Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1955–1958, March 1994
Genetics

Lack of telomere shortening during senescence in *Paramecium*

(DNA damage/clonal life span/telomere maintenance)

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Contributed by Elizabeth H. Blackburn, November 24, 1993

ABSTRACT **_Paramecium tetraurelia_ cells have a limited clonal life span and die after ≈200 fissions if they do not undergo the process of autogamy or conjugation. To test the possibility that cellular senescence of this species is caused by telomere shortening, we analyzed the genomic DNA of the macronucleus during the clonal life span of _P. tetraurelia_. We found that telomeric DNA sequences were not shortened during the interval of decreased fission rate and cellular death, defined as senescence in these cells. However, the mean size of the macronuclear DNA was markedly decreased during the clonal life span. We present a model that expands upon previous proposals that accumulated DNA damage causes cellular senescence in _P. tetraurelia_.**

Cellular and organismal aging have been correlated with accumulated DNA damage (1, 2). A more specific model for the limited life span of dividing cells has been proposed to be gradual shortening of telomeres, leading eventually to impaired chromosome maintenance (3–6). The ciliated protozoan *Paramecium* has been studied extensively as a model for clonal cellular aging (7–9). The single-celled eukaryote *Paramecium* contains two types of nuclei: the germ-line micronucleus and a polygenomic somatic macronucleus, generated from a micronuclear division product after either self-fertilization (autogamy) or sexual reproduction (conjugation). When *Paramecium tetraurelia* stock 51 cells are maintained in a state of continuous logarithmic division and are prevented from undergoing autogamy or conjugation, they have a limited clonal life span and die after ≈200 fissions. However, senescence can be averted by allowing the cells to undergo either autogamy or conjugation.

In *Paramecium*, as in other ciliated protozoans, cellular functions during clonal fissions are carried out under the direction of the macronuclear genome (10). During each asexual or clonal fission the micronucleus divides by mitosis and the macronucleus divides by amitotic division. The highly polygenomic macronuclear DNA is formed by processing the diploid micronuclear chromosomes into shorter acentric macronuclear chromosomes, which are amplified to ≈1500 copies in *P. tetraurelia* during macronuclear development in autogamy or conjugation. The average size of these high copy number linear chromosomes is ≈300 kb in *P. tetraurelia* (11). Previous studies of aging of *Paramecium* have shown that replacing the macronucleus with a newly developed macronucleus (by autogamy or conjugation) is necessary to prevent clonal senescence, although the efficiency of this process decreases in aged *Paramecium* cells. These findings have suggested that loss of macronuclear function underlies *Paramecium* aging. Cells remain capable—at least for many fissions—of producing a young macronucleus, implying that the genetic content of the micronucleus is not compromised, although eventually during clonal aging this capability also becomes impaired.

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In contrast to *Paramecium*, clonally dividing cells of the related ciliated protozoan *Tetrahymena thermophila* are effectively immortal and show no comparable clonal aging. However, it has been shown that delayed cellular lethality of *T. thermophila* cells can be directly caused by mutating the RNA moiety of the ribonucleoprotein enzyme telomerase (12). Telomerase is a specialized cellular reverse transcriptase that synthesizes one strand of telomeric DNA, using as the template a short sequence in the telomerase RNA (13). One particular telomerase RNA mutation caused telomere shortening and cell death after several cell divisions (12). This result showed that normal telomerase function is required for telomere maintenance and complete replication. Similarly, in the yeast *Saccharomyces cerevisiae*, mutations of the *EST1* gene cause progressive telomere shortening and cell death (5). In human tissues and derived cell lines, telomere shortening has been observed to correlate with increasing numbers of cell fissions undergone and has also been proposed to contribute to senescence, although a direct causal relationship has not been demonstrated (6).

A distinction exists between the senescence observed with both human cells in culture and *Paramecium* and the limited clonal life span imposed by mutations causing loss of telomere maintenance in yeast and *Tetrahymena*. In the former case, senescence is gradual (14), with cell fission rates continuously declining for many divisions before loss of the ability to divide. In contrast, in *est1*$^-$ yeast and *Tetrahymena* cells that fail to maintain telomeres because of certain mutations in the telomerase RNA, the loss of division capability is relatively precipitous and is not preceded by a gradual decline in fission rate (5, 12).

We tested the possibility that cellular aging in *P. tetraurelia* is caused by telomere shortening in the macronucleus. Our analysis of macronuclear DNA shows that shortening of telomeric DNA sequences was not associated with the decreasing fission rates and eventual cell death during the course of the clonal life span of *P. tetraurelia*. However, we found that the mean size of macronuclear DNA was dramatically decreased during aging. A model is presented in which it is proposed that aberrant repair and consequent accumulated DNA damage cause aging in *Paramecium*.

MATERIALS AND METHODS

Strains and Culture Conditions. Wild-type cells were *P. tetraurelia* stock 51. The macronuclear deletion mutant d48 was originally obtained from stock 51 (wild type) by x-ray mutagenesis and surface antigen antiserum selection (15). This strain is deleted for macronuclear surface antigen *A* gene sequences near the start of *A* gene transcription (15). Telomeric sequences have been shown to be added at this point of deletion during macronuclear development (16).

Cells were cultured in 0.15% Cerophyl (Pine Brothers, Kansas City, MO) supplemented with 0.1 g of Bacto yeast extract per liter, 1 mg of stigmasterol per liter, and 0.45 g of

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Na_2HPO_4 per liter and inoculated with *Klebsiella pneumoniae* 24 hr before use.

Aging of Paramecia. The method of aging paramecia was essentially performed as described by Sonneborn (7). Exautogamous *P. tetraurelia* stocks 51 and d48 were aged by daily transfer of single cells into separate depressions containing fresh culture medium. Cells were checked for autogamy by daily monitoring of fission rates and staining with 4',6-diamidino-2-phenylindole. The possibility that cell death was caused by poor medium or a diffusible substance in the medium was eliminated by transferring young cells into medium in which old cells had senesced and observing that the young cells divided normally.

Southern Blot Analysis. The blot shown in Fig. 1 was hybridized at 42°C in 5× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate)/50 mM NaH_2PO_4/50% formamide/0.5% SDS/3× Denhardt's solution/10% dextran sulfate (average molecular weight, 500,000)/denatured salmon sperm DNA (200 μg/ml). The filter was washed twice with 2× SSC/0.1% SDS at room temperature for 10 min and then washed twice with 0.1× SSC/0.1% SDS at 68°C for 20 min.

Optimal hybridization conditions for the blot shown in Fig. 2 were determined to be 50°C in 5× SSC/20 mM sodium phosphate (pH 7.0)/10× Denhardt's solution/7% SDS/100 μg of denatured salmon sperm DNA per ml/10% dextran sulfate. The filter was washed first at 55°C for 1 hr in 3× SSC/5% SDS and then for 1 hr at 55°C in 1× SSC/1% SDS.

RESULTS

Absence of Telomere Length Shortening During Senescence. To examine telomere length during clonal aging of *P. tetraurelia*, cells that had freshly undergone autogamy were kept dividing continuously by clonal fissions until they senesced and died. The absence of autogamy or conjugation was ensured as described in *Materials and Methods*. DNA was prepared at intervals throughout the clonal growth period and analyzed for telomere length, the amount of total telomeric DNA sequence, and overall native molecular weight. Table 1 shows the fission rates and points of cellular death for representative cell lines. The findings were similar to previous results reported with *P. tetraurelia*, including the slow decline in fission rate over the course of the divisions and the somewhat variable number of fissions before cellular death (up to ≈200 cell fissions) (7).

Telomere lengths were examined by Southern blotting analysis of genomic DNA from cells that had gone through increasing numbers of clonal fissions after autogamy (Fig. 1). Telomere length has been shown in many systems to be regulated coordinately among all telomeres. Thus, the mean length of any one telomere reflects that of all the telomeres in that cell (13). The hybridization probe chosen contains a portion of the surface antigen *A* gene, which is located very close to a macronuclear telomere. The map in Fig. 1 shows the telomeric region in the strain used and the probe (15). The sharp bands corresponded to the expected chromosomal internal genomic restriction fragment upstream of the *A* gene region; the fainter, cross-hybridizing bands are probably derived from related surface antigen genes (10). The disperse, fuzzy band has been shown to be the telomeric restriction fragment possessing a terminal structure of 100–500 bp of telomeric G_4T_2/G_3T_3 repeats (16). Its mean size decreased somewhat during the first fissions, but thereafter it increased slightly. The initial small size decrease was not accompanied by any significant decrease in the fission rate or viability of the cells in this line. The same result was obtained with all cell lines analyzed. It should be emphasized that the design of the experiment prevented the overgrowth of the clonally dividing cultures by fast-growing minor variant cells: each cell line was propagated by daily single cell isolation from the previous transfer, which contained a maximum of 16–32 cells. We conclude that telomeric DNA sequences are not shortened during the interval of decreased fission rate and cellular death, defined as senescence in these cells.

Table 1. Fission rates of representative aging cell lines

	d48 cell line				51 cell line		
Days*	1	2	3	Days*	1	2	3
2–20	3–4 fissions†			2–15	2–3 fissions†		
25	2.8	3.4	3.2	20	2.2	2.5	(1.2)
30	2.4	3.4	3.6	25	2.4	2.1	
35	2.2	3.6	3.8	30	1.9	2.0	
40	1.8	3.1	3.4	35	2.4	2.5	
45	(1.8)	3.1	2.9	40	2.9	2.0	
50		2.7	2.5	45	3.0	1.8	
55		2.4	2.4	50	2.9	1.9	
60		2.4	2.3	55	2.3	(1.4)	
65		1.2	1.2	60	2.6		
70		(1.3)	(0.7)	65	(1.7)		

Numbers in parentheses indicate average number of fissions within this 5-day period in which death occurred.
*Days after autogamy.
†Average fission rate per day during this time period.

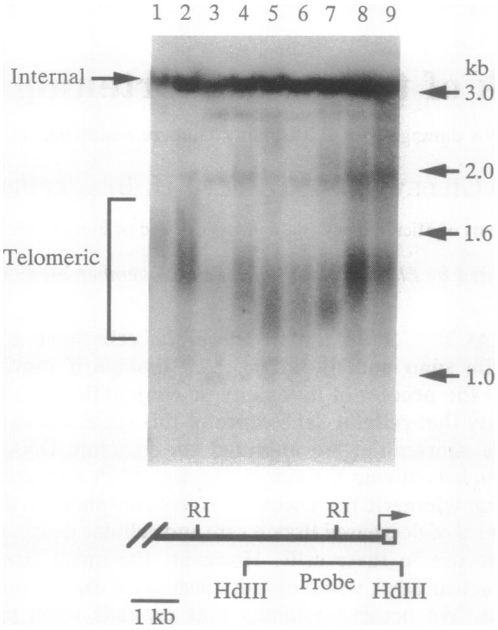

FIG. 1. Southern blot analysis of the terminus of a specific macronuclear chromosome during the aging process from d48 cell line 3. Total genomic DNA from d48 cell line 3 was isolated at specific points after autogamy and 10 μg of DNA was digested with *Eco*RI and then resolved on a 1.0% agarose gel. This blot was probed with the ^{32}P-labeled *Hin*dIII fragment shown below the partial restriction map. Lanes (average fission rate per day and days postautogamy): 1, 3–4 fissions per day, 4 days; 2, 3–4 fissions per day, 7 days; 3, 3–4 fissions per day, 17 days; 4, 3.2 fissions per day, 23 days; 5, 3.6 fissions per day, 30 days; 6, 3.4 fissions per day, 37 days; 7, 2.9 fissions per day, 45 days; 8, 2.3 fissions per day, 60 days; 9, 0.7 fission per day, 66 days. d48 cell line 3 died 68 days after autogamy. Shown is a partial restriction map of the macronuclear chromosomal region of interest. Arrow indicates start and direction of transcription of the wild-type surface antigen *A* gene. Box indicates region of telomeric sequence. RI, *Eco*RI; HdIII, *Hin*dIII.

Accumulation of Macronuclear DNA Damage Without Loss of Telomeric Sequences During Aging. The macronuclear DNA (which comprises the vast majority of total cell DNA) was examined by nondenaturing agarose gel electrophoresis. The mean length of macronuclear DNA from cells allowed to undergo repeated autogamies has been determined previ-

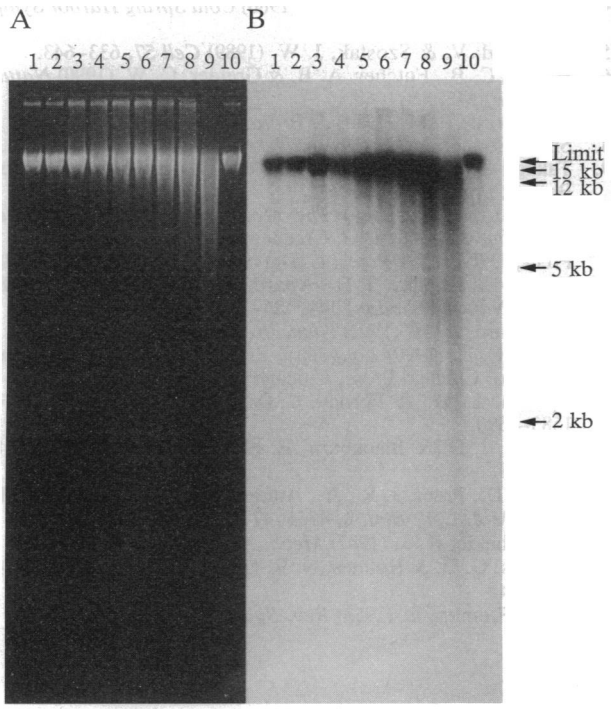

FIG. 2. Progressive breakage of aged DNA without loss of telomeric sequences. (*A*) Total genomic DNA from d48 cell line 3 at incremental points of aging resolved on a 1.0% agarose gel stained with ethidium bromide. Note that DNA greater than ≈20 kb will migrate as a compact band at the limit mobility (marked "limit"). Lanes and days postautogamy: 1, 4 days; 2, 7 days; 3, 17 days; 4, 23 days; 5, 30 days; 6, 37 days; 7, 45 days; 8, 60 days; 9, 66 days; 10, 4 days. (*B*) DNA transferred to nylon and probed with a ^{32}P 5′-end-labeled $(C_4A_2)_4$ *Paramecium* telomeric oligonucleotide.

ously to be ≈300 kb (11). Strikingly, the mean size of the extracted DNA progressively decreased in all lines during clonal aging, with the mean size of the last DNA sample, four fissions before senescence, as shown in Fig. 2, being ≈12 kb. Control experiments done by preparing DNA in parallel from old and young cell cultures eliminated the possibility that degradation of DNA during the extraction procedure could account for these findings. We cannot formally eliminate the possibility that the DNA does not exist in the macronucleus in such fragmented form and that instead, during the process of cell lysis, nuclease activity degrades DNA. However the susceptibility of macronuclear DNA to such nuclease activity, or the level of nuclease activity itself, would have to increase progressively with clonal fission number. We conclude that over the course of clonal aging the mean size of macronuclear DNA decreases by more than an order of magnitude.

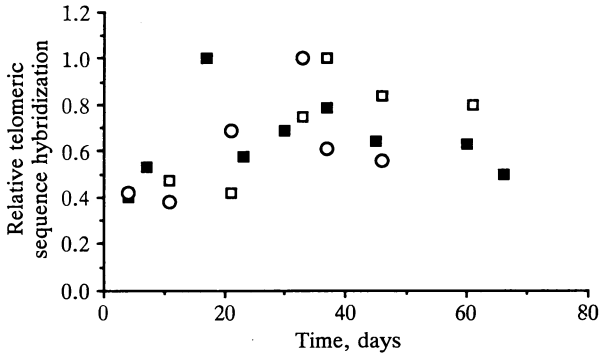

FIG. 3. Relative genomic $(C_4A_2)_4$ hybridization with increasing age. Total genomic DNA from aged cell lines was immobilized onto nylon membranes using a slot blot apparatus. The probe was a ^{32}P 5′-end-labeled $(C_4A_2)_4$ telomeric oligonucleotide. Relative hybridization intensities were obtained by PhosphorImager (Molecular Dynamics) analysis and then normalized to α-tubulin macronuclear sequences. More than 90% of the $(C_4A_2)_4$ hybridization was due to DNA sequences located within 0.5 kb of chromosomal termini, as determined by BAL-31 digestion and Southern blotting (data not shown). Solid squares, d48 cell line 3; open squares, 51 cell line 1; open circles, 51 cell line 2 (see Table 1 for fission rates).

Are the shortened macronuclear DNAs healed by addition of new, full-length telomeres, as has been shown for DNA transfected into *Paramecium* macronuclei a few fissions after autogamy (17)? As shown above, mean telomere length remains essentially constant throughout clonal aging, while the mean macronuclear chromosomal molecular weight decreases by >10-fold. Therefore, if all newly fragmented DNA was healed by the addition of full-length telomeres, an order of magnitude increase in total telomeric sequence content would be predicted. However, quantitative slot blot analysis (Fig. 3) showed no significant upward trend throughout clonal aging.

DISCUSSION

In our attempt to study whether the mechanism underlying senescence in *Paramecium* might be telomere shortening, we examined genomic DNA from cells at different stages of clonal aging. Analysis of a specific macronuclear terminus and total macronuclear genomic termini showed that telomeric DNA sequences are not shortened during the interval of decreased fission rate and cellular death. However, the mean size of macronuclear genomic DNA extracted at various times during clonal aging decreased dramatically. Macronuclear DNA damage has been proposed previously to cause clonal aging in *Paramecium* (8, 9, 18). Previous work has also provided evidence for increased frequencies of nicks in macronuclear DNA during clonal aging (19). Our results reveal that the extent of DNA damage may be much greater than previously suspected.

The results in the present work, as well as previous observations, suggest a model that satisfactorily accounts for the many observations that have been found associated with the aging process of *Paramecium*. Breaks in DNA could be the result of incomplete DNA repair after excision of DNA bases altered by oxidative damage or other forms of DNA damage. If one nick or gap remained unrepaired in a macronuclear DNA molecule before the replication fork passed through the break during the next round of DNA synthesis, then one parental strand would give rise to two shorter daughters, and the other unnicked strand would give rise to a daughter duplex of the original size. The mean size of macronuclear DNA would therefore decrease as the average length of the DNA fell. However, the impact of such unrepaired breaks on mean DNA size would become progressively less if they occurred at a fixed frequency per cell fission. Yet we observed that the mean size continued to fall dramatically even late in the clonal fissions (Fig. 2, lanes 8 and 9). This could be accounted for by the decreasing efficiency of repair during late stages of clonal aging, consistent with previous findings of decreased efficiency of diverse cellular functions during clonal aging (10).

How can *Paramecium* cells continue to function through many cell divisions in the face of such extensive DNA damage? The macronuclear genome is present in ≈1500 copies in G_1, so considerable damage to many gene copies may be tolerated before the copy number of any given essential gene falls below that required for function or cell viability. The gradual course of aging, with its progressive decline in fission rate, is consistent with a model in which genic balance could be upset in various ways that would

impair cell functions, gradually slowing the fission rate, before the eventual loss of functional gene copies below the threshold level leads to cell inviability.

It was more than 40 years ago that Faure-Fremiet (20) proposed that an important function of autogamy and conjugation is to replace the old macronucleus. Here we have shown that macronuclear telomere maintenance is not noticeably impaired during clonal aging. However, our and others' results showing the accumulation of extensive general DNA damage during clonal fissions can well explain why such replacement is necessary.

We thank members of the laboratory and Dr. John R. Preer, Jr., for helpful conversations and critical readings of this manuscript. This work was supported by the National Institutes of Health Grant GM 26259 (E.H.B.) and the American Cancer Society (D.G.).

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