Plasmid Accumulation Reduces Life Span in *Saccharomyces cerevisiae* *

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Aging in the yeast *Saccharomyces cerevisiae* is under the control of multiple pathways. The production and accumulation of extrachromosomal rDNA circles (ERCs) is one pathway that has been proposed to bring about aging in yeast. To test this proposal, we have developed a plasmid-based model system to study the role of DNA episodes in reduction of yeast life span. Recombinant plasmids containing different replication origins, cis-acting partitioning elements, and selectable marker genes were constructed and analyzed for their effects on yeast replicative life span. Plasmids containing the *ARS1* replication origin reduce life span to the greatest extent of the plasmids analyzed. This reduction in life span is partially suppressed by a *CEN4* centromeric element on *ARS1* plasmids. Plasmids containing a replication origin from the endogenous yeast 2 μ circle also reduce life span, but to a lesser extent than *ARS1* plasmids. Consistent with this, *ARS1* and 2 μ origin plasmids accumulate in ~7-generation-old cells, but *ARS1/CEN4* plasmids do not. Importantly, *ARS1* plasmids accumulate to higher levels in old cells than 2 μ origin plasmids, suggesting a correlation between plasmid accumulation and life span reduction. Reduction in life span is neither an indirect effect of increased ERC levels nor the result of stochastic cessation of growth. The presence of a fully functional 9.1-kb rDNA repeat on plasmids is not required for, and does not augment, reduction in life span. These findings support the view that accumulation of DNA episodes, including episomes such as ERCs, cause cell senescence in yeast.

The yeast *Saccharomyces cerevisiae* has proved to be a valuable model organism for investigating mechanisms of cellular aging (recently reviewed in Refs. 21, 45, and 48). Central to the biology of aging in *S. cerevisiae* is an asymmetric cell division process that gives rise to mother and daughter cells with different characteristics. Mother cells have a limited capacity to produce daughter cells, and the decline in this capacity with each generation is referred to as replicative aging. The limited replicative potential of yeast mother cells has been recognized since the 1950s (35). Pioneering studies in the Jazwinski and Guarente laboratories (13, 24) postulated the existence of a senescence factor/substance that accumulates in mother cells (reviewed in Refs. 44–46).

More controversial is the role ERCs play in the aging process. Are ERCs “mediators” or “markers” of yeast aging? Certain findings link ERC production with regulation of life span and support a “mediator” role for ERCs. One of the first life span-extending mutations characterized in yeast (*SIR4–42*), was found to redirect Sir (silent information regulator) protein complexes to the rDNA locus and limit recombination (25, 26). Expression of *SIR2*, which encodes a nucleolar NAD-dependent histone deacetylase, correlates with longevity. Sir2p binds to rDNA and suppresses rDNA recombination and ERC production (14, 17, 47). Deletion of *SIR2* shortens life span, whereas overexpression of *SIR2* extends life span (29). *FOB1* encodes a nucleolar “fork blocking” protein that binds to the replication fork barrier site in rDNA and in so doing halts DNA replication in the direction opposite of pre-35 S rRNA transcription (4, 5, 29). The replication fork barrier site and the overlapping *HOT1* site promote rDNA recombination (29, 49). Mutations in or deletion of *FOB1* reduces rDNA recombination, lowers ERC levels, and extends life span (12). Recombination of replication forks stalled at replication fork barrier sites is suppressed by Sir2p (2), which explains, at least in part, the role of Sir2-de-
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Pendant silencing in extending life span. Also, the introduction of a plasmid carrying a stretch of rDNA, as an "artificial" ERC, was shown to reduce life span (46). On the other hand, ERCs have been interpreted as a "marker" of aging that are a consequence, not a cause, of aging. Mutations that impair DNA replication, recombination, or repair have been observed to reduce life span without concomitant accumulation of ERCs (31, 33, 38). However, reduction in life span may be the result of the combined effects of age-dependent and age-independent processes at work in certain mutants. hrm1Δ mutants, which affect rDNA recombination, age prematurely due to a combination of the normal aging process and a G2-like cell cycle arrest (33). Similarly, sgs1Δ mutants exhibit a shortened life span because of the combined effects of the normal aging process and cell cycle arrest due to defective recombination (22). Some petite mutants have been shown to have elevated ERC levels (10) but extended life spans (28). However, to our knowledge, both elevated ERC levels and extended life span in petite mutants have not been demonstrated side by side in the same strain. A sir2Δ mutant with an extended life span was reported to have normal ERC levels (27). More generally, the effects of Sir2 on life span have been attributed to altered patterns of gene expression, including altered transcription of rDNA, which may lead to an imbalance in ribosome synthesis (19, 20). Thus, although there is agreement that the rDNA locus plays a key role in the yeast aging process, the precise role of extrachromosomal DNAs remains controversial.

To shed light on this controversy, we have developed a plasmid-based model system to investigate the role of episomal DNAs in reduction of yeast life span. Here we present the first comprehensive test of the ERC model of yeast aging proposed by Sinclair and Guarente (46). We have constructed three types of recombinant plasmid for this purpose: ARS plasmids, ARS/CEN plasmids, and 2 μ origin plasmids. ARS plasmids are most like ERCs in that they are circular DNA molecules with a replication origin but lack a cis-acting partitioning sequence. Classic pedigree analysis studies by Murray and Szostak (36) showed that ARS plasmids exhibit a strong bias to be retained in mother cells during mitosis. Thus, ARS plasmids are predicted to accumulate in mother cells like ERCs, but this has not yet been demonstrated. ARS/CEN plasmids contain a centromeric DNA region that acts in cis to attach plasmid DNA to the mitotic spindle and ensure efficient delivery to daughter cells during mitosis. ARS/CEN plasmids should not accumulate in mother cells. 2 μ origin plasmids typically contain a DNA replication origin, a cis-acting REp3/StB element, and one copy of an inverted repeat that regulates plasmid copy number (20–40 copies/cell) (6). The REp3/StB element actively partitions plasmid DNA to daughter cells during mitosis in cis plus yeast strains (i.e. in strains that contain the endogenous 2 μ circle DNA plasmid that encodes proteins that interact in trans with REp3/StB) (6). 2 μ origin plasmids are not predicted to accumulate in mother cells, although the 2 μ plasmid partitioning machinery is not predicted to exhibit the fidelity of a centromere-based partitioning machinery. We have also constructed a series of plasmids containing functional rDNA repeat units and tested their effects on life span. This represents a significant improvement over a previously reported experiment (46), which employed a nonfunctional stretch of rDNA (i.e. rDNA incapable of being transcribed to yield full-length 35 S pre-rRNA; see "Results").

Our studies show that yeast plasmids accumulate in mother cells and reduce replicative life span. The effect of plasmids on life span appears to be a direct effect and not an indirect effect on ERC levels in mother cells. A functional rDNA repeat unit is not required for reduction in life span, and the presence of a functional rDNA repeat does not augment reduction in life span by plasmids. Thus, plasmids containing ARS elements appear to "mimic" ERC-mediated reduction in life span. These findings provide strong evidence that replicative aging in S. cerevisiae is caused by accumulation of episomal DNA. The fact that functional rDNA sequences are not required for reduction in life span argues that expression of rDNA genes from plasmid ERCs is not a causative process in yeast aging. This indicates that accumulation of episomal DNAs, such as ARS plasmids and ERCs, is one mechanism by which yeast life span is regulated.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—W303AR5 (MATa leu2−3,112 his3−11,15 ured−1 ade2−1 trp1−1 can1−1 100 RAD5 ADE2:rDNA, [cir−]) (46) was obtained from D. Sinclair. yaF5 and yaF6 were constructed by integrating linearized pRS305 and pRS306 (43), respectively, into the leu2−3,112 or ured−1 loci of W303AR5, respectively, and genotypes were confirmed by Southern blotting. Plasmids were transformed into W303AR5 using a standard lithium acetate method (16). All experiments were done with freshly prepared, independently isolated, colony-purified transformants. Unless otherwise noted, yeast were grown on synthetic dextrose medium (45). Descriptions of plasmids are provided in Table I. A 200-bp fragment containing ARS1 was amplified by PCR with primers 5′-GGAAAGCCCGTGCTGGCCTGTTACATTATCGGG-3′ and 5′-CCGGAATTTCTGTTACATTATCGGG-3′ using template YRp17. A 200-bp fragment containing the rDNA ARS was amplified by PCR with primers 5′-CCGAATTCCTGTTACATTATCGGG-3′ and 5′-CCGGAATTTCTGTTACATTATCGGG-3′ using template YCP50. A 1346-bp region of 2 μ circle DNA, containing the REp3/StB cis-acting stability element and a single 599-bp repeat region, was amplified by PCR with primers 5′-GGAAATCCCAAGGAAGATG-3′ and 5′-CCGAATTCCTGTTACATTATCGGG-3′ using template pRS424 as template. rDNA repeats were amplified by PCR using PCR primers 5′-GGATCCCTGTTACATTATCGGG-3′ and 5′-CCGAATTCCTGTTACATTATCGGG-3′ using template YCP50. SD agar plates were weighed at the beginning of each replicated life span determination to avoid insert instability.
agarose gels, and capillary-transferred to positively charged nylon yeast cells using a glass bead/phenol method, digested with restriction of different sizes that hybridized to 32P-labeled probe generated by obtained.

30 μl adenine hemisulfate and 5 μg/ml adenine hemisulfate and 5 μg/ml histidine to enhance red color production.

Southern Blot Analysis and Quantitation—DNA was extracted from yeast cells using a glass bead/phenol method, digested with restriction enzymes according to the supplier (New England Biolabs), separated on agarose gels, and capillary-transferred to positively charged nylon membrane under alkaline conditions using standard methods (50). For each plasmid copy number and ERC monomer level determination, five plasmid transformants were analyzed in parallel. Digestion with BamHI or PstI yielded single plasmid-specific or genome-specific bands of different sizes that hybridized to 32P-labeled probe generated by random-primer labeling (New England Biolabs). PstI and BamHI do not cleave rDNA. Genomic bands were used as internal standards for measurements of plasmid levels. Chromosomal rDNA bands were used as internal standards for measurements of ERC monomer levels. Blots were hybridized first to URA3 or LEU2 probe, followed by stripping and hybridization to rDNA probe. Data from the same blots were used to prepare Figs. 2 and 3B. Southern data were acquired with a Typhoon PhosphorImager and analyzed using ImageQuant software (Amersham Biosciences).

Cell Sorting—Old yeast cells were collected following biotinylation and sorting with magnetic beads as described (46). Transformants were grown to midlog phase (OD600 < 0.5) in SD liquid medium under selection prior to biotinylation. Following biotinylation, cells were grown overnight in selective SD medium, from which both young and old cells were obtained.

RESULTS

Roles for Different Cis-acting Plasmid Sequences in Reduction of Yeast Replicative Life Span—To study the effects of plasmids on yeast replicative life span, we generated two series of plasmids based on commonly used integrating vectors pRS306 and pRS305 (43). In each plasmid, we inserted ARS1, or ARS1 and CEN4, or the 2 μ circle origin (see "Experimental Procedures"). ARS1 (autonomous replicating sequence 1) is a nuclear genomic DNA replication origin whose function and domain organization have been studied in detail (reviewed in Ref. 3). Centromeric DNA from chromosome IV (CEN4) has been mapped and functionally dissected (reviewed in Ref. 7). The region of the 2 μ circle plasmid extending from REP3 through the adjacent 599-bp repeat functions as a replication origin as well as a cis-acting plasmid partitioning element (reviewed in Refs. 6 and 41). The plasmids used in this study are summarized in Table I.

TABLE I

| Plasmid | Origin, Insert | Marker | Backbone |
|---------|----------------|--------|----------|
| pJPA105 | 2 μ, rDNA repeat (XmaI end points) | TRP1 | pAF15 |
| pJPA106 | 2 μ, rDNA repeat (AhdI end points) | TRP1 | pAF15 |
| pJPA107 | 2 μ, rDNA repeat (PstI end points) | TRP1 | pAF15 |
| pJPA113 | ARS1 | URA3 | pRS306 (43) |
| pJPA114 | rDNA ARS | URA3 | pRS306 |
| pJPA116 | ARS1, CEN4 | URA3 | pRS306 |
| pJPA117 | rDNA ARS, CEN4 | URA3 | pRS306 |
| pJPA135 | 2 μ | URA3 | pRS305 (43) |
| pJPA136 | ARS1, CEN4 | LEU2 | pRS305 |
| pJPA148 | 2 μ | LEU2 | pRS305 |

*Derived from pRS424 (8), see "Experimental Procedures."
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The number of daughter cells (generations) produced per mother cell are plotted as a function of mother cell viability. A, life span curves of strain W303AR5 (46) grown on SD (synthetic dextrose) and S+D (dextrose added after autoclaving) media at 30 °C or 14 °C. The number (n) of mother cells analyzed per curve is as follows: SD 4 °C, n = 60; SD 14 °C, n = 59; S+D 14 °C, n = 60. B, life span curves of W303AR5 transformed with plasmid pJPA113 (ARS1), pJPA116 (ARS1, CEN4), or pJPA138 (2 μ ori) and control strain yAF5 (URA3) (n = 55, 47, 57, and 58, respectively). C, life span curves of W303AR5 transformed with plasmids pJPA133 (ARS1), pJPA136 (ARS1, CEN4), pJPA148 (2 μ ori), and control strain yAF5 (LEU2) (n = 38, 33, 41, and 59, respectively). D, life span curves of W303AR5 transformed with pJPA116 (ARS1, CEN4) determined on SD and YPD (n = 45 and 49, respectively). Life spans of control strains yAF6 and W303AR5 were determined on YPD (n = 50 and 55, respectively). Plasmids are described in Table I.

![Life span analysis of plasmid-transformed yeast.](https://example.com/figure1.png)

**FIG. 1.**

Table II: Life span data summary

| Plasmid/Strain | Average life span | Maximum life span | n* |
|---------------|-------------------|-------------------|----|
| pJPA113       | 12.4 ± 1.8        | 21.8 ± 2.2        | 4  |
| pJPA116       | 23 ± 1.4          | 39 ± 2.7          | 4  |
| pJPA138       | 16.3 ± 1.8        | 31.3 ± 0.6        | 3  |
| yAF6          | 33.2 ± 3.0        | 42 ± 1            | 3  |

* Number of separate life span experiments.

In contrast to the W303AR5 controls, the number of daughter cells (generations) produced per mother cell were significantly reduced as a function of mother cell viability in transformants containing plasmids pJPA116 (ARS1, CEN4) and pJPA136 (ARS1, CEN4) (Fig. 1C), similar to what was observed with the URA3 plasmid pJPA138 (2 μ ori) (Fig. 1B).

The reduction in average life span by ARS1, CEN4 plasmids pJPA116 and pJPA136 was unexpected. A similar plasmid had previously been reported to have no effect on life span when grown on YPD medium (46). One possible explanation for this difference was that ARS1, CEN4 plasmids are occasionally lost from mother cells, causing them to cease division on selective medium prior to senescence, which would result in a reduction in average life span. To test this, ARS1, CEN4 plasmid transformants were analyzed on nonselective YPD medium as done previously (46). On YPD, transformants carrying pJPA116 (ARS1, CEN4) were as long lived as control strains yAF6 (URA3) and W303AR5 (Fig. 1D). pJPA116 transformants analyzed in parallel on selective SD medium showed a reduction in average life span (Fig. 1D), as expected. These findings support the interpretation that ARS1, CEN4 plasmids, which are present at near unit copy number in transformants (see below), are occasionally lost from mother cells, rendering them unable to divide at a point in their life span prior to normal senescence.

We have also examined the effects of two well-known plasmids that carry the TRP1-selectable marker. pTV3 carries the 2 μ origin, whereas pRS314 carries ARSH4 and CEN6 (41, 43). Life spans of transformants containing each plasmid were analyzed on medium lacking tryptophan. pTV3 transformants had an average life span of 18.7 and a maximum life span of 32, both values of which are in good agreement with corresponding values for the 2 μ origin plasmids pJPA138 and pJPA148 (see above and Table II). pRS314 had average and maximum life spans of 21 and 41, respectively, which are in good agreement with values obtained with the ARS1/CEN4 plasmids pJPA116 and pJPA136 (see above and Table II). These data allow us to exclude a specific role for ARS1 and CEN4 in life span reductions presented above (Fig. 1).

Plasmid Inheritance Correlates with Reduction in Yeast Life Span—The plasmids used in this study were constructed to explore relationships between plasmid inheritance and effects on life span. Mitotic stability and plasmid copy number are widely used measures of plasmid DNA inheritance. Mitotic stability is defined as the proportion of a population of cells grown under selection that contains plasmid. We determined the mitotic stability and plasmid copy number of the plasmids used in life span experiments. Included in our studies were plasmids containing the rDNA ARS. rDNA repeats contain a single, relatively weak ARS (34). pJPA114 and pJPA117 contain the rDNA ARS at the same position as ARS1 in pJPA113 and pJPA116, respectively (see Table I and “Experimental Procedures”).

Plasmid pJPA113 (ARS1) was found to have a mitotic stability of ~20% (Fig. 2A), which is typical of yeast replicating plasmids containing ARS1, which exhibit a mother cell partitioning bias (36). pJPA116 (ARS1, CEN4) exhibited a much higher mitotic stability, ~90%, which is consistent with the presence of CEN4 centromeric DNA, and agrees with the mitotic stability of pRS316 (ARSH4, CEN6) (Fig. 2A). pJPA138 (2 μ ori) showed a high degree of mitotic stability, ~90% (Fig. 2A). The 2 μ origin plasmid pRS424 had a somewhat lower mitotic stability by comparison (Fig. 2A). pJPA114 (rDNA ARS) has a very low mitotic stability, <1% (Fig. 2A). The presence of CEN4 with the rDNA ARS in pJPA117 improves mitotic stability to ~35% (Fig. 2A). These results with pJPA114 and pJPA117 are consistent with the low efficiency of the rDNA ARS (34). Not surprisingly, it was impractical for us to carry out life span analyses of transformants containing pJPA114.
Plasmid copy number was determined using Southern blot analysis. Copy number determinations using two different restriction enzymes gave comparable results (Fig. 2B). pJPA113 (ARS1) exhibited the highest plasmid copy number (Fig. 2C). Plasmids pJPA116 (ARS1, CEN4) and pJPA117 (rDNA ARS, CEN4) exhibited near unit copy number values (Fig. 2C), which is typical of centromeric plasmids (7), such as pRS316 (ARSH4, CEN6) (43). pJPA138 (2 μ ori) exhibited a copy number of -33 (Fig. 2C), which is in the range of copy number values reported for other 2 μ origin plasmid vectors (41). The high copy number of pJPA113 is primarily due to the asymmetric inheritance of this plasmid and its accumulation in mother cells rather than ARS strength per se. We reach this conclusion because pJPA114, which contains a weak (rDNA ARS) replication origin, achieves a copy number almost as great as pJPA113, which contains a strong (ARS1) replication origin (Fig. 2C). Thus, pJPA113 demonstrates a correlation between extent of recombination of transformant life span (Fig. 1B) and tendency to be inherited asymmetrically and attain a high copy in yeast cells (Fig. 2C).

Plasmids Do Not Significantly Increase ERC Levels—The results presented above suggest that reduction in life span by the ARS1 plasmid pJPA113 is due to asymmetric inheritance and accumulation in mother cells. An alternative explanation is that pJPA113 increases ERC levels in transformed cells and thereby reduces life span indirectly. To address this possibility, we measured recombination at the rDNA locus using an ADE2 marker loss assay and measured ERC levels in transformed cells by Southern blotting.

To analyze the frequency of recombination at the rDNA locus, we took advantage of the fact that W303AR5 contains ADE2 integrated at the rDNA locus (46). Recombination between flanking rDNA repeats results in loss of ADE2 and a change in colony color. The frequency of half-red sectored colonies is a measure of rDNA recombination rate (events per cell division). Transformation of yeast with plasmid results in a small increase in rDNA recombination as measured by ADE2 marker loss. For W303AR5, we find that ADE2 marker loss occurs at a frequency of -1.3 per thousand cell doublings (Fig. 3A), which is in good agreement with frequencies reported by others (23, 31, 33). The rate of ADE2 marker loss from yAF6 (URA3) occurs at -2.7 per thousand (Fig. 3A). Transformants containing the three plasmids used in this study, pJPA113 (ARS1), pJPA116 (ARS1, CEN4), and pJPA138 (2 μ ori), exhibited marker loss rates of 4.1, 4.5, and 4.1 per thousand cell doublings, respectively. The differences between transformants and yAF6 represent increases of less than 2-fold. Higher levels of ADE2 marker loss are typically observed in strains with reduced life spans. For example, short lived sir2Δ mutants exhibit ADE2 marker loss rates >10-fold higher than isogenic SIR2 strains (23).

To directly compare ERC levels, yeast transformants and
control strains were analyzed by Southern blotting, and ERC monomer bands were quantitated (see “Experimental Procedures”). ERC monomers consist of a single 9.1-kb rDNA repeat and were chosen for purposes of quantitation because they are well resolved from chromosomal rDNA and other ERC bands on Southern blots. ERC monomer levels in transformants were not significantly different from ERC monomer levels in control strains. Control strains W303AR5 and yAF6 (URA3) have \( \sim 0.0007 \) and \( \sim 0.0015 \) ERC monomers per total chromosomal rDNA, respectively (Fig. 3B). Transformants bearing pPJAl13 (ARS1), pPJAl16 (ARS1, CEN4), and pPJAl38 (2 \( \mu \) ori) have ERC monomer levels of \( 0.0014, 0.001, \) and \( 0.001, \) respectively (Fig. 3B). These values are within the error of measurements and are not significantly different (Fig. 3). For comparison, we examined yAF5 (LEU2), which contains a copy of pRS305 integrated at the \( \text{leu2–113} \) locus, and found that the ERC monomer level was \( 0.001, \) which is intermediate between W303AR5 and yAF6 (Fig. 3B). Quantitation of slower migrating ERC multimer bands did not reveal significant differences in levels between transformant and control strains (data not shown). We conclude that plasmids do not have a significant effect on ERC levels.

Plasmid Accumulation Correlates with Reduction in Yeast Life Span—If plasmids reduce life span in a manner analogous to ERCs, then plasmid DNAs should accumulate in old mother cells. To test this prediction, we used a biotinylation and magnetic sorting approach to isolate \( \sim 7\)-generation-old yeast cells (see “Experimental Procedures”). Plasmid DNA levels in young and old cells were measured by quantitative Southern blotting. The ages of old and young (unsorted) cells were determined by counting bud scars stained with Calcofluor (40). From single sort experiments, the average ages of yeast transformed with pPJAl13, pPJAl16, pPJAl38, and yAF6 were 6.9, 7.0, 6.1, and 6.2 generations, respectively (Fig. 4A). Young cells from the same cultures were an average of 1.5, 1.4, 1.1, and 1.1 generations old, respectively (Fig. 4A). Inspection of the Southern blot clearly reveals increases in relative amounts of pPJAl13 (ARS1) and pPJAl38 (2 \( \mu \) ori) in old cells (Fig. 4B). pPJAl16 (ARS1, CEN4) did not accumulate in old cells, and it yields bands similar in their intensities to corresponding bands from yAF6 (Fig. 4B). In a striking illustration of the accumulation of pPJAl13 and pPJAl38 in old cells, the linearized plasmid DNA bands can be observed by ethidium bromide staining (Fig. 4D). ERC levels in young and old cells were also analyzed by Southern blotting. Hybridization to rDNA probe revealed ERC bands and a broad band corresponding to the rDNA locus on chromosomal rDNA (Fig. 4E). ERC monomer levels were also quantitated in young and \( \sim 7\)-generation-old transformants and yAF6. ERC monomer levels in young cells were equal or close to 0.001 (Fig. 4G), which agrees with measurements presented above (Fig. 3B). In old cells, however, ERC monomer levels were appreciably higher and exhibited increases between \( \sim 20\)- and \( \sim 70\)-fold (Fig. 4H). The levels of ERCs we observe in \( \sim 7\)-generation-old cells appear comparable with ERC levels in sorted cells of similar age reported by others (e.g. Refs. 23 and 46), although quantitative analysis of ERC levels in young and old yeast cells is not commonly reported in the literature.

In the experiment shown in Fig. 4, ERC monomer levels in yAF6 (URA3) are higher than in transformants (Fig. 4H). This raises the question of whether the presence of plasmid reduces ERC levels. In a separate experiment, ERC monomer levels in young cells were equal or close to 0.001 (ERC monomer/chromosomal rDNA), and ERC monomer levels in old transformants containing pPJAl13, pPJAl16, and pPJAl38 and in old yAF6 cells were determined to be 0.083, 0.075, 0.045, and 0.081, respectively (data not shown). The similar ERC levels in yAF6 and transformants in this experiment suggest that plasmid vectors do not appreciably affect ERC monomer levels (see also Fig. 5).

Does the extent of ERC accumulation in old cells in Fig. 4 agree with predictions based on our estimates of rates of recombination within the rDNA locus (see above and Fig. 3)? If we assume that extrachromosomal rDNA repeats are generated at a rate of 0.5/cell/generation and that ERCs are retained in mother cells, then 6–7 generations should yield an increase of between 32- and 64-fold, which is similar to the observed range of increase from 20- to 70-fold (Fig. 4, G and H).

We have also quantitated the relative amount of all ERCs (i.e. monomers, multimers, and concatemers) found in old transformants containing pPJAl13, pPJAl16, and pPJAl38 and in old yAF6 cells. We found levels of 0.140, 0.136, 0.086, and 0.238, respectively (extrachromosomal rDNA/chromosomal rDNA; data not shown). These values mirror levels of accumulation of ERC monomers presented in Fig. 4H. Thus, ERC monomers comprise about one-fourth to one-third of all extrachromosomal rDNA repeats and are present at similar levels relative to all ERCs in old transformed and untransformed cells.

To extend these studies, yeast sorting experiments were done with transformants containing the LEU2 plasmids pPJAl33 (ARS1), pPJAl36 (ARS1, CEN4), and pPJAl48 (2 \( \mu \) ori) and with the LEU2 strain yAF5. The average ages of sorted yeast transformed with pPJAl33, pPJAl36, pPJAl48, and yAF5 are 7.1, 7.0, 7.6, and 7.9 generations, respectively (Fig. 5A). Young cells from the same cultures were an average of 1.7, 1.0, 1.6, and 1.6 generations, respectively (Fig. 5A). pPJAl33 and pPJAl48 attain copy number levels of 119 and 39, respectively, in \( \sim 7\)-generation-old cells (Fig. 5C). This represents an increase in copy number between young and old cells of \( \sim 30\)- and \( \sim 8\)-fold for pPJAl33 and pPJAl48, respectively. pPJAl36 did not show a significant increase in old cells (Fig. 5C). In comparison with pPJAl33 and pPJAl38, pPJAl33 and pPJAl48 reached lower absolute levels of plasmid in \( \sim 7\)-generation-old cells. However, pPJAl33 and pPJAl48 accumulated to similar extents in terms of -fold increase. To resolve whether this difference in absolute levels of plasmids in old...
cells was due to experimental error, sorting experiments with transformants and the control strain were repeated, followed by Southern analyses. The repeat experiment gave results very similar to first experiment, in terms of both absolute level of plasmid in young and old cells and -fold increase in young and old cells (Fig. 5, B and C). This indicates that plasmids with identical ARS1 origins and CEN4 elements, but with different backbones and selectable markers, are maintained at different

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**Fig. 4. Plasmid DNA and ERC levels in young and old cells.** A conveys the *cis*-acting elements present in each plasmid (see also Table I). Plasmids are abbreviated by numbers in B–H. All plasmids carry URA3. Control strain yAF6 (URA3) did not contain plasmid. Old cells were harvested using a biotinylation and magnetic sorting approach (see “Experimental Procedures”). A, age profile histograms of young and old cells. The number of cells is plotted as a function of the number of bud scars (n > 40 for each histogram). B, Southern blot of plasmid DNAs. *Pst*I-digested genomic DNA yields a 3.67-kb URA3 band. Other bands are plasmid-derived. Genomic URA3 DNA in lane Old 113 migrated as two bands due to partial overdigestion of this sample. C, Southern blot of ERCs. D, ethidium bromide-stained agarose gel corresponding to the blot in B and C. DNA marker sizes (in kb) are shown. E and F, plasmid levels in young and old cells (quantitation of data presented in B). G and H, ERC monomer levels in young and old cells (quantitation of data presented in C). E–H, ratios of episome (plasmid or ERC monomer) band intensity divided by chromosomal rDNA band intensity (× 1000) are plotted (on a semilog scale). See Fig. 1B for corresponding life span data. Comparable results were obtained from similar cell sorting and Southern blotting experiments and are discussed under “Results.”

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Terminal Cell Morphology—Currently, in the field of yeast aging, there are few approaches available to directly address the senescent phenotype in old nondividing cells. To address this issue indirectly, we scrutinized the "terminal" morphology of cells at the end of their life span. The rationale for this approach is that cell morphology is a phenotypic indicator of cell cycle stage and can serve as a basis to compare senescent cells (32). If cell morphology in terminal transformed cells is very different from the morphology of terminal wild type cells, this would imply that different mechanisms may bring about the senescent phenotype in transformed and untransformed cells.

To examine terminal yeast cells, images of terminal cells were collected from three different life span experiments. Three different cell morphologies were scored: un budded cells, single budded cells with small buds, and single budded cells with large buds (32). Bud emergence in S. cerevisiae correlates with entrance into S phase, and small buds are indicative of early S phase, whereas large buds are indicative of late S/G2 or mitotic arrest. Un budded cells are in G1 phase. Between 10 and 15% of the terminal cells, transformed or untransformed, had multiple buds (data not shown) and were omitted from this comparison. For pJPA113 (ARS1) and pJPA116 (ARS1, CEN4) transformants and W303AR5, more than 50% of terminal cells were un budded (Fig. 6). Typically, between 50 and 60% of senescent yeast cells have been found to be un budded (32, 33). pJPA116 transformant cells consistently yielded the highest proportion (~65%) of un budded cells (Fig. 6). yAF6 (URA3) and pJPA138 (2 μ ori) transformants ceased dividing with a predominance, yet a lower percentage, of unbudded cells (Fig. 6). Thus, the majority of pJPA113 transformants, like W303AR5 cells, senesced in G1, as expected. In addition, similar proportions of small budded and large budded terminal cells in senes-
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Fig. 6. Terminal morphology of senescent cells. Cells at the end of life span experiments were classified according to budding pattern as described (32). Small buds were defined as having a diameter less than 25% of the diameter of the mother cell. All other buds were classified as large. Average and S.D. values from three independent experiments are shown (n > 40 for each transformant or control strain in each experiment).

Fig. 7. Life span analysis of yeast transformed with plasmids containing rDNA repeats. Number of daughter cells (generations) produced per mother cell are plotted as a function of mother cell viability. Life span analysis was done as described in the legend to Fig. 1 using W303AR5 carrying plasmids pJPA105 (n = 45), pJPA106 (n = 43), or pJPA107 (n = 46) and control plasmid pAF15 (n = 46). Plasmids pJPA105, pJPA106, and pJPA107 contain full-length (9.1-kb) rDNA repeats with different endpoints (see Table I and “Experimental Procedures”). pJPA105 contains an rDNA insert with XmaI endpoints, which has been shown to be functional in vivo (37).

cent pJPA113 transformants and W303AR5 cells (Fig. 6) indicate that similar proportions of these cells arrested in similar phases (S or G2/M) of the cell cycle. Thus, this analysis supports the interpretation that pJPA113 (ARS1) reduces life span by a normal aging process.

Do Functional rDNA Transcription Units Play a Role in Reduction of Life Span?—Although plasmids without rDNA sequences reduce yeast life span, it is important to consider a potential role for rDNA sequences in life span reduction. It is possible that ERCs reduce life span in a manner that is mechanistically more complex than the manner in which plasmid episomes reduce life span. There are significant differences in coding potential between plasmids and ERCs. The 9.1-kb rDNA repeat carries genes for rRNA precursors as well as the gene *TAR1*, which lies on the strand opposite the 25 S rRNA and encodes a mitochondrial protein (9). One way to address this issue is to ask whether or not a plasmid vector carrying an rDNA repeat unit has a more pronounced effect on life span than plasmid vector alone. It is important to note this issue was not completely addressed in a previous study employing the rDNA-containing plasmid pDS163 (46). Plasmid pDS163 does not contain a functional 9.1-kb rDNA repeat unit. The rDNA on pDS163 consists of a 12.1-kb insert extending from an EcoRI site within the coding sequence of 5.8 S rRNA to the 5′-most EcoRI site in the 25 S rRNA coding region (data not shown). The 12.1-kb fragment does not carry a full-length 35 S pre-rRNA transcription unit and is capable of producing only a truncated 35 S pre-rRNA transcript, which if processed would be incapable of yielding mature 25 S rRNA.

To determine whether an episomal rDNA repeat influences life span, we constructed three plasmids containing 9.1-kb rDNA repeats and used them in life span experiments. The three plasmids, pJPA105, pJPA106, and pJPA107, contain 9.1-kb repeats with different endpoints in the plasmid pAF15, which contains a 2 μ origin (see “Experimental Procedures” and Table I). Plasmid pJPA105 contains a repeat with XmaI end points, which has been shown by Nomura and colleagues to functionally complement an rDNA deletion in vivo (37). pJPA106 and pJPA107 contain repeats with AhdI and PsiI end points, respectively, which should not interfere with rDNA gene expression. A 2 μ origin plasmid was used, because plasmids constructed with rDNA inserts whose replication relied solely on the rDNA ARS were found to integrate into the chromosomal rDNA locus with high frequency (as determined by Southern blot analysis; data not shown). Life span determinations of W303AR5 transformants containing pAF15, pJPA105, pJPA106, and pJPA107 were done as described above (see Fig. 1). pJPA105, pJPA106, pJPA107, and pAF15 transformants gave very similar life span curves, indicating that the presence of a functional rDNA repeat does not have a dramatic effect on life span (Fig. 7). All four plasmids affect life span to an extent similar to the 2 μ origin plasmids pJPA138 and pJPA148 (Fig. 1, B and C), although the average life spans for pJPA105, pJPA106, pJPA107, and pAF15 (13.3, 11.8, 11.7, and 12.2 generations, respectively) are lower than the average life spans for pJPA138 and pJPA148 transformants (15.5 and 16.3 generations, respectively; Fig. 1 and Table II). Life span curves for pJPA106 and pJPA107 transformants did not show a statistically significant difference from pAF15 transformants based on the Wilcoxon signed pair rank test (p < 0.05). Only transformants carrying pJPA105 and pAF15 exhibited a statistically significant difference (p < 0.05), but this represents a small increase in life span of transformants carrying pJPA105. These findings support the conclusion that the presence of a full-length rDNA repeat per se is not required for, and does not necessarily augment, reduction in yeast life span.

**DISCUSSION**

Budding yeast is an excellent system in which to study cell-autonomous mechanisms of aging. Mechanisms linked to genome stability, metabolic damage, and metabolic regulation have been found to regulate yeast replicative life span (1, 11, 21, 30, 45, 48). Sinclair and Guarente (46) have proposed that a key regulator of life span is the cellular level of ERCs. To study this proposal, we have used plasmids to model ERC inheritance and accumulation, two processes that govern ERC levels in yeast cells. Our work shows that plasmid DNAs bring about significant reductions in yeast life span. We find that
ARS1 and 2 μ origin plasmids specifically accumulate in old yeast cells and that the level of accumulation of ARS1 and 2 μ origin plasmids in old cells correlates with the extent of reduction in life span. This is the first demonstration to our knowledge of an inverse relationship between DNA episome level in old cells and reduction in life span. We find that plasmids have a direct effect on life span and do not indirectly reduce life span by increasing recombination at the rDNA locus and increasing ERC levels in transformed cells. Analysis of the "terminal" morphology of senescent cells indicates that plasmids do not cause a stochastic arrest in the cell cycle, which is consistent with a normal aging process. Reduction in life span does not require that plasmids carry rDNA repeat sequences, and the presence of a full-length, functional 9.1-kb rDNA repeat on a plasmid does not augment reduction in life span. These findings confirm the work of Sinclair and Guarente (46) and provide significant new support for their ERC model by directly demonstrating a relationship between plasmid inheritance, plasmid accumulation, and reduction in life span. Our studies also highlight the value of plasmids as tools to investigate properties of ERCs that are relevant to the aging process in yeast.

Why do ARS plasmids accumulate in mother cells? It has long been appreciated that ARS plasmids are inherited asymmetrically and accumulate in mother cells (36). This accounts for the high copy number and low mitotic stability of ARS plasmids. However, accumulation of ARS plasmids in cells that are multiple generations old has not been directly demonstrated. Our studies are the first to directly demonstrate that ARS1-containing plasmids accumulate to high levels in old yeast cells. Although ARS1 plasmid partitioning bias is well known, little is understood about its underlying mechanism. One possibility is that plasmid partitioning bias is due to the nature of cell and nuclear division in budding yeast. During closed mitosis in yeast, an intact nucleus elongates along the axis of the mitotic spindle and adopts an elongated "dumbbell" shape due to constriction of the nucleus at the bud neck. Chromosomes pass through the constriction at the bud neck by virtue of their attachment to the mitotic spindle, which is able to exert force on chromosomes. In the absence of spindle attachment, passage of DNA molecules through the constriction at the bud neck may be limited. Consistent with this notion, the relatively small (1.45-kb) TRP RI plasmid has been shown to be inherited efficiently and to exhibit high mitotic stability (51). The small size of the TRP RI plasmid may allow it to readily distribute between mother and daughter cells through the bud neck constriction. Commonly used yeast recombinant DNA vectors are typically larger than the TRP RI plasmid and require cis-acting sequences and trans-acting factors to be stably inherited.

Why do budding yeast exhibit a mother cell plasmid segregation bias? One possibility is that mother cell segregation bias is a mechanism to protect progeny cells from potential "parasitic" effects of episomal DNAs acquired from the environment. The 2 μ circle is a "commensal" episomal DNA that Futter et al. (15) have depicted as a sexually transmitted selfish DNA. The 2 μ circle depends on its capacity to overcome mother cell segregation bias (see below) in order to survive in a host population in the absence of any selective value. Another possibility is that mother cell segregation bias is a mechanism to increase the longevity of progeny cells by limiting transmission of ERCs.

Why do ARS1 plasmids bring about cellular senescence more rapidly than do ERCs? One possibility is that virgin mothers contain at least one ARS plasmid but probably contain on average 0.5 ERC/cell. The difference in origin strength between ARS1 and the rDNA ARS may also be important. ARS1 is a relatively "strong" ARS and capable of supporting rapid plasmid accumulation in mother cells. ERCs contain a comparatively "weak" ARS that is likely to support only relatively slow accumulation in mother cells. The rDNA ARS contains an ACS (ARS consensus sequence) that departs from the consensus at position 1, a change that has been shown to reduce ARS function, primarily by limiting DNA unwinding (34). This difference in strength could explain why ARS1 plasmids bring about senescence in mother cells more rapidly than do ERCs. ARS1 plasmids are replicated more efficiently than ERCs, which increases the rate of ARS1 plasmid accumulation in mother cells compared with ERCs.

Do cis-acting sequences that counteract mother cell segregation bias suppress reduction in life span by ARS1 plasmids? Yes, ARS1/CEN4 plasmids reduce life span to a lesser extent than ARS1 plasmids, which is consistent with results of Sinclair and Guarente (46). However, inclusion of CEN4 on ARS1 plasmids suppresses the reduction in maximum life span by ARS1 plasmids but does not fully suppress the reduction in average life span. Our studies also directly show that ARS1/CEN4 plasmids do not accumulate in 7-generation-old mother cells. The reduction in life span is not specific for the combination of ARS1 and CEN4. The combination of ARSH4 and CEN6 (in pRS314) (43) reduces average life span with a minimal effect on maximum life span. The fact that centromeric DNA elements suppress reduction in maximum life span supports the conclusion that ARS1 plasmids exert their effect by accumulation in mother cells, as discussed above.

Why do 2 μ origin plasmids reduce life span? One clue emerges from the observation that endogenous 2 μ circles do not accumulate in old cells (data not shown). Although both 2 μ origin plasmids and 2 μ circles contain the REP3/STB cis-acting stability element, 2 μ origin plasmids contain a single 599-bp segment, whereas 2 μ circles contain two 599-bp segments arranged as an inverted repeat (6, 41). More efficient autoregulation of 2 μ circle copy number and inheritance is likely to prevent accumulation in old cells. It is important to note that 2 μ circles can be toxic to cells when present at high copy number. Constitutive expression of the 2 μ amplification machinery results in high copy number and has deleterious effects on cell growth (6). Similarly, mutations in NIB1/ULP1 result in unusually high levels of 2 μ circles, formation of large inviable or mitotically arrested cells, and clonal lethality (18). Studies by Dobson and co-workers indicate that a modified form of Rep2p, a 2 μ circle-encoded plasmid partitioning protein, accumulates in ulp1 mutants, suggesting that ULP1 is involved in partitioning of 2 μ circles during mitosis. This suggests that high levels of 2 μ circles in nib1/ulp1 mutants may result from asymmetric inheritance. In this sense, phenotypes associated with nib1/ulp1 defects may share mechanistic underpinnings with senescent phenotypes associated with asymmetric inheritance of plasmids and ERCs.

Why do 2 μ origin plasmids have an intermediate effect on life span? Although 2 μ origin plasmids accumulate in 7-generation-old mother cells, they attain levels approximately half that observed with ARS1 plasmids. As mentioned above, comparison of results with 2 μ origin and ARS1 plasmids supports an important inverse relationship; the extent of plasmid accumulation in old cells correlates with the extent of reduction in life span.

Our findings raise the question of by what mechanism(s) plasmids, and by implication ERCs, reduce life span in yeast. It is clear that asymmetric inheritance of plasmid DNAs has the...
potential to burden mother cells with high DNA content. If we assume that a 5-kb plasmid is replicated once each S phase and uniformly inherited by the mother cell during M phase, then 12 doublings will yield a plasmid DNA content in excess of the nuclear genomic DNA content (5 × 212 = 20.5 Mb of plasmid DNA content > −13 Mb of nuclear genomic DNA content). Of course, this estimate is an oversimplification and omits factors such as origin firing frequency and segregation efficiency. However, we note that after 12 generations, 90% of pJPA133 (4.8 kb of ARS1 plasmid) transformants were senescent, and after 20 generations, 90% of pJPA133 (4.8 kb of ARS1 plasmid) transformants were senescent. The fact that significant percentages of senescent mother cells arise between 10 and 20 generations may prompt further research into the mechanisms that govern plasmid accumulation. We are likely to be useful tools for exploring possible mechanisms by which ERCs reduce life span. The study of plasmid accumulation may also provide insights into the mechanisms by which episomal DNAs accumulate during yeast aging. This, in turn, should shed additional light on the role ERCs play in the yeast aging process.

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