Evidence for the Involvement of a Cytoplasmic Factor in the Aging of the Yeast Saccharomyces cerevisiae

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The life spans of individual Saccharomyces cerevisiae cells were determined microscopically by counting the number of buds produced by each cell to provide a measure of the number of cell generations (age) before death. As the cells aged, their generation times increased five- to sixfold. The generation times of daughter cells were virtually identical to those of their mothers throughout the life spans of the mothers. However, within two to three cell divisions after the daughters were detached from their mothers by micromanipulation, their generation times reverted to that characteristic of their own age. Recovery from the mother cell effect was also observed when the daughters were left attached to their mothers. The results suggest that senescence, as manifested by the increase in generation time, is a phenotypically dominant feature in yeast cells and that it is determined by a diffusible cytoplasmic factor(s) that undergoes turnover. This factor(s) appeared to be transmitted by a cell not only to its daughter, but also indirectly to its granddaughter. In separate studies, it was determined that the induced deposition of chitin, the major component of the bud scar, in the yeast cell wall had no appreciable effect on life span. We raise the possibility that the cytoplasmic factor(s) that appears to mediate the "senescent phenotype" is a major determinant of yeast life span. This factor(s) may be the product of age-specific gene expression.

The budding yeast Saccharomyces cerevisiae possesses a limited life span (6). Yeast cells produce a finite number of daughters during their life spans, which are elaborated on the surface of mother cells as buds. The number of buds produced by the mother, one during each cell division cycle, is the metric of the life span. Chronological age does not play a role (9). Yeast aging can be described by the Gompertz equation (15). Morphological and physiological changes accompany the aging process. These include an increase in cell size (6), an increase in generation time one to three divisions before cell death (6), and a decrease in the ability to mate (8). Individual cells display a wide range of life spans. The mean and the maximum life spans for a given yeast strain are characteristic for that strain, but they vary from one strain to another (4, 6-9). As yeast cells age, they accumulate bud scars on their surface due to bud abscission. These bud scars are the product of the deposition of the polysaccharide chitin in the cell wall. It has been suggested that the accumulation of bud scars on the yeast cell surface may result in its senescence (4, 6).

Yeast cells appear to be an excellent experimental system for the study of aging. Besides ease of cultivation, yeast cells possess the advantage that they are well suited to genetic manipulation, both classical and molecular (19). In addition, life span studies can be readily carried out on individual cells and pedigrees can be constructed, due to the asymmetric mode of cell division. However, nothing is known about the molecular determinants of life span in yeasts. Before attempting a genetic and molecular dissection of the aging process, it is important to examine the nature of the determinants of life span in these organisms.

In this communication, evidence is presented that the senescent phenotype prevails in yeast cells and that it is determined by a diffusible cytoplasmic factor(s) that undergoes turnover. We also add data to the body of evidence contradicting the idea that accumulation of bud scars may be a major determinant of life span in yeast cells.

MATERIALS AND METHODS

Strains and growth conditions. S. cerevisiae X2180-1A (a SUC2 mal mel gal2 CUP1), 124 (a ade1 ade2 ural his7 bys2 tyr1 gall cdc7-1), here called cdc7, and 182-6-3 (a ural tyr1 arg4 thr4 ade1 ade2 gall his cdc24-1), here called cdc24, were all from the Yeast Genetic Stock Center, Berkeley, Calif. Strains were cultured in 2% peptone–1% yeast extract–2% glucose (Difco Laboratories, Detroit, Mich.). Solid medium contained 1.5% agar (Difco). For cdc7 and cdc24, the medium was supplemented with 0.003% adenine sulfate. Liquid cultures were grown on a rotary shaker. Cells were cultured at 30°C, or at 22°C in the case of the cdc mutants.

Life span determinations. Life span determinations were carried out essentially as described before (6). A Nikon Labophot microscope with a ×40 long-working-distance objective and a micromanipulator attachment (17) was used. A thin slab of solid medium about 0.5 by 2 in. (1.27 by 5.08 cm) was cut and placed on a glass slide (1 by 3 in. [2.54 by 7.62 cm]). A sample of mid-logarithmic-phase yeast cells was streaked along one edge of the slab with an inoculating loop, and the slide was placed upside down on a chamber (1 by 3 in.) (17). Mature buds were removed from budding cells and deposited in isolated positions on the slab with the aid of the micromanipulator. These virgin cells that had never budded before were used to initiate the experiment. The cells located at the designated spots on the slab were examined hourly or every 30 min when necessary. The position and size of buds relative to the mother cells were recorded. (This facilitated the determination of generation times). Buds either were removed from these cells immediately at maturity by micromanipulation or were allowed to remain attached for a few cell generations before removal.

In several experiments, generation times (periods between the appearance of the first and second consecutive buds on a
given cell) were determined for the cells and also for their progeny. The measurements were carried out on 6 to 19 cells for each experimental point, except for the 36-generation points in Fig. 2 in which two cells each were analyzed. Cells removed by micromanipulation were deposited at least 0.5 cm away from the cells whose life spans were being determined, except when their generation times were measured. In the latter case, they were deposited 2 mm away from the mother cell to maintain their identity. (Granddaughter and great-granddaughter cells, when they were separated from their mothers and analyzed, were placed in an array 2 mm away from the original mother cell.) In each case in which buds were allowed to remain attached to the mother cells for a period of time, the pedigrees of the cells at the designated spots on the slab were unequivocal (10).

At night, the cells were kept at 5°C to slow growth. As shown previously, this treatment has no effect on the life span of S. cerevisiae (9). When generation times were measured after storing at 5°C, two generations were allowed to pass after returning cells to the elevated temperature before measurements were made, so that the cells would be dividing at the rate characteristic for that temperature. All microscopy associated with these experiments was carried out at room temperature, 5°C below the incubation temperature, for 20 cells at a time. These operations took 8 to 10 min, that is, no more than 15% of the generation time. In fact, we have determined that the effect on generation time amounts to no more than 2%, taking into account the generation times at room temperature and 30°C.

**Determination of chitin accumulation in cells.** Virgin cells were isolated from stationary-phase cultures. Approximately 10^8 cells were sonicated briefly (15 s, 80 W) and loaded onto 50-ml 10 to 30% linear sucrose gradients at 4°C. The gradients were centrifuged at 1,400 rpm for 4 min in an IEC Centra 8R swinging-bucket rotor (model 216). The faint upper band, which contained the small virgin cells, was removed. The cells were washed with distilled water twice and suspended in liquid medium at 10^7 cells per ml. These suspensions were transferred to a shaker at 36°C, and samples were withdrawn after incubation for various lengths of time.

The cells were stained with Cellulfluor (Polysciences), which is equivalent to Calcofluor (17). Fluorescence readings were taken from individual virgin cells. A Nikon Microphot FX epifluorescence microscope with a Nikon P1 photometer attachment was utilized for quantitation of cell surface fluorescence. Readings were taken at ×1,000 magnification with a UV-2A filter cube. The results are given in arbitrary fluorescence units. (Background fluorescence was adjusted to zero.)

**Statistics.** The t test was used for determining significance of differences in generation time and mean life span. The χ^2 test was used to evaluate significance of maximum life span differences. Comparisons were made between cell populations by selecting the life expectancy of 90% of the shorter-lived population as the criterion for longevity.

**RESULTS**

The senescent phenotype. Yeast cells display a limited life span in culture. An examination of population survival curves reveals that the mortality rate of this organism increases exponentially with age (15). The mean life span for S. cerevisiae X2180-1A, which was used in most of the experiments described in this paper, was 24 ± 9 (standard deviation) generations, and 50% survival was also 24 generations. The maximum life span was 39 generations. These parameters were established in life span determinations on 43 cells. For all of the strains we have examined, the mean life spans correlated with the maximum life spans (data not shown).

Yeast cells undergo a series of changes as they age. Among these changes was an increase in the generation time. To quantify this effect, the generation times of mother cells were recorded during the course of their life spans. At various corresponding points in the life spans of these mother cells, the generation times of their daughters were determined. The results of this experiment (Fig. 1A) clearly show that generation time increased with age, especially after 18 to 20 generations. Furthermore, the daughter cells displayed a very similar pattern of increase in generation time. For older mother cells, this resulted in growth of the daughter well beyond the usual size observed for young cells prior to budding (data not shown). Most important, the generation time of the daughters decreased dramatically (from 80 to 190 min) to a value characteristic of young cells (60 min), after they completed two to three cell cycles, a period of "recovery." In the case of the 26-generation-old mother cells, this recovery may not have been complete (to 80 min) after three cell cycles.

That a period of recovery was required is evidenced by the time course of recovery of daughter cells (Fig. 1B). Daughters detached from 24-generation-old mother cells required three cell divisions to resume dividing at the same rate as the daughters separated from 2-generation-old mother cells. Furthermore, this recovery occurred even when the daughters remained attached to their mothers. In the latter case, it was not possible to examine recovery beyond three divisions, because of the difficulty in distinguishing the original daughter from the other cells which now formed a microcolony. These results indicate the presence of a factor(s) elaborated by aging mother cells that can pass from these cells to their daughters. That daughter cells recover even when left attached to their mothers suggests that the factor(s) is cytoplasmic rather than extracellular.

The question arises whether the age-specific factor(s) postulated above can be transmitted through consecutive cell generations. This was examined by recording the generation times of the daughters, granddaughters (the daughters of the mothers), and great-granddaughters produced by individual mother cells of various ages (Fig. 2). As expected, the generation time of the mother cell increased with age. Immediately after separation at maturity by micromanipulation, the daughters and granddaughters displayed generation times similar to those of the mothers. However, the great-granddaughters showed a considerably shorter generation time, characteristic of young cells, regardless of the age of the mothers from which they were derived. The generation time of the great-granddaughters derived from the 36-generation-old mother cells was somewhat longer (about 75 min) than that found for those derived from the younger mothers (about 60 min). This observation is consistent with the earlier daughter recovery patterns (Fig. 1). In this experiment, the generation times of the mother cells were generally shorter than those observed for the mother cells in the experiment described in Fig. 1A, for any given age. This may be because the life spans of the mother cells studied in this experiment fell into a broader range (18 to 40 generations) than those in the former experiment (27 to 30 generations). Because each point represents the mean of determinations on 2 to 19 cells, cells with longer life spans would "dilute" the effect of age on generation time. The longer the life span
of an individual cell, the later in that life span the lengthening of the generation time began (data not shown). The results indicate that the factor produced by old mother cells was transmitted through at least two subsequent generations to progeny cells, but that it was ultimately subject to depletion (dilution or degradation or both).

In these studies, the pattern of a moderate increase in generation time between the ages of 10 and 20 generations was always followed by a sharper increase after generation 20. It should be noted that in all experiments only generations up to the two preceding cell death were considered. During the last two cell divisions, generation times as long as 360 min were not uncommon. In yeast strains with different life spans, the senescent phenotype, evidenced by the lengthening of the generation time, appeared at different ages: the longer the life span of a particular strain, the later this lengthening occurred (data not shown). This suggests that aging in this organism possesses a genetic component.

Cell wall chitin and aging. When yeast cells bud, the mother cell is permanently marked by the deposition of chitin in the cell wall in the form of a ring structure known as the bud scar (2). It has been proposed that accumulation of bud scars on the surface of a mother cell may lead to a decrease in the ratio of active surface to volume, which could eventually result in cell death (4, 6). Several arguments against this exist; however, the effects of actual chitin accumulation in the cell wall on yeast life span have not been examined. To address this question, the cell division cycle mutant cdc24 was chosen. At the nonpermissive temperature, cdc24 cells arrest in the cell cycle as unbudded cells and accumulate chitin in the cell wall randomly (18). For comparison, a different mutant, cdc7, was chosen. This mutant arrests at a functionally different, although temporally similar, point in the cell cycle at restrictive temperature (16) and does not accumulate chitin. CDC7 is required for traversal of the G1/S boundary, while CDC24 is required for budding (16).

The time course of chitin accumulation, at nonpermissive temperature, was examined in the two mutants (Fig. 3A). First, it is obvious that the amount of total chitin was much higher (fivefold) in cdc24 than in cdc7, even prior to exposure to the restrictive temperature. Second, cdc24 showed a dramatic increase in chitin at nonpermissive temperature. The amount of chitin nearly doubled in 2 h. At this time, there was no detectable increase in chitin in cdc7. After 2 h at restrictive temperature, the amount of chitin present in the cdc24 cell wall was about 10-fold higher than in cdc7. This corresponds to an increase in chitin equivalent to about five bud scars (Fig. 3B). It should be noted that fluorescence is given in arbitrary units due to the nature of fluorescence measurements. All determinations shown in Fig. 3 reflect increases in chitin levels relative to virgin cells that possess no bud scars.

If chitin accumulation is a major determinant of life span in yeast cells, exposure of cdc24 cells to a period of incubation at nonpermissive temperature, which results in chitin accu-

FIG. 1. Effect of age of mother cells on generation times of their daughters. (A) The generation times of individual mother cells were recorded (●). At various points in their life spans, the generation times of their daughters were also determined. First, the generation time for a daughter was measured during its first cell division, while the daughter remained attached to the mother cell (●). Then, it was recorded after the daughter had been separated from the mother and allowed to recover for two to three generations (●). The maximum life spans of the mother cells used in this experiment were in the range of 27 to 30 generations. The differences in the mean generation times between mother cells and daughters that remained attached were not significant at 18 and 26 generations. However, they were significant for the daughters after separation and recovery. At 18 generations, the significance level was P < 0.005, while at 26 generations it was P < 0.025. (B) Daughter cells were separated from individual 2 (●) and 24 (●)-generation-old mother cells. The generation times of these daughter cells were recorded during the first through fourth cell divisions after separation. Daughter cells were also left attached to 24-generation-old (△) mother cells, and their generation times were recorded during the first through third cell divisions following their appearance. The maximum life spans of the mother cells ranged from 27 to 32 generations.
mulation, should shorten the life span. A 2-h exposure to the nonpermissive temperature was chosen to maintain an initial cell viability (75 to 80%) comparable to that of cells maintained at permissive temperature, and the life span of both kinds of cell was determined (Fig. 4A). The survival curves of cells in which chitin was permitted to accumulate and those in which it was not were virtually identical. The mean life span was 12 generations in unexposed cells, and it was 11 in the case of exposed cells. The maximum life spans were 21 and 19 generations, respectively. This difference was not significant. In contrast, a significant difference was found in the survival curves of cdc7 cells (Fig. 4B). The mean life spans were 13 and 9, while the maximum life spans were 20 and 16 generations, for unexposed cells and cells incubated for 2 h at nonpermissive temperature, respectively. Thus, the expression of the cell cycle defect in cdc7 cells affected their life spans, while in cdc24 cells it did not. This result is significant because at permissive temperature cdc7 displays a survival curve virtually identical to that of cdc24. The presence in the cell wall of chitin at levels that would normally accumulate in a cell during five generations (Fig. 3), which is equivalent to about 40% of the mean life span, had no effect on survival in cdc24 (Fig. 4A). Even a relatively small difference in life span can readily be detected after exposure of cdc7 to nonpermissive temperature.

The initial viability of cdc7 cells was similar (75 to 80%) to that of cdc24 cells. The low initial viability of cdc mutant cells, as compared with wild-type cells for which it is close to 100%, was apparently due to the cell cycle defect as such, rather than to accumulation of chitin in a substantial fraction of the initial cell population. Even in cdc24 cell populations, cells displaying random chitin accumulation at permissive temperature were rare, and only a moderate increase was observed in older cells in culture (data not shown). In summary, although the amount of chitin doubled in cdc24 cells after 2 h at nonpermissive temperature, there was no appreciable change in the life span. These results do not support the notion that chitin deposition in the cell wall per se plays the sole or even a major role in the determination of yeast life span. They also lend further support to the

FIG. 2. Diminution of mother cell effect in successive generations. Generation times were recorded for mother cells (○) and their daughters (△), granddaughters (●) (daughters of the daughters), and great-granddaughters (▲) immediately after separation at maturity by micromanipulation, without any period of recovery. The maximum life spans of the mother cells were in the range of 18 to 40 generations. For 24-, 30-, and 36-generation-old mother cells, the differences in generation times between mothers and daughters were not significant. The same was the case for the differences between granddaughters and their 24- and 30-generation-old mothers. However, for 36-generation-old mothers, the differences in generation times between mothers and granddaughters and mothers and great-granddaughters were significant at levels of P < 0.05 and P < 0.01, respectively.

FIG. 3. Chitin accumulation in yeast cells. (A) The deposition of chitin in the cell walls of cdc7 (●) and cdc24 (○) cells was determined as described in Materials and Methods after shift-up to 36°C. (B) Chitin levels in exponentially growing X2180-1A cells were determined by fluorescence microphotometry, as described in Materials and Methods.
conclusion that accumulation of bud scars is not the determinant of life span.

**DISCUSSION**

The results presented here indicate the presence of a factor(s) elaborated by aging mother cells that can pass from these cells to their daughters (Fig. 1 and 2). This putative factor(s) exerted at least two effects on these daughters. First, it appeared to lengthen the generation time, while allowing the cell to continue to grow in size. This effect is reminiscent of arrest subsequent to the point at which the nutritional block operates in the G_1 phase of the cell cycle (16). Second, it appeared to synchronize the cell cycle of the daughter cell with that of the mother at least in terms of the point at which the cell buds. This point is temporally associated with traversal of the G_1/S boundary under normal conditions (16). Whether or not the two effects listed are related to each other is not clear at present. The postulated factor(s) appeared to be labile, and it seemed to undergo turnover, at least in young cells (Fig. 1 and 2). No reversal of the effect of the factor(s) was observed in mother cells (Fig. 1 and 2), suggesting that the mothers continuously generate this material or cannot degrade it, while the daughters only respond to it. The factor(s) was transmitted via the cytoplasm rather than extracellularly (Fig. 1B), and this transmission occurred from a cell to its progeny through at least two generations but was ultimately depleted (Fig. 2). The results suggest that continued attachment of the daughter to the mother cell is not required to elicit these effects (Fig. 1). They also suggest that attachment as such is not their cause (Fig. 2). Inasmuch as the length of the generation time can be viewed as a marker for progress through the life span, the elaboration of this factor(s) and its effects may be associated with aging. Finally, the results suggest that senescence, as manifested by lengthened generation time, is a phenotypically dominant feature in yeast cells.

In addition to an increase in generation time, yeast cells are marked as they progress through the life span by a chitinous bud scar at each generation. It has been suggested that it is the accumulation of these scars on the cell surface that determines life span (6). Yeast cells rarely exhaust even one-half of the 100 or so sites available for bud scars on their surface (1). The observation that old yeast cells are not “rescued by hybridization” with young cells, even though the bud scars occupy only a fraction of the surface area after hybridization (8), argues that accumulation of bud scars is not the cause of death in *S. cerevisiae* cells. It does not seem that the acquisition of bud scars affects the life span by altering active surface/volume ratio in the cell (7). The lack of effect on life span (Fig. 4) of the induced deposition of chitin (Fig. 3) lends further support to the conclusion that the accumulation of bud scars is not the dominant of life span, although it is important to keep in mind that random deposition of chitin in the cell wall is not equivalent to formation of bud scars. Most important, the evidence for a degradable cytoplasmic factor(s) that determines the senescent phenotype argues against this hypothesis. The foregoing discussion indicates that the number of bud scars on the surface of the yeast cell is an accurate measure of age, but not of aging. On the other hand, the extension of generation time may provide a marker for the aging process.

Aging, in yeasts, does not appear to be determined by the acquisition of mutations throughout the life span, because at the beginning of each lineage the cells are at equilibrium with respect to mutational burden (6). In addition, the cells do not appear to accumulate mutations or mitochondrial defects.
throughout the life span (7). Limited life span in this organism does not appear to involve an error catastrophe (13) either, because daughter cells recover from the manifestations of the senescent phenotype (Fig. 1 and 2) and lead a normal life span (4, 7). These facts pose some difficulty for application of damage theories to yeast aging in general. They also indicate that the accumulation of amplified sequences derived from the mitochondrial genome that is associated with senescence in the fungus _Podospora anserina_ (3) is an unlikely candidate for the cause of cellular aging in _S. cerevisiae_.

The mechanism of action of the cytoplasmic factor(s) indicated by our studies is not clear at present. However, cell fusion studies with mammalian cells indicate that the senescent phenotype is dominant in higher eukaryotes (11). This dominance may be at least partially explained by the elaboration of a membrane-associated protein that inhibits DNA synthesis (14). This inhibitory effect can be duplicated by the microinjection of mRNA prepared from senescent cells into proliferating cells (5). Normal diploid fibroblasts arrest at the G_1/S boundary of the cell cycle when they reach the limits of their population doubling capacity (12). Interestingly, an impairment in the normal mechanisms of cell cycle progression, such as that caused by the cdc7 mutation which leads to arrest at the G_1/S boundary, results in reduction of life span (Fig. 4). The senescent phenotype of yeast cells, as described here, can readily be explained by the activity of one or more products of age-specific gene expression. Identification and characterization of these molecules will be the next step in the analysis of the aging process in _S. cerevisiae_.

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