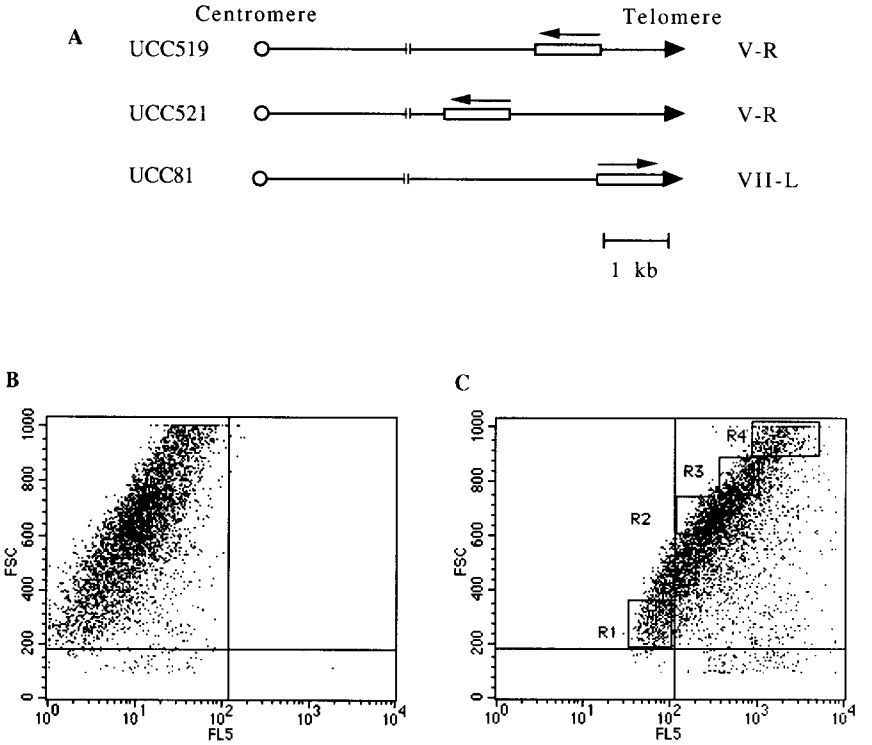

FIG. 1. (A) Representation of parts of the chromosomes in which the *URA3* gene (open rectangles) in a 1.1-kb *Hin*dIII DNA fragment is located in the strains used in this study. The arrows above the rectangles indicate the direction of transcription. The open circles and the dark arrow heads represent centromeres and telomeric repeats, respectively. **(B, C)** Distribution of individual unstained **(B)** and stained **(C)** cells of UCC519 (shown as an example) displayed by the FACS according to their cell size (y axis in linear scale) and fluorescence intensity (x axis in logarithmic scale). Approximately 5×10^4 cells were collected for subsequent experiments from the cells enclosed by each of the boxes labeled R1, R2, R3, and R4.

UCC519 (Fig. 3A) and UCC521 (Fig. 3B). Thus, there was an age-dependent decline in silencing of *URA3* at two sites, 1.0 and 2.4 kb from the telomere on chromosome V-R. Note that the strength of *URA3* silencing was greater nearer the telomere (compare Fig. 3A with 3B), as expected from previous studies (14,15). This gradient of transcriptional silencing, and previous studies that establish the nature of the silencing of *URA3* at this subtelomeric location (14,15), indicate that we are dealing with a decline in telomeric silencing on chromosome V-R during aging in yeast. Since the level of transcriptional silencing at both sites declined coordinately during the yeast life span, these data argue that the telomeric silencing gradient or "cone" on this chromosome is maintained but progressively extinguished with replicative age.

Because of the availability of a yeast strain (UCC81) with the *URA3* gene directly adjacent to the telomere on chromosome VII-L, we have also determined the degree of silencing at this site during the yeast life span (Fig. 3C). In contrast to the situation at the subtelomeric region of chromosome V-R, there was little or no change with age in the expression of *URA3* found at this site. This result indicates that general transcription factors that act on the *URA3* promoter are not the source of the change seen with age at the telomere of chromosome V-R. However, this result raises the question of why *URA3* expression at the telomere of chromosome VII-L does not decline as it does at the two subtelomeric sites of chromosome V-R. Virtually no change was seen at VII-L even when the loss of silencing at V-R was approximately 50%, and the loss of silencing at V-R began after the first generation (Fig. 3A and B). It could be that the position of *URA3* directly

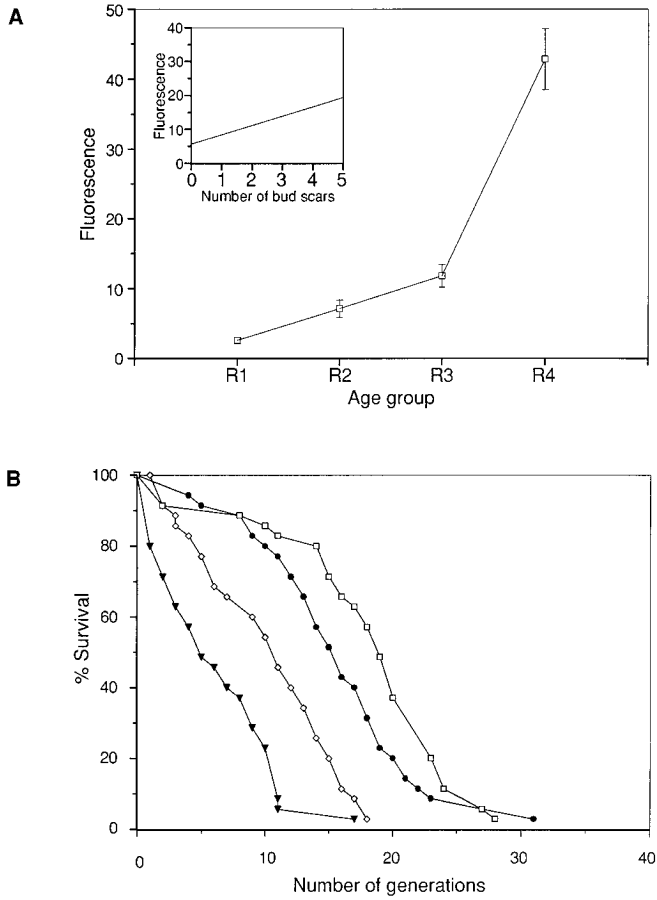

FIG. 2. Determination of replicative ages. **(A)** Fluorescence measurements of 10 individual cells in each age group of UCC519, shown as an example. The plot shows the means and standard errors. The inset shows the linear regression relating measurements of fluorescence intensity and bud scar number of unsorted UCC519 cells. **(B)** Survival curves of the four age groups of sorted UCC519 cells. The number of buds that individual cells generated during their remaining life span was plotted against the percentage of live cells at each generation. The open squares are for R1 (virgin) cells (median life span, 18), closed circles for R2 (median remaining life span, 15), open diamonds for R3 (median remaining life span, 10), and closed triangles for R4 (median remaining life span, 4). These remaining life spans correspond to replicative ages of 3, 8, and 14 generations for R2, R3, and R4.

TABLE 1
Determination of Replicative Age of Sorted Cells in Individual Age Groups

		Replicative age of sorted cells (SE)	
Strain	Method	R1 (virgin)	R4 (old)
UCC81	Bud scar number	0.6 (±0.2)	7.0 (±0.8)
	Remaining life span	0	7
UCC519	Bud scar number	0.0 (±0.0)	13.5 (±1.6)
	Remaining life span	0	14
UCC521	Bud scar number	0.0 (±0.0)	13.9 (±2.0)
	Remaining life span	0	11

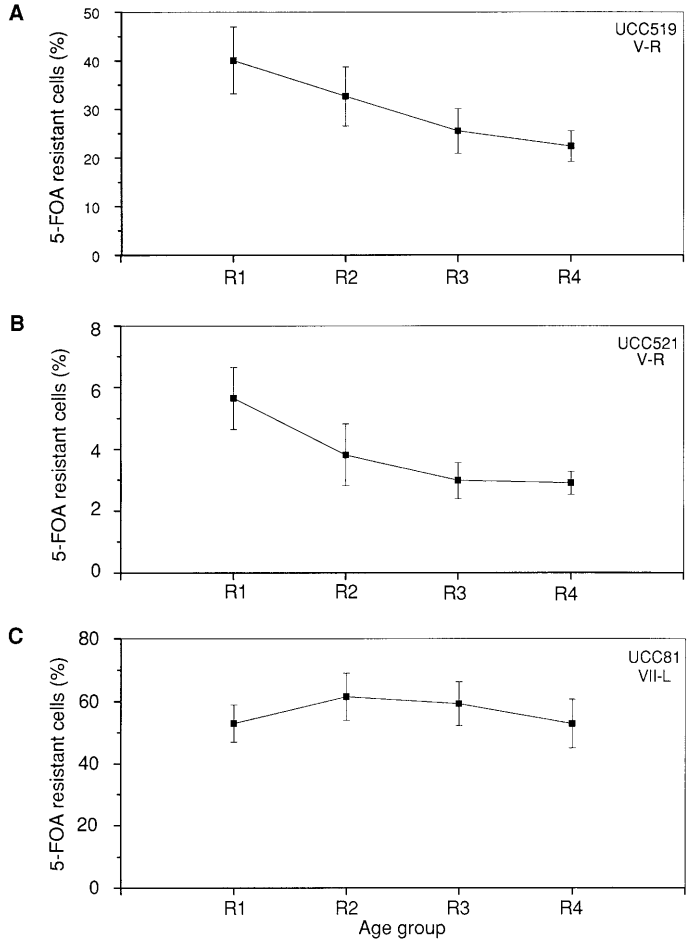

FIG. 3. Change in the proportion of FOA-resistant cells with replicative age. The abscissa and the ordinate denote respectively age group and relative frequency of colony-forming units on FOA plates compared to plates containing uracil but without FOA. Decreased viability on 5-FOA indicates loss of silencing. The means of several (n) independent experiments with the SE are plotted. Two-tailed t-tests (paired) were used to determine the significance (p) of the differences between the age groups. **(A)** UCC519 (n = 9). R1 vs. R2 ($p = 0.047$), R1 vs. R3 ($p = 0.002$), R1 vs. R4 ($p = 0.005$), R2 vs. R3 ($p = 0.036$), and R2 vs. R4 ($p = 0.019$) are significant, while R3 vs. R4 is not ($p = 0.266$). **(B)** UCC521 (n = 5). Differences between R1 and R3 and R1 and R4 are significant (R1 vs. R2, $p = 0.066$; R1 vs. R3, $p = 0.048$; R1 vs. R4, $p = 0.047$; R2 vs. R3, $p = 0.277$; R2 vs. R4, $p = 0.388$; R3 vs. R4, $p = 0.862$). **(C)** UCC81 (n = 6). Differences between R1 and R2 ($p = 0.041$) and between R1 and R3 ($p = 0.043$) are significant, while other combinations are not (R1 vs. R4, $p = 0.980$; R2 vs. R3, $p = 0.423$; R2 vs. R4, $p = 0.106$; R3 vs. R4, $p = 0.056$).

adjacent to the telomere on chromosome VII-L is the reason for the stable silencing, such that the high degree of silencing of genes this close to the telomere is little affected by age. Alternatively, the telomeric transcriptional silencing may not decline with replicative age on all yeast chromosomes in the same fashion.

As indicated in Fig. 1A, UCC81 and UCC519 differ in the distance of the *URA3* gene from the telomere and its orientation, and by the presence of a mutation in UCC519 (*ppr1*) which reduces somewhat the transcription of *URA3*. These differences appear to have little, if any, discernible effect on the silencing of *URA3* in virgin cells in these strains. Any difference in the degree of silencing exerted on *URA3* on chromosome V-R and VII-L in UCC519 and UCC81 is likely balanced by the presence of *ppr1* in UCC519 and by the reverse orientation of *URA3* that places

its promoter at about the same distance from the telomere in both strains. Indeed, it has been shown that it is specifically the distance of the promoter of the gene that determines the degree of telomeric silencing (14,15). The results shown here with the *URA3* marker and selection on FOA were confirmed by plating on uracil-lacking medium (data not shown). The lack of any distinct age changes in telomeric silencing on chromosome VII-L as compared to V-R was confirmed using the *ADE2* marker (data not shown). We have also examined the yeast strain UCC513, in which the *URA3* gene is located 10.5 kb from the chromosome V-R telomere, as a function of replicative age. On FOA-containing medium the plating efficiency was $\leqslant 10^{-6}$, indicating high levels of expression and providing an estimate of the sensitivity of the assay. The high and constant levels of *URA3* expression in this strain were confirmed on uracil-lacking plates, indicating that the silencing on chromosome V-R does not reach 10 kb in from the telomere. This provides support for the conclusion that the changes in expression found at sites close to this telomere are due to telomeric silencing and not simply to promoter-specific factors. Thus, there is no indication of age change in the transcription of the *URA3* gene at sites far away from the telomere.

The fact that the *ura3-52* mutation that is present in the background of all of the *URA3* strains does not contribute to the results obtained was ascertained by the fact that the control strain plated at high and constant efficiency on FOA throughout its life span. The strains UCC81, UCC519, and UCC521 lack the X and Y′ subtelomeric repeats on chromosomes V-R and VII-L (14,15). This renders the extension of our conclusions to chromosomes V-R and VII-L in other strains difficult, but it does not alter the significance of the decrease in silencing at V-R and its comparative absence at VII-L. It should be noted that all these strains age normally (Fig. 2B), displaying an exponential increase in mortality with age.

Our studies open the prospect of a causal link between the age-dependent change in silencing at certain telomeres and yeast aging. Some evidence could support such a relationship. The involvement of *CDC7* has been implicated in yeast longevity (18). The *CDC7* gene, which is required for initiation of DNA replication, is also involved in transcriptional silencing at the silent mating type locus, *HMR* (19). Recently, the *SIR4* gene, known for its role in silencing (20), has also been implicated in yeast aging (21). The involvement of *CDC7* and *SIR4* in yeast life span suggests that there might be a connection between attenuated telomeric silencing and aging in yeast. One can imagine that dysregulated expression of certain genes caused by decreased telomeric silencing might precipitate yeast aging. One could also entertain the notion of a genetic program put into play, although no evidence exists for such an alternative.

In sum, our data indicate that the transcriptional status of subtelomeric genes can change with age in yeast. This occurs despite the lack of telomere attrition in this organism. These results raise the possibility that in other organisms a similar change in telomeric transcriptional silencing without attendant loss of telomeric sequences may pertain. This would afford the possibility to extend the telomere hypothesis of aging (9–11) to postmitotic cells, such as cardiac myocytes and neurons, in which telomere loss is not expected to occur.

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