Telomere Length Constancy during Aging of
*Saccharomyces cerevisiae*

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It has been proposed that a decrease in the length of telomeres with the successive rounds of DNA replication that accompany mitotic division could play a causal role in the aging process. To investigate this possibility, telomeres from cells of the budding yeast *Saccharomyces cerevisiae* that varied in replicative age were examined. No change in the lengths of the telomeres was observed in cells that had completed up to 83% of the mean life span. We conclude that the length of the telomeres is not a contributing factor in the natural aging process in individual yeast cells.

Eukaryotic chromosomes terminate in specialized structures called telomeres that serve a dual function. They stabilize the chromosome and sustain the ends of the chromosome by facilitating complete replication (34). Although the specific telomere sequence pattern is unique for an organism, the strand oriented 5' to 3' toward the end of the chromosome is always G rich. Experiments have shown that the telomere sequences obtained from diverse organisms, ranging from protozoans to humans, act as telomeres in yeast cells (3, 7, 25), suggesting that telomere function is highly conserved.

Recent studies have elucidated the role of the repeats present in the telomere, and several models (2) have been proposed concerning the interaction of these repeats with telomerase, the enzyme that adds the repeating units to the telomeres. However, very little is known of the overall size and stability of telomeres during development and aging. We have examined the lengths of telomeres in aging yeast cells as part of our studies on the aging process of the budding yeast *Saccharomyces cerevisiae*.

The life span of yeast cells is finite, since the cells exhibit a limited replicative capacity (22). The measure of the life span in yeast cells is not chronological age but the number of times the cell divides (23). Several of the characteristics that accompany aging in yeast cells reflect those exhibited by normal human diploid fibroblasts as they age in culture (14). Fibroblasts also display a finite replicative capacity and arrest at the G1/S boundary of the cell cycle when they reach the maximum population-doubling level (14). It has been proposed that shortening of telomeres occurs with successive rounds of DNA replication during somatic differentiation (6). Such shortening has been shown to occur in normal human diploid fibroblasts in culture as cumulative population-doubling levels increase (12). However, in vivo studies in mice, which exhibit hypervariable ultralong telomeres, have shown no significant shortening of telomeres during development and aging of the animal (15). In colorectal carcinomas, telomeres decrease in length on average compared with telomeres in normal colonic mucosa obtained from the same source (13). The change in telomere length, whether increase or decrease, appears clonal in nature. We undertook this study to ascertain whether telomeres shorten during the life span of a unicellular organism and whether such a change might be a determinant of the cell’s limited capacity to proliferate.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *S. cerevisiae* X2180-1A (MATa SUC2 mal mel gal2 CUP1) was obtained from the Yeast Genetic Stock Center, Berkeley, Calif. Cells were cultured in YPD medium (2% peptone, 1% yeast extract, 2% glucose), YPDG medium (2% peptone, 1% yeast extract, 0.04% glucose, 1.6% glycerol), or YM1' medium (0.67% yeast nitrogen base; 1% succinic acid; 0.6% NaOH; 1% glucose; 0.001% adenine sulfate; 0.001% uracil; 0.004% each of tyrosine, lysine, histidine, and leucine [pH 5.8]) on a rotary shaker at 30°C.

**Life span determination.** Life span was determined microscopically with a 40× long-working-distance objective on a Nikon Labophot microscope as described before (9). Cells were streaked along one edge of an agar slab (YPD medium with 1.5% agar), and buds were removed from mother cells by micromanipulation and deposited at isolated spots on the slab. The agar slab was incubated at 30°C. The cells were observed periodically, and buds were removed and discarded by micromanipulation. With each budding or cell division, these cells were considered one generation older. A cell was considered to have completed its replicative life span if no cell division was observed for several days. Such cells frequently lysed.

**Preparation of age-synchronized yeast cells.** Age-synchronized yeast cells were prepared according to Eglmez et al. (9). A 100-μl inoculum from a logarithmic-phase culture was used to initiate a culture in 100 ml of YPD medium. This culture was incubated at 30°C for 4 days to ensure that the cell concentration was >4 × 10⁶/ml. At stationary phase, about 50% of the cells are virgins (small, unbudded cells that have never divided), while the rest are large, unbudded cells whose number decreases exponentially as their age (number of divisions undergone) increases. To obtain the virgin cells, the culture was sonicated and subjected to rate-zonal sedimentation in 20 sucrose gradients (10 to 30%). Of the two bands in the gradient, the upper band, containing the virgin cells, was retained. By this procedure, 10¹⁰ virgin cells were obtained. A sample of 10⁸ virgin cells was saved for extraction of DNA. After being washed, the cells were suspended

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in YPD medium with 400 nM α-factor to synchronize them. (This was the only step at which α-factor was used.) They were washed with water to remove the α-factor and suspended in YPDG medium at 4 x 10^7 cells per ml. The culture was incubated at 30°C until growth arrest in stationary phase at 1.2 x 10^8 cells per ml. The culture thus contained the original virgin cells (mother cells) that had budded twice, which we call G2 or two-generation-old cells, and the resultant buds (daughters) of these cells (two from each cell). Because of the increase in size of the mother cells with each division, they could be separated from their daughters by rate-zonal sedimentation. Two bands were observed on the gradients. The lower band, representing the G2 cells, was collected. A sample containing 10^8 of the G2 cells was saved.

The rest of the cells were suspended in YPDG medium at 2.5 x 10^7/ml. They were cultured at 30°C for three generations until the cell number had increased fivefold at stationary phase (1.2 x 10^9 cells per ml). In this step, the mother cells budded three times, and their first buds budded once. This pattern of division was dictated by the fact that daughter cells must grow to a certain size before they can divide (27). Because of the increasing size of the mother cells, three bands were evident after sedimentation in sucrose gradients. The lowest band, which contained the cells of interest, now five generations old (G5), was fractionated. From this, a sample containing 10^9 cells was stored for extraction of DNA. The five-generation-old cells were resuspended in YPDG medium at a concentration of 2.5 x 10^7/ml and cultured so that the cell number would increase fivefold. The G5 cells were eight generations old at this step. The rate-zonal sedimentation of these and subsequent cultures produced four bands in the gradients, because the second buds produced by the mother cells formed a separate band. The rate-zonal sedimentation and growth in YPDG medium was reiterated until 11-, 14-, 17-, and 20-generation-old cells were obtained. The final yield was 3.25 x 10^8 20-generation-old (G20) cells. At each step of the procedure, the percentage of daughter cells containing the age-synchronized mother cells was determined and found to be no more than 2.5% (standard deviation = 1.1%). This determination was done by microscopically scoring the large, aged mother cells and the small daughter cells in the preparations. The procedure has no detrimental effect on the aging characteristics of the cells (9). Moreover, the colony-forming ability of the cells obtained remains constant and high (approximately 80%) throughout the procedure (9). The accuracy of the protocol has been determined by examining several characteristics such as bud scars and increase in cell size and by determination of the remaining life spans of preparations of age-synchronized cells of different nominal ages (9).

Determination of telomere length. DNA was extracted from the cells by a Zymolyase-sodium dodecyl sulfate (SDS) method (1). Briefly, the yeast cells were treated with Zymolyase to produce spheroplasts. The spheroplasts were lysed with SDS. SDS, proteins, and cellular debris were precipitated with potassium acetate, and the DNA was recovered from the supernatant. DNA (2 μg) from each sample was digested with the restriction enzyme XhoI (BRL Life Technologies, Inc.) according to instructions supplied by the manufacturer, and electrophoresis was performed on an 0.8% agarose gel in Tris-borate buffer. The DNA was transferred to nitrocellulose by utilizing the Posiblot pressure blotter (Stratagene Cloning Systems). The probe, poly(dA-dC)·poly(dG-dT) (Pharmacia LKB Bio-Technology AB), was labeled with [α-32P]dATP (3,000 Ci/mmol) by extending it with the Klenow fragment of DNA polymerase I (BRL Life Technologies) as recommended by the supplier. Prehybridization was done for 4 h, and hybridization was done for 16 h at 55°C in 6x SSPE (1x SSPE is 0.18 M NaCl, 10 mM sodium phosphate [pH 7.7], and 1 mM EDTA) with 5x Denhardt's solution (1x Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin). The membrane was washed twice for 20 min per wash with 0.1x SSC (1x SSC is 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature and subjected to autoradiography.

Analysis of chromatid segregation. Procedures for obtaining age-synchronized cells were essentially as described above, except that the cells were labeled for 10 generations in YM1' medium supplemented with [2-14C]uracil at 1 μCi/ml prior to preparation of virgin cells. After being washed, the cells were allowed to complete one division at each step compared with increments of two to three generations in the original protocol (9) in unlabeled medium. Samples of the cells (1 x 10^7 to 5 x 10^7) were withdrawn in triplicate and subjected to alkaline hydrolysis. The volume for each sample was adjusted to 1 ml with water, and 50 μl of calf thymus DNA (2 mg/ml) was added as carrier. After sodium hydroxide was added to 1 N, the samples were incubated overnight at 37°C. HCl was added to 1 N to neutralize the samples.) The DNA was precipitated by adding 1 ml of 30% trichloroacetic acid–0.1 M sodium pyrophosphate and storing the solution on ice for 15 min. The precipitates were collected by filtration, washed with 1 N HCl–0.1 M sodium pyrophosphate and then with ethanol, and counted in a scintillation counter.

RESULTS AND DISCUSSION

Telomere length in age-synchronized yeast cells. S. cerevisiae X2180-1A was chosen for the analysis of telomere length during aging of individual yeast cells. The rate of death increases exponentially with the age of a yeast population. At 20 generations in this particular strain, the cells had completed 83% of their mean life span (Fig. 1), and approximately 25% of the cells had already died. In a few more generations, the population reached the median life span (50% survival). Following repeated cycles of synchronous growth alternating with fractionation on sucrose gradients, yeast cells from 0 through 20 generations of age were obtained. The age-synchronized yeast preparations used for
this analysis contained cells at various points in their individual life spans but few if any dead cells (9). DNA was extracted from the cells and digested with the restriction enzyme XhoI. After electrophoretic separation on an 0.8% agarose gel, the DNA was transferred to nitrocellulose and probed with a labeled copolymer, poly(dA-dC)·poly(dG-dT), to detect the telomere repeats (32). This probe hybridizes to the dC13-dA repeats characteristic of yeast telomeric ends as well as to internal (dC-dA) repeats, which appear as high-molecular-weight bands (5, 31). The broad band indicated in Fig. 2 represents the XY′ class of telomeres. The XY′ telomeres cap one or both ends of the majority of the chromosomes in this strain, as deduced from the following. X2180-1A is the MATα sporulation product of a spontaneous diploid that arose in strain S288C. Strain AB972 is an ethidium bromide-induced rho0 product of the MATα sporulation derivative X2180-1B-trp10Δ of the same S288C diploid. Both X2180-1A and AB972 trace directly to S288C with no intervening outcrosses (21). Twelve of the 16 chromosomes in AB972 possess one or two XY′ telomeres (16). The average size of the XY′ telomeres is 1.3 kb, making changes in their lengths particularly easy to detect. Telomeres that lack the Y′ element belong to the X telomere category and produce several bands of higher molecular weights (5, 31). The patterns of migration of the bands on all lanes indicate that there was no detectable change in the lengths of the telomeres as the yeast cells aged. The same results were obtained with four different sets of age-synchronized cells. Thus, no gradual loss of telomeric DNA repeats like that detected in a similar assay of normal human diploid fibroblasts aging in tissue culture was observed (12). Telomere shortening in yeast cells can be readily detected with these procedures (17). It is possible that a catastrophic loss of telomere repeats that would not be detected in these experiments might occur just prior to cell death.

Chromatid segregation during successive cell divisions. One way in which telomere length could remain constant in aging yeast cells would be for chromatids of the same replicative age always to cosegregate to the mother cell instead of to the bud or daughter cell during mitosis. (This, of course, might pose problems for the daughter cell.) It had been concluded that cosegregation of chromatids of similar replicative ages occurs in yeast cells, but no evidence for any preference for mother or daughter cell was presented (33). In order to address this question, virgin cells with uniformly labeled DNA were passaged one generation at a time for a total of four generations. There was no significant bias reproducibly detected in the distribution of the labeled DNA among the progeny, as shown in Fig. 3. In view of the fact that chromatids of similar replicative ages appeared to cosegregate in yeast cells (33), we conclude that these chromatids show little or no consistent preference for the mother or the daughter cell. Thus, the factors that balance telomere shortening and lengthening must operate in both the mother and the daughter cells, and no mechanisms peculiar to either need be postulated to explain telomere length constancy and the aging patterns of mother and daughter cells. This would not be the case if preferential segregation to either mother or daughter cells was occurring. It is worth noting that no changes in telomere length were observed in daughters derived from cells 17 generations old (Fig. 2). After this study was completed, a more-exhaustive analysis (24) clearly demonstrated lack of cosegregation of chromatids of similar replicative ages in yeast cells.

We conclude, therefore, that the lengths of the telomeres do not normally limit the proliferative capacity of individual yeast cells. During aging, the telomeres were stable and were maintained at a constant length by the balance of activities (29) that lengthen or shorten the telomeres. The lengthening process is due either to recombination (26) or to the activity of telomerase (10, 11, 30). During culture of yeast cells, clonal variations in telomere length do occur (29). Yeast cultures are immortal, which is the case for populations of
other organisms and in contrast to the mortality of the individual. However, 97% of the individual yeast cells in such cultures are less than five generations old. Our findings indicate that telomere loss does not occur in individual, mortal yeast cells of much greater age, a fact that would be difficult to glean from studies of exponential cultures. The decrease in telomere length observed in senescent normal human diploid fibroblasts could be due to the inability of cells to perform in vitro a metabolic process that effects the maintenance of telomeres in vivo (15). In addition, interclonal variability in telomere length could well occur, as it does for DNA methylation (28).

A paradoxical situation exists in the case of the observed shortening of telomeres that has been demonstrated in immortal tumor cells (8). The decrease in the lengths of telomeres has been attributed to the number of divisions the cell has undergone. However, it is assumed that normal cells lack telomerase activity, while transformed cells, such as HeLa cells, are known to possess such activity (20). Therefore, during the transformation of the normal cell, an induction or increase in telomerase activity has been postulated (13). We surmise, then, that tumor cells exhibit telomeres whose lengths reflect the point at which the normal cell was transformed. Hence, these telomeres should be either shorter or longer than telomeres in normal cells. In most cases examined by Hastie et al. (13), a decrease in telomere length was observed in the transformed cells. An alternative explanation offered is that cells with shorter telomeres were selected, since they had a growth advantage over those with longer telomeres (13). This argues against the dynamic-equilibrium model, i.e., a balance between activities that lengthen and shorten the telomere that has been shown to pertain to yeast cells and that is assumed to operate on the replicative descendants of cells (29). According to this model, instead of a decrease in the length of telomeres during clonal expansion, a broader size distribution is predicted.

We propose that during the life span of an organism, telomere shortening does not play a role in the normal aging process. However, mutations or epigenetic changes that affect the activity of the telomerase, like any other genetic change, might affect the lifespan of the individuals in which they occur. In yeast cells, several genes whose mutations affect telomere length have been identified: RAP1, CDC17, TEL1, TEL2 (4, 18, 19), and, most recently, EST1 (17). As expected, est1 mutants exhibit clonal senescence, in which the entire population dies, a feature distinct from the normal aging process in yeast cells. It is not likely that telomerase activity decreases with age, because as we have shown, no shortening of telomeres occurs in old yeast cells.

In summary, the telomere shortening with age observed in human diploid fibroblasts may not be a universal phenomenon. Further studies are required to examine telomere length and telomerase activity not only in different cell types as they age but also in the same cell type in different organisms with differing life spans. This would indicate whether telomere shortening plays a causal role in the senescence of a particular cell type or organism.

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