Journal of Cellular Biochemistry 39:75–85 (1989)

Augmented Nuclease Activity During Cellular Senescence In Vitro

Mark A. Dayton, Piruz Nahreini, and Arun Srivastava

Division of Hematology/Oncology, Departments of Medicine (M.A.D., P.N., A.S.) and Microbiology and Immunology (P.N., A.S.) and Indiana Elks Cancer Center, Indiana University School of Medicine, Indianapolis, Indiana 46223

The molecular correlates of the limited proliferative potential of normal human diploid fibroblasts and extensive single-strand breaks in the genomic DNA of these cells were examined by transfection analyses in which DNA replication could be uncoupled from DNA damage and repair. Both supercoiled (fmI), and restriction endonuclease-cleaved, linear (fmIII) molecules of a well-defined bacterial plasmid DNA, pBR322, were transfected into, and subsequently recovered from, early and late passage fibroblasts. Southern blot analysis revealed that fmI DNA was converted by random nicks into fmII DNA slightly more rapidly in late passage cells compared with cells at early passage. Similarly, fmII and fmIII DNAs also sustained multiple random nicks and no appreciable net religation of free ends of fmIII DNA could be detected at either passage. In addition, the efficiency of in vitro ligation of fmIII DNA recovered from late passage cells was also reduced, compared with that from early passage cells, as determined by Southern blotting. These data suggest that in the absence of DNA replication, a putative nuclease activity may contribute to DNA damage observed in senescent cells, which, in turn, may be causally related to their limited replicative potential.

Key words: DNA damage and repair, DNA transfection, single-strand breaks, DNA ligase, limited replicative lifespan

It is now firmly established that normal human diploid fibroblasts have a limited replicative lifespan in vitro [1–3]. While the precise molecular basis of the limited proliferative potential (senescence) of these cells remains obscure, a number of attractive mechanisms have been proposed by several investigators [4–10]. In particular, the well-documented propensity for single-strand DNA breaks in senescent fibroblasts is of significant interest because of its relevance to impaired DNA replication observed in these cells [11–13]. The single-strand DNA breaks could result from the activity of a putative nicking enzyme in senescent cells, or, alternatively, could be due to decreased amounts and/or activities of DNA repair enzymes [14–16]. Since repair enzymes are likely to be required for DNA replication, [17], and a great

Received August 12, 1987; accepted July 22, 1988.

majority of senescent cells fails to enter S phase of the cell cycle [18, 19], it is possible that the single-strand DNA breaks in senescent cells may be independent of DNA replication. In order to test this possibility directly, we utilized the well-characterized bacterial plasmid pBR322 in DNA-mediated transfection experiments in which DNA replication could be uncoupled from DNA damage and repair. In this report, we document that in the absence of DNA replication, the plasmid DNA suffers rapid, multiple random single-strand breaks in fibroblasts at early and late passage, and suggest that a putative nuclease activity may be responsible for the DNA damage observed in senescent cells.

MATERIALS AND METHODS

Cultured Fibroblasts

Human diploid fibroblast strain WI-38 was obtained from the NIA Aging Cell Repository (Camden, NJ). Cells were grown and maintained in Eagle's minimal essential medium supplemented with 15% fetal calf serum (MEM). Cultures were routinely passaged, and their cumulative number of mean population doublings (MPD) was determined, as described previously [20].

Isolation of DNA

The bacterial plasmid pBR322 obtained from the American Type Culture Collection (Rockville, MD) was propagated in *E. coli* HB101 [21] and purified by centrifugation on cesium chloride-ethidium bromide density gradients essentially as described previously [22]. Low molecular weight DNA from fibroblasts was isolated by the method described by Hirt [23], except that the cell lysate was treated with 100 μg/ml of proteinase K, as described previously [24]. Total genomic DNA was isolated essentially as described by Wigler et al. [25].

DNA Transfection

Early and late passage fibroblasts were transfected by the DEAE-dextran method, as described previously [26]. Briefly, unless otherwise stated, 70% confluent dishes (100 mm) were washed once with phosphate-buffered saline (PBS), pH 7.0, and incubated in a 1.0-ml solution containing 50 mM HEPES, pH 7.01, 500 μg DEAE-dextran, and 1 μg of pBR322 DNA for 30 min at room temperature. The transfection solution was removed, cells were washed once with PBS and lysed immediately, or incubated at 37°C for various times in MEM, as described above. Both covalently closed, supercoiled pBR322 (fmI) and restriction enzyme-cleaved linearized (fmIII) DNAs were transfected under identical conditions. The plasmid was linearized by digestion to completion with either Bam H1 or Pst I restriction endonucleases under the conditions recommended by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, MD). Mock transfections without pBR322 DNA or treatment of pBR322 DNA with Hirt extraction protocol were also carried out as controls.

Southern Blotting and Hybridization

Low molecular weight DNA samples isolated from fibroblasts after transfections were electrophoresed, with or without digestion with restriction endonucleases, on 1.5% agarose horizontal slab gels and transferred to nitrocellulose filters (0.45

μm, Schleicher and Scheull, Keene, NH), as described previously [27]. Hind III-cleaved PM2 DNA fragments were coelectrophoresed to serve as size markers. Baked filters were hybridized in 5 × SSC, 50% formamide at 42°C for 16–24 h with the ^{32}P-labeled pBR322 DNA probe (specific activity ranging from 0.2 to 1 × 10^9 cpm/μg DNA) radiolabeled by the hexanucleotide primer labeling method described by Feinberg and Vogelstein [28]. Filters were washed under stringent conditions (0.1 × SSC at 68°C) and autoradiographed at −70°C, usually for 2–4 h.

Statistical Analysis

Autoradiographs were scanned at various exposures using a densitometer, and standard deviations of area-integration were calculated. The statistical significance of the data was determined by the use of two-sided Student's *t* test.

RESULTS

We transfected normal human diploid fibroblasts at early (MPD < 25) and late (MPD > 50) passage with equivalent amounts of covalently closed supercoiled (fmI) and Bam H1- or Pst I-linearized (fmIII) pBR322 DNA and, at various time points, isolated the low molecular weight DNA. This was electrophoresed on agarose gels, Southern blotted, and probed with ^{32}P-labeled pBR322. A representative Southern blot for such an experiment is depicted in Figure 1. Both fmIII (lane 1) and fmI (lane

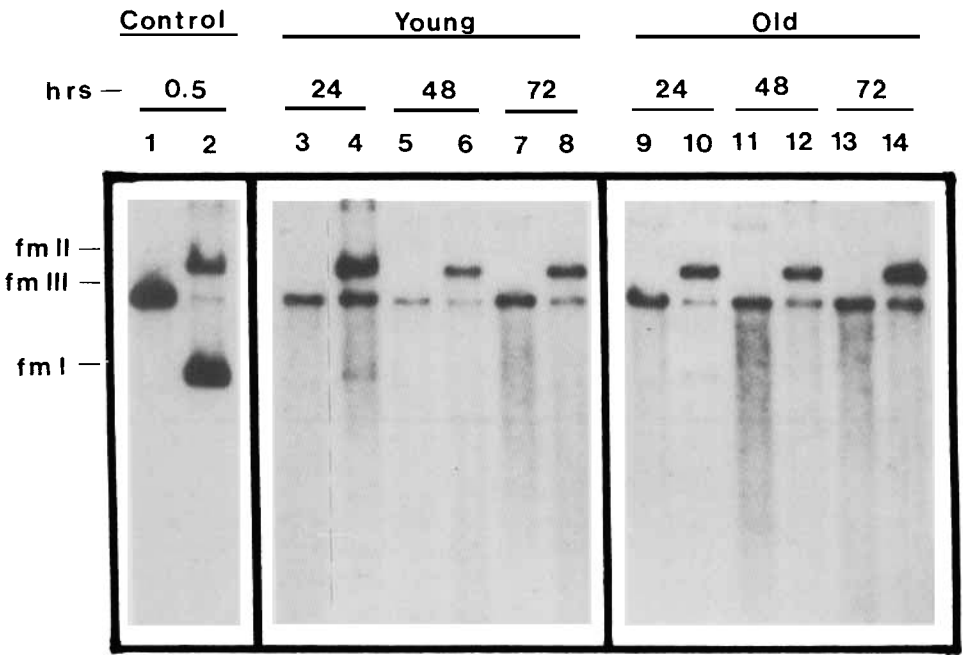

Fig. 1. Southern blot analysis of the fate of the bacterial plasmid pBR322 DNA transfected into and recovered at various times from normal human diploid fibroblast strain WI-38 at 24 MPD **(young)** and 52 MPD **(old)**. Lanes **1, 3, 5, 7, 9, 11,** and **13** represent Bam H1-linearized fmIII DNA, and lanes **2, 4, 6, 8, 10, 12,** and **14** represent fmI DNA. Approximately equal numbers of cells (70% confluence) were transfected with 1 μg of pBR322 without any carrier DNA and analyzed as described under Materials and Methods.

2) pBR322 DNA when transfected into, and immediately recovered from, fibroblasts at early passage remained unchanged except for low-level conversion of fmI DNA to fmII DNA. A similar pattern was obtained with fibroblasts at late passage. Untransfected fmI DNA, when subjected to the Hirt extraction procedure, remained unaltered (data not shown). After transfection, when cells were incubated at 37°C for 24 h, fmIII molecules remained electrophoretically unaltered in both early (lane 3) and late passage (lane 9) fibroblasts. FmI molecules, however, were converted to fmII molecules by 24 h in both early (lane 4) and late passage (lane 10) fibroblasts. By 48–72 h, there was quantitative conversion of fmI molecules to fmII and fmIII in fibroblasts at both early (lanes 6 and 8) and late (lanes 12 and 14) passage. FmIII molecules remained unaltered throughout, although with time there appeared to be an increasing degree of degradation of these molecules, as evidenced by the "smear" running ahead of the main band. No discrete size fragments were detected, suggesting that the degradation was random. Southern blot analysis of the total genomic DNA isolated from both early and late passage fibroblasts transfected with pBR322 failed to detect any hybridization with the plasmid probe (data not shown).

We next carried out a short time-course experiment in which low molecular weight DNA was isolated from early and late passage cells at 1, 2, 4, and 8 h post-transfection and subjected to the same analysis as described above. The results of these experiments are shown in Figure 2. Once again, while fmIII DNA remained unaltered in cells at early (lanes 1, 3, 5, and 7) and late (lanes 9, 11, 13, and 15) passage for up to 8 h, most of the fmI DNA was converted into fmII DNA by 8 h in fibroblasts at early passage (lanes 2, 4, 6, and 8). It is interesting to note that the conversion rate appeared to be more rapid in cells at late passage (lanes 10, 12, 14, and 16). By 24 h, however, fmI DNA disappeared completely in both early (lane 8°) and late (lane 16°) passage cells, as observed before. Quantitation of the conversion of fmI to fmII DNA molecules in early and late passage fibroblasts was carried out by densitometric scanning of autoradiographs from several experiments. These data are presented in Table I. It is evident that the ratio of fmII/fmI increased with time in both early and late passage cells, but the rate of this conversion, on an average, was

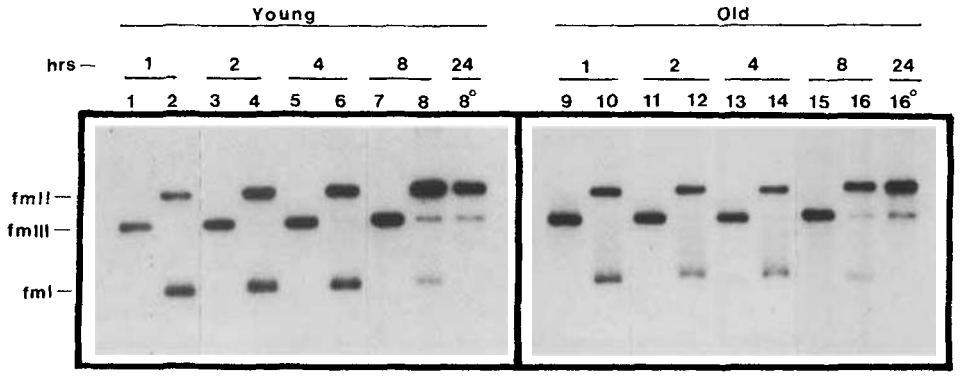

Fig. 2. Southern blot analysis of short-term plasmid pBR322 DNA transfections into fibroblasts at early and late passage. These experiments were carried out exactly as described under the legend to Figure 1, except that the low molecular weight DNA was isolated from transfected cells 1, 2, 4, 8, and 24 h post-transfection. Lanes **1, 3, 5, 7, 9, 11, 13,** and **15** represent Pst I-linearized fmIII DNA, and lanes **2, 4, 6, 8, 10, 12, 14,** and **16** represent fmI DNA.

TABLE I. Quantitative Analysis of Various Forms of pBR322 DNA Recovered From Early and Late Passage Fibroblasts

Time post-transfection (hours)	% Distribution of pBR322 DNA[a]							
	Young				Old			
	fmI	fmII	fmIII	fmII/fmI	fmI	fmII	fmIII	fmII/fmI
0.5	57.7	40.2	2.1	0.70 ± 0.12	42.4	57.6	—	1.36 ± 0.22*
1	44.5	50.9	4.5	1.14 ± 0.20	39.0	61.0	—	1.56 ± 0.18*
2	35.1	60.2	4.7	1.72 ± 0.17	30.0	62.0	8.0	2.10 ± 0.86**,[b]
4	26.7	66.3	7.0	2.48 ± 0.51[b]	22.1	65.7	12.2	2.99 ± 0.41**
8	14.7	73.4	11.9	4.99 ± 1.14	11.6	71.0	17.4	6.12 ± 0.87**
24	—	81.6	18.4		—	79.9	20.1	

[a]Percent distribution of various forms of pBR322 DNA was determined from densitometric scanning of autoradiographs at various exposures from several experiments (n = 3). fmII/fmI = ratio ± standard deviations are indicated. —, not detectable.
[b]n = 2.
*$P < 0.02$, young vs. old.
**$P \geqslant 0.05$, young vs. old.

approximately 20% faster in late passage cells. These results suggest the possibility that senescent fibroblasts possess an augmented level of a putative nuclease activity responsible for the observed differences.

It remained possible, however, that the observed differences in the rate of conversion of fmI to fmII pBR322 DNA molecules were a direct consequence of cell cycling status of the two populations examined, especially in early passage cells, which are capable of active cellular growth. Such a possibility was investigated in experiments in which early passage cells were plated at various cell densities to obtain early-log, mid-log, late-log, and confluent stages of growth. Low molecular weight DNA isolated from 100%, 75%, 50%, and 25% confluent WI-38 cells (MPD = 26) at 0.5, 4, and 8 h post-transfection were analyzed on Southern blots essentially as described above. Such a blot is depicted in Figure 3. It is interesting to note that the cellular growth rate had no significant effect on the extent of single-strand DNA breaks, and the total amount of fmI DNA was gradually reduced to nearly the same extent in cells at various stages of the growth curve. Similar results were obtained with cells at late passage (data not shown). These results document that the observed differences were senescence-specific and were independent of the cycling status of the cell populations examined.

It then became imperative to document the existence of single-strand breaks in the plasmid DNA transfected into, and recovered from, fibroblasts at early and late passage. Low molecular weight DNA was isolated from these cells 48 and 72 h post-transfection of fmI and fmIII DNA, and equivalent amounts of DNA samples were electrophoresed with and without prior digestion with single-strand specific nuclease S1 under conditions in which native, double-stranded DNA is relatively resistant to S1 digestion [29]. These samples were analyzed on a Southern blot; such a blot is presented in Figure 4. Once again, no fmI DNA could be detected after 48 or 72 h (lanes 2, 4: early passage; lanes 6, 8: late passage), and random degradation of fmII and fmIII DNA molecules was apparent. It is interesting to note, however, that the same DNA samples were extremely sensitive to degradation by S1 nuclease (lanes 1° to 4°: early passage; lanes 5° to 8°: late passage), strongly suggesting that both fmII and fmIII DNAs contained extensive single-strand breaks. It is also noteworthy that

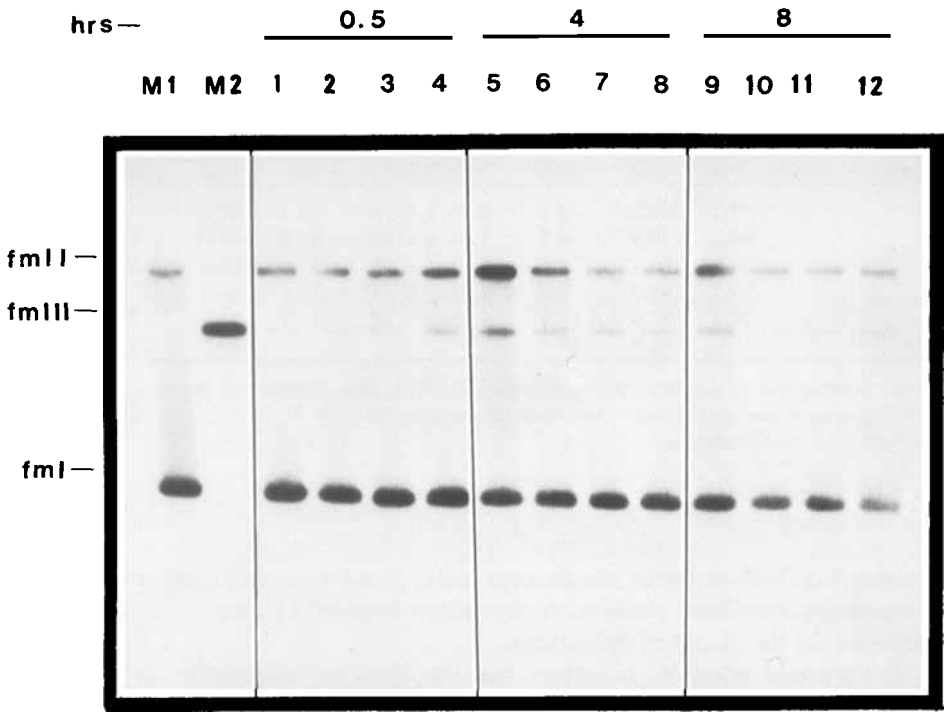

Fig. 3. Southern blot analysis of pBR322 DNA transfected into and recovered from fibroblast strain WI38 (MPD = 26) at various stages of the growth curve. Cells were plated at 100% confluence (lanes **1, 5,** and **9**), 75% confluence (lanes **2, 6,** and **10**), 50% confluence (lanes **3, 7,** and **11**), and 25% confluence (lanes **4, 8** and **12**). Low molecular weight DNA was recovered from these cells at 0.5 h (lanes 1–4), 4 h (lanes 5–8), and 8 h post-transfection (lanes 9–12) and analyzed essentially as described above. Lanes marked **M1** and **M2** represent untransfected fmI and fmIII pBR322 DNA molecules, respectively, coelectrophoresed to serve as controls.

there was a marked diminution in the hybridization intensity of the "smear" following the S1 nuclease treatment, indicating that even the shorter, nested DNA fragments contained single-strand breaks. Similar results were obtained when DNA samples were subjected to electrophoretic fractionation on alkaline agarose gels. A representative denaturing gel is shown in Figure 5. pBR322 DNA recovered 4 h post-transfection from both early and late passage fibroblasts produced significant smearing, and although the overall intensity of the smear decreased with time, these results document the extensive single-strand breaks suffered by transfected DNA molecules within these cells.

We also found it intriguing that upon transfection into and recovery of fmIII molecules from fibroblasts, no net ligation of free ends could be detected, despite a 5-bp sequence complementarity generated by Bam H1 or Pst I restriction endonucleases. In order to examine the structural integrity of the free ends of fmIII DNA, equivalent amounts of DNA transfected into, and recovered from, early and late passage fibroblasts 0.5–4 h post-transfection were incubated with T4 DNA ligase in vitro and analyzed on Southern blots essentially as described above. Such a blot is presented in Figure 6. It is evident that while the control, untransfected fmIII plasmid DNA ends could be religated in vitro (not shown), fmIII DNA recovered from early

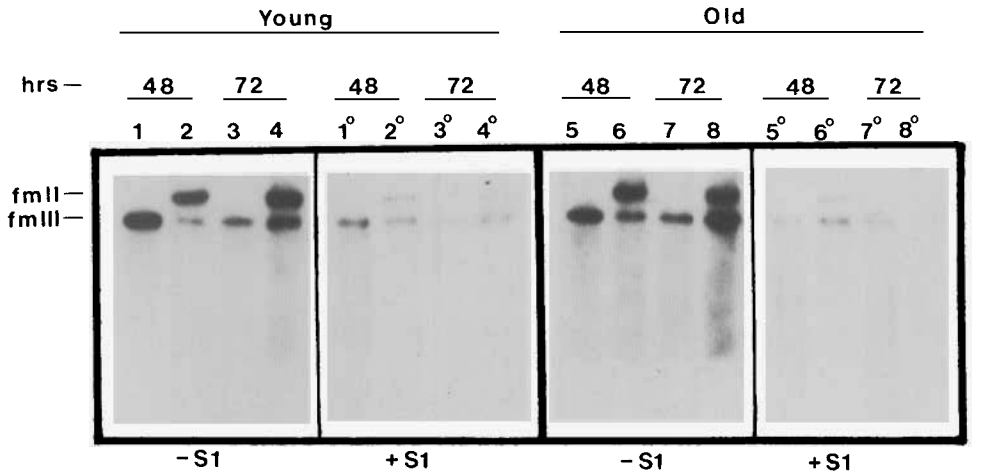

Fig. 4. Southern blot analysis of S1-nuclease sensitivity of plasmid pBR322 DNA transfected into and recovered from early and late passage human diploid fibroblasts. Equivalent amounts of low molecular weight DNA samples recovered at 48 and 72 h were electrophoresed on agarose gels with and without digestion with 1 unit of S1 nuclease in 0.2 M NaCl, 33 mM sodium acetate, pH 4.5, 0.03 mM zinc sulfate at 37°C for 30 min as described previously [29].

passage fibroblasts soon after transfection could be only partially religated into fmII and various concatemeric forms, and the efficiency of religation diminished progressively with time. Similar results were obtained with fmIII DNA recovered from late passage cells, except that the religation efficiency was reduced even further. Quantitation of conversion to fmII molecules upon DNA ligation in vitro was also carried out by densitometric scanning of the relevant areas of autoradiographs from several experiments. These data are presented in Table II. While approximately 85% of the untransfected fmIII molecules could be completely religated in vitro, the extent of religation of these molecules upon transfection was significantly reduced with time, and the effect appeared to be more pronounced in fmIII DNA molecules recovered from senescent fibroblasts. These results document that in addition to the internal single-strand breaks in transfected plasmid DNAs, the free ends are also susceptible to degradation by the putative nuclease function, and this activity may be augmented during cellular senescence in vitro.

DISCUSSION

Although the existence of extensive single-strand breaks in the genomic DNA of senescent cells has been proposed as a possible etiology for the limited replicative potential of normal human diploid fibroblasts [30–33], the following scenarios must be considered. First, single-strand breaks occur randomly throughout the cell cycle, and when the cell enters the S phase, these breaks are repaired by DNA ligases following DNA replication [34–37]. This process may indeed occur in fibroblasts at early passage. Since senescent cells fail to enter the S phase, the single-strand breaks remain unrepaired [18–20]. Second, DNA repair by ligases may be independent of DNA replication, and senescent cells may be deficient in DNA ligases. A cogent example of a DNA ligase deficiency in Bloom's syndrome cells has indeed been

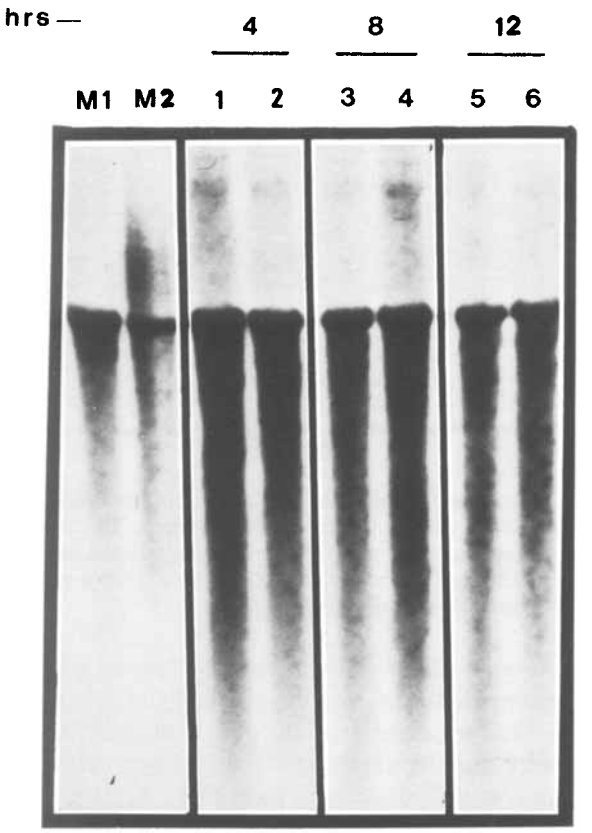

Fig. 5. Southern blot analysis of pBR322 DNA transfected into and recovered from early and late passage fibroblasts and electrophoresed on alkaline-agarose gels. Low molecular weight DNA from early passage cells (lanes **1, 3,** and **5**) and from late passage cells (lanes **2, 4,** and **6**) was isolated at 4 h (lanes 1 and 2), 8 h (lanes 3 and 4), and 12 h post-transfection (lanes 5 and 6), and equivalent amounts were denatured and electrophoresed on alkaline agarose gels exactly as described previously [22]. Lanes marked **M1** and **M2** represent untransfected fmI and fmIII pBR322 DNA molecules, respectively.

described recently [15, 16]. Third, a putative nuclease activity may be augmented in senescent cells that may or may not be correlated with DNA replication.

In pursuit of answers to these questions, it was first necessary to uncouple DNA replication from DNA damage and repair. We chose the well-characterized bacterial plasmid pBR322 for our experiments to circumvent several potential complications. pBR322 does not replicate in eukaryotic cells nor does it have any sequence homology with the human genome, and it does not integrate into the genome. Therefore, even small differences in either ligation or nicking would not be obscured by the production of newly replicated plasmid DNA, nor would the disappearance of the plasmid be attributable to integration with the high molecular weight genomic DNA. DNA-mediated transfections of pBR322 without the use of any carrier DNA into, and its almost quantitative recovery from, early and late passage human diploid fibroblasts would thus reflect on differences in the extent of modification of the plasmid DNA in these cells.

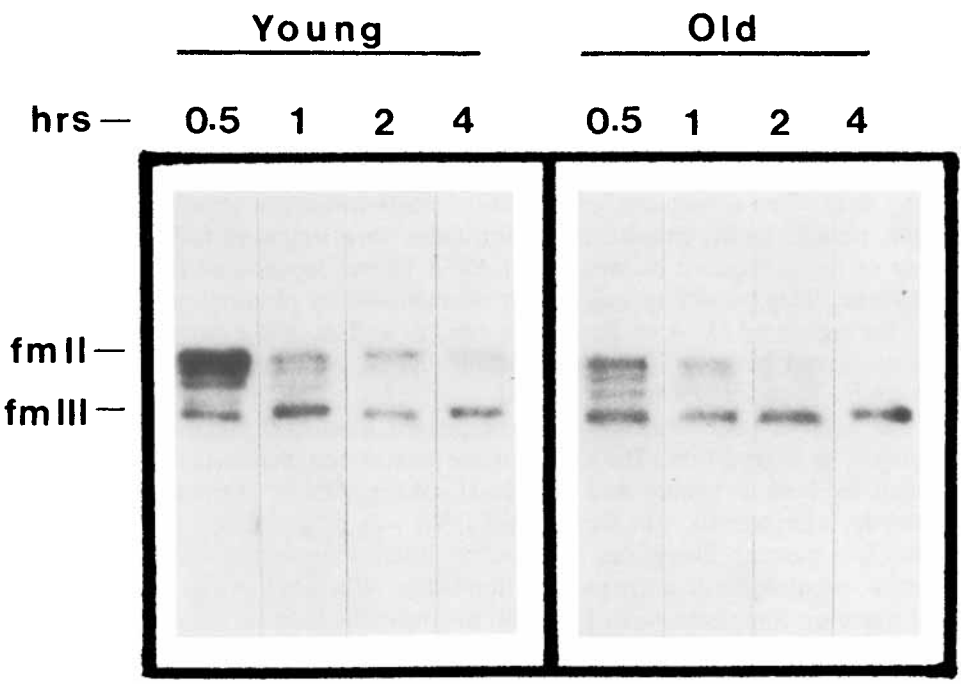

Fig. 6. Southern blot analysis of in vitro ligation of free ends of fmIII DNA recovered from fibroblasts at early and late passage 0.5 to 4 h post-transfection. Equivalent amounts of low molecular weight DNA isolated from transfected cells were incubated with 1 unit of T4 DNA ligase at 15°C for 16 h and analyzed as described under Materials and Methods.

TABLE II. Quantitative Analysis of the Extent of In Vitro Religation of Linear, fmIII pBR322 DNA Recovered From Early and Late Passage Fibroblasts

Time post-transfection (hours)	% Religation of fmIII DNA[a]	
	Young	Old
0.5	74.9 ± 0.4	62.3 ± 0.4*
1	59.6 ± 0.8	50.4 ± 1.3*
1.5	44.6 ± 1.6	53.8 ± 3.2**
2.5	36.7 ± 1.3	34.6 ± 1.6***
4	30.2 ± 1.4	24.2 ± 0.4*
8	22.4 ± 1.0	21.9 ± 2.1***

[a]Percent religation of fmIII DNA (± standard deviations) was determined by densitometric scanning of the relevant areas of autoradiographs at various exposures from several experiments (n = 4 for young, n = 3 for old).

*$P < 0.001$, young vs. old.

**$P < 0.01$, young vs. old.

***$P \geq 0.05$, young vs. old.

In our experiments, no religation of free ends of linearized plasmid DNA could be detected in both early and late passage fibroblasts even with extended incubation, whereas the supercoiled fmI DNA was rapidly converted into its relaxed, circular fmII. The rate of this conversion was independent of the cycling status of the cells and seemed to be more rapid in late passage fibroblasts, compared with early passage fibroblasts. The mechanism of conversion of fmI to fmII molecules evidently involved random nicks. The subsequent generation of fmIII molecules appeared to involve multiple, random nicks, consistent with fortuitous linearization of fmII molecules as a result of the juxtaposed nicking of the DNA strand opposite an existing single-strand break. This possibility was further substantiated by electrophoretic fractionation of the recovered DNA on denaturing gels, as well as by the extreme sensitivity of the recovered plasmid DNA to digestion with S1 nuclease under conditions in which double-stranded DNA was resistant.

The ends of restriction endonuclease-cleaved linearized plasmid DNAs were also subject to degradation. The extent of the loss of complementary end integrity, evaluated by both structural and functional assays, further documented that the potential for circularization of the plasmid DNA was progressively lost with time, and that late passage fibroblasts appeared to inflict more rapid damage on these molecules, compared with early passage fibroblasts. Whether this was because of the loss of sequence complementarity per se or, alternatively, because of a 5′ phosphatase activity, remains to be examined.

Despite the documentation of a putative nuclease activity in human diploid fibroblasts as measured by deliberate introduction of foreign DNA into these cells, several questions still remain unanswered and must be addressed. For example, our data do not indicate the intracellular site of the putative nuclease action. Also, it is unlikely that the transfected DNA is present as a DNA-protein complex and, therefore, it may be more susceptible to degradation, compared with the chromosomal DNA. Finally, whether specific DNA sequences capable of intracellular DNA replication would undergo similar alterations and modifications remains to be elucidated.

In summary, several lines of evidence document an increased propensity for single-strand DNA breaks in senescent fibroblasts. We postulate that in the absence of DNA replication, a putative nuclease function may be responsible for the DNA damage observed in senescent cells, which, in turn, may be causally related to their limited capacity for proliferation.

ACKNOWLEDGMENTS

This research was supported in part by an American Cancer Society Institutional Grant I-161A, a grant from the Phi Beta Psi sorority, and a Grant-in-Aid from the American Heart Association, Indiana Affiliate, Inc., to A.S. M.A.D. was supported by a National Institutes of Health Training Grant, IT32 AM 07519. We thank Sandra Jackson for expert technical assistance, and Stephanie Moore for excellent secretarial assistance during the preparation of this manuscript.

REFERENCES

1. Hayflick LE, Moorehead PS: Exp Cell Res 25:585–621, 1961.
2. Finch CE, Hayflick LE: "Handbook of the Biology of Aging." New York: Van Nostrand, 1977.

3. Sohal RS, Birnbaum LS, Cutler RG. "Molecular Biology of Aging: Gene Stability and Gene Expression." New York: Raven Press, 1985.
4. Orgel LE: Proc Natl Acad Sci USA 49:517–521, 1963.
5. Bell E, Marek LF, Levinstone DS, Merrill C, Sher S, Young IT, Eren M: Science 202:1158–1163, 1978.
6. Smith JR, Lumpkin CKL Jr: Mech Ageing Dev 13:387–392, 1980.
7. Bunn CL, Tarrant GM: Exp Cell Res 727:385–396, 1980.
8. Burmer GC, Zeigler CJ, Norwood TH: J Cell Biol 94:187–192, 1982.
9. Maciera-Coelho A: Mech Ageing Dev 27:257–262, 1984.
10. Hayflick LE: Mech Ageing Dev 28:177–185, 1984.
11. Price GB, Modak SP, Makinodan T: Science 171:917–920, 1971.
12. Wheeler KT, Lett JT: Proc Natl Acad Sci USA 71:1862–1865, 1974.
13. Turner DR, Morley AA, Seshadri RS, Sorrell JR: Mech Ageing Dev 17:305–309, 1981.
14. Rothstein M: "Biochemical Approaches to Aging." New York: Academic Press, 1982.
15. Willis AE, Lindahl T: Nature 325:355–357, 1987.
16. Chan JYH, Becker FF, German J, Ray JH: Nature 325:357–359, 1987.
17. Arrand JE, Willis AE, Goldsmith I, Lindahl T: J Biol Chem 261:9079–9082, 1986.
18. Cristofalo VJ, Sharf BB: Exp Cell Res 76:419–427, 1973.
19. Olashaw NE, Kress ED, Cristofalo VJ: Exp Cell Res 149:547–554, 1983.
20. Srivastava A, Norris JS, Reis RJS, Goldstein S: J Biol Chem 260:6404–6409, 1985.
21. Bolivar F, Backman K: Methods Enzymol 68:245–267, 1979.
22. Maniatis T, Fritsch EF, Sambrook J: "Molecular Cloning—A Laboratory Manual." New York: Cold Spring Harbor Press, 1982.
23. Hirt B: J Mol Biol 26:365–369, 1967.
24. Srivastava A, Lusby EW, Berns KI: J Virol 45:555–567, 1983.
25. Wigler M, Sweet R, Sim G-K, Wold B, Pellicer A, Lacy E, Maniatis T, Silverstein S, Axel R: Cell 16:777–785, 1979.
26. Samulski RJ, Srivastava A, Berns KI, Muzyczka N: Cell 33:135–143, 1983.
27. Southern EM: J Mol Biol 98:503–517, 1975.
28. Feinberg AP, Vogelstein B: Anal Biochem 132:6–13, 1983.
29. Vogt VM: Methods Enzymol 65:254–271, 1979.
30. Epstein J, Williams JB, Little JB: Biochem Biophys Res Commun 59:850–857, 1974.
31. Benn PA: Am J Hum Genet 28:465–473, 1976.
32. Maciera-Coelho A: Gerontol 26:276–282, 1980.
33. Dutkowski RT, Lesch S, Staianocoico L, Thaler H, Darlington GJ, Weksler ME: Mutat Res 149:505–512, 1985.
34. Soderall S, Lindahl T: FEBS Lett 67:1–7, 1976.
35. Mezzina M, Sarasin A, Polite N, Bertazzoni U: Nucleic Acids Res 12:5109–5122, 1984.
36. Teraoka H, Sumikawa T, Tsukada K: J Biol Chem 261:6886–6892, 1986.
37. Niedermuller H, Hufecker C, Skalicky M: Mech Ageing Dev 29:221–238, 1985.