Senescent Human Fibroblasts Have a Post-transcriptional Block in the Expression of the Proliferating Cell Nuclear Antigen Gene*

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The product of the proliferating cell nuclear antigen (PCNA) gene is the co-factor of DNA polymerase δ, which is required for cellular and viral DNA replication. Its steady-state mRNA levels are growth-regulated in young human diploid fibroblasts (HDF) as well as in many other cell types. In senescent HDF, PCNA mRNA is not detectable. However, the PCNA gene is transcribed in senescent HDF as efficiently as in young cells. Furthermore, PCNA hRNA is easily detectable by reverse transcriptase-polymerase chain reaction in both senescent and young HDF, and the levels are essentially similar. These results indicate that in senescent HDF which are incapable of synthesizing cellular DNA, one of the genes coding for a protein of the DNA-synthesizing apparatus is still transcribed, but the product fails to be processed into mature mRNA.

Cellular senescence of human diploid fibroblasts (HDF) in culture is characterized by reproductive failure. Senescence occurs after a period of replication, the number of population doublings being inversely proportional to the age of the fibroblasts' donor (1-4). One of the most studied strains of HDF are WI-38 cells (5), which are capable of a period of high proliferative activity followed by a gradual decrease in growth rate and an increase in the fraction of cells arrested in the non-replicative phase (6).

The expression of growth-regulated genes, i.e. genes whose expression is regulated by mitogenic stimuli, is in general an excellent indicator of the proliferative state of a cell population (7). In young WI-38 cells as in many other cell types, steady-state levels of several growth-regulated mRNAs (for instance, c-myc, c-fos, ADP/ATP translocase, vimentin, calmodulin, ornithine decarboxylase, and others) markedly decrease or become undetectable when proliferating cells become quiescent (8). In the case of thymidine kinase, another growth-regulated gene, even the hRNA is no longer detectable in quiescent cells, despite the use of a very sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) (9), as previously reported (10). It would seem reasonable that in senescent cells, the expression of growth-regulated genes was to be decreased, as in young non-proliferating cells, but this is not always the case. Cristofalo and co-workers (11) had noted that thymidine kinase activity remained high in senescent WI-38 cells, and Rittinger et al. (12) were the first to show that mRNA levels of several growth regulated genes (c-myc, vimentin, ornithine decarboxylase, thymidine kinase, and others) were as high in serum-stimulated senescent cells as in young cells. This was confirmed by Paulsson et al. (13) who, using the AG1523 strain of HDF, added c-fos to the list of genes inducible by growth factors in senescent cells. However, more recently, Seshadri and Campisi (14), using WI-38 cells, while confirming that c-myc and ornithine decarboxylase mRNAs were equally expressed in young and senescent cells, reported that c-fos was not expressed in the latter cells and that the failure of expression was at the transcriptional level.

The proliferating cell nuclear antigen (PCNA) is a co-factor of DNA polymerase δ, necessary for cellular and viral DNA replication (15-18) and for cell cycle progression (19, 20). The steady-state PCNA mRNA levels are growth-regulated (21-23), as they are increased by serum and platelet-derived growth factor but not by insulin or platelet-poor plasma. Although intron 4 plays a role in its regulation (24), the increase in mRNA levels that occurs in serum-stimulated cells is largely post-transcriptionally regulated (25). In this paper, we have investigated the expression of PCNA in senescent WI-38 cells.

**MATERIALS AND METHODS**

**Cell Line**—WI-38 human diploid fibroblasts were grown in our laboratories as previously described (4, 6, 8). Young cells used in these experiments were at population doubling level (PDL) 42 (6), while senescent cells were used at PDL 63 and 64. Cells were made quiescent by serum deprivation for 4 days and subsequently restimulated either with 10% fetal calf serum or the indicated growth factors. Percentage of labeled cells was determined by autoradiography.

**RNA Blots**—The RNA was extracted by the method of Chomczynski and Sacchi (26), and Northern blots were prepared by standard techniques (27, 28).

**Run-on Transcription**—The method used was that of Groudine et al. (29) and Greenberg and Ziff (30). The number of nuclei was the same for both young and senescent cells (10 x 10⁶ nuclei/assay), and the same number of counts was hybridized to the filter (8 x 10⁶ cpm) containing the probes (2 μg of DNA/ slot).

**Probes**—The PCNA, histone H3, and β-actin probes were the same as in Ref. 25, as was the negative control, λ DNA. The only exception is in Fig. 1 where the probe used for PCNA is described in the legend. The calycicin (2A9) probe has been described in Ref. 8.

**RT-PCR**—The RT-PCR was carried out essentially as described by Rappollee et al. (9). Exclusion of DNA and quantitation have been described in detail in a previous paper (10). In addition, absence of DNA contamination was confirmed by the fact that no signal was obtained if the reverse transcriptase step was omitted. The input RNA (1 μg) and the number of cycles (40) were kept constant for young and senescent cells. The amplimers used have been described in Ref. 25. In our modification of the procedure (10), the amplification

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products are displayed on a Southern blot filter, to which a specific probe (in our case, from intron 1 of the PCNA gene, Ref. 25) is hybridized. The specific activity of the probe was also kept constant. Under these conditions, the intensity of the signal is reproducible within a factor of 2.

**Growth Factors—**Epidermal growth factor (EGF, Collaborative Research, Inc.) and IGF-1 (AmGen Biologicals) were used at concentrations of 25 and 100 ng/ml, respectively. The dexamethasone was used at a concentration of 55 ng/ml.

### RESULTS

Fig. 1 shows that PCNA mRNA is much decreased in quiescent young WI-38 cells (PDL 42, lane 2) but is clearly detectable in cells serum-stimulated for 24 h (lane 3). In other experiments, PCNA mRNA was altogether not detectable in G0 cells. No PCNA mRNA is detectable in senescent WI-38 cells, whether in G0, or serum-stimulated, or stimulated by a combination of growth factors (lanes 4–6). Faint bands are detectable in lanes 7 and 8 (see below for an explanation). Lane 1 is a positive control with a cell line (Balb/c 3T3 carrying a transfected human PCNA gene) (25), known to express human PCNA vigorously; the difference between lanes 1 and 3 is negligible. In these experiments, we used subconfluent populations of cells; in senescent cells, the fraction of proliferating cells was less than 2%, while in young cells it was 80%. The same amounts of RNA (20 μg) were loaded in each lane; two control probes were used, histone H3 and calcyclin (31), both of which are known to be expressed in senescent HDF (12). Both probes detect mRNAs from growth-regulated genes, increasing when cells are stimulated to proliferate. Since PCNA is also growth-regulated (21–23), this experiment has been repeated, using different PCNA probes, and the results have been the same; PCNA mRNA is detectable in growing young WI-38 cells (and in exponentially growing HL-60 cells, not shown), but not in quiescent or senescent cells (PDL 63). In the latter case, it does not matter whether the cells have been stimulated or not.

We have examined PCNA transcription in senescent WI-38 cells by the technique of run-on transcription (29,30). Fig. 2 shows that the PCNA gene is transcribed in senescent WI-38 cells; the level of transcription (based on the PCNA/β-actin ratio) is roughly the same as in young WI-38 cells or in Balb/c 3T3 cells carrying a human PCNA gene (25), i.e. approximately 1:10. The important point here is that the PCNA gene is transcribed in senescent cells. The incorporation of the precursors into nascent RNA in the run-on assay is 98% inhibited by the addition of 2 μg/ml α-amanitin (data not shown).

To detect transcriptional activity, we have also used RT-PCR, which is very sensitive (9, 10, 25) and essentially gives steady-state levels of hRNA. RT-PCR does not measure transcription rates like run-on assays but gives hRNA levels, determined in turn by both transcription and processing. We reasoned that if PCNA transcripts are detectable in senescent cells, it would confirm the run-on assay, that PCNA is still transcribed. In Fig. 3, the amplifiers used were from exon 1 and intron 1 of the human PCNA gene (25), and the amplification products were hybridized to a Smal probe from intron 1 of the human PCNA gene (this Smal fragment is 5’ to the second amplifier). Lane 1, exponentially growing Balb/c 3T3 cells carrying a human PCNA gene (25); lane 2, unstimulated WI-38 cells; lane 3, stimulated with EGF, IGF-1, and dexamethasone for 16 h; lane 4, with EGF and IGF-1 for 16 h; lane 5, stimulated with EGF only for 16 h; lane 6, stimulated with IGF-1 only for 16 h; lane 7, stimulated with dexamethasone for 16 h. bp, base pairs.

### FIG. 2. Run-on transcription of the PCNA gene in senescent and young WI-38 cells. Run-on assay was carried out as previously described (29, 30) using the same probes reported in Ref. 25. λ DNA was used as the negative control. Panel A, senescent WI-38 cells; panel B, young WI-38 cells.

### FIG. 3. Levels of PCNA hRNA in young, WI-38 cells (PDL 42). hRNA levels were measured by RT-PCR (6, 7). The RNA (1 μg) was amplified with two amplifiers, from exon 1 and intron 1 of the human PCNA gene (25) for 40 cycles (10). After amplification, the amplification products were blotted and hybridized to a Smal probe from intron 1 of the human PCNA gene (this Smal fragment is 5’ to the second amplifier). Lane 1, exponentially growing Balb/c 3T3 cells carrying a human PCNA gene (25); lane 2, unstimulated WI-38 cells; lane 3, stimulated with EGF, IGF-1, and dexamethasone for 16 h; lane 4, with EGF and IGF-1 for 16 h; lane 5, stimulated with EGF only for 16 h; lane 6, stimulated with IGF-1 only for 16 h; lane 7, stimulated with dexamethasone for 16 h. bp, base pairs.

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Fig. 4 shows that PCNA hRNA was also detectable in senescent WI-38 cells, whether unstimulated (lane 2), serum-stimulated (lanes 3 and 4), or growth factor-stimulated (lanes 5 and 6), although at 24 h the signal is very weak. The amplifiers and the probe used were the same as in Fig. 3.

### FIG. 4. Levels of PCNA mRNA in young and senescent WI-38 cells. RNA blots were hybridized to a PstI fragment of the PCNA gene which extends from +4480 on exon 5 of the human PCNA gene to the PstI on the poly linker of pGEM-3. The probe is specific for human PCNA. Lane 1, RNA from exponentially growing Balb/c 3T3 cells carrying the human PCNA gene; lane 2, RNA from young WI-38 cells (PDL 42) in G0; lane 3, RNA from young WI-38 cells serum stimulated for 24 h; lanes 4–8, RNA from senescent cells, respectively, in G0, stimulated for 16 or 24 h with EGF, IGF-1, and dexamethasone, or for 16 h with serum. The senescent cells were from PDL 64. The blot was also hybridized to a cell cycle-specific histone H3 probe (25) and to calcyclin cDNA (8).
Panel C.

**Fig. 4.** PCNA hnRNA levels in senescent WI-38 cells (PDL 63). The procedures were exactly the same as in Fig. 3, except that the RNA came from senescent cells. Lane 1, as lane 1 in Fig. 3; lane 2, unstimulated senescent cells; lane 3, senescent cells serum stimulated for 16 h; lane 4, senescent cells serum stimulated for 24 h; lanes 5 and 6, senescent cells stimulated with EGF, IGF-1, and dexamethasone, respectively, for 16 and 24 h. bp, base pairs.

Panel A, B, B.

**Fig. 5.** PCNA hnRNA and mRNA levels in WI-38 cells. Panel A, hnRNA in young cells; panel B, hnRNA in senescent cells; panel C, mRNA in young cells; panel D, mRNA in senescent cells. Lanes 1–4 refer to 10, 20, 30, and 40 cycles of amplification. For hnRNA, primers and probe were the same as in Figs. 3 and 4. For mRNA, the amplimers were from exons 5 and 6, and the PCNA cDNA (23) was used as the probe. Exposure times: 8 h for hnRNA, 20 min for mRNA. bp, base pairs.

Indeed, the conditions (amount of RNA, number of amplification cycles, specific activity of the radioactive probes, etc.) were all kept strictly the same for both young and senescent cells. The important point is that PCNA transcripts are detectable in senescent cells roughly in the same amounts as in younger cells. The cells used in Fig. 4 were at PDL 63. The experiment was repeated with cells from PDL 64, and the results were the same (data not shown).

The results of Figs. 3 and 4 have been confirmed by comparing (still using RT-PCR) the levels of hnRNA and mRNA for PCNA, with different cycles of amplification (Fig. 5). Panels A and B show the levels of PCNA hnRNA in young and senescent cells after 10, 20, 30, and 40 cycles of amplification. After 30 cycles, there is slightly more hnRNA in young than in senescent cells, but the difference is small. Surprisingly, if the Southern blot is overexposed, a faint signal appears in the 10-cycle lane of senescent cells, but not in the corresponding lane of young cells. Thus, while there may be a difference in PCNA hnRNA levels between young and senescent cells, such a difference is no more than 2-fold, far below the difference in the fraction of proliferating cells (40-fold).

When the mRNA levels are compared, it is clear that PCNA mRNA is abundant in young cells but barely detectable, even by RT-PCR, in senescent cells, regardless of the number of cycles (panels C and D, respectively). The comparison, it should be stressed, is only horizontal. The ratio mRNA/hnRNA cannot be calculated directly because different probes and different times of exposure were used.

**DISCUSSION**

The validity of our conclusion depends essentially on two technical aspects: 1) the residual fraction of proliferating cells in the senescent population; and 2) the accuracy of the two methods used to measure transcription rates and transcripts.

After growth factor stimulation, the percentage of labeled cells was 2% in senescent cells and 90% in young cells; if transcription of PCNA in senescent cells were due to the small fraction of cycling cells, the signals from run-on transcription and RT-PCR should be roughly 2% those of young cells. Although neither run-on assays nor RT-PCR are strictly quantitative, they can easily detect a 2-log difference. The signals, instead, have essentially the same intensity with both methods. In addition, in our RT-PCR, contamination with DNA is carefully excluded and the measurements of hnRNA levels are quantitative within a factor of 2 (10) see also "Materials and Methods". On this basis, the PCNA hnRNA levels are roughly the same in senescent and young cells (the signal from Balb/c 3T3 cells carrying a human PCNA gene serves as a common denominator for the two experiments), and yet, the mRNA is not detectable in senescent cells, while abundant in younger cells. Regardless of the presence or not of a small subpopulation of cycling cells, if transcription and hnRNA amounts are the same and the mRNA amounts are strikingly different this can only indicate the existence of a post-transcriptional block in senescent cells (incidentally, when amplimers from exon 5 and 6 of the PCNA gene were used, together with an intron 5 probe in the RT-PCR, the same results were obtained as in Fig. 3 (25)). It is fair to state, therefore, that in senescent WI-38 cells, PCNA mRNA is not detectable (although two other growth-regulated mRNAs are, and one of them even in higher amounts than in young cells), while transcription rates and transcripts are essentially the same in both populations.

Our results show that the PCNA gene behaves, like c-fos (14), to that category of growth-regulated genes whose mRNA is not detectable in senescent WI-38 cells, which is at variance with several other growth-regulated genes whose expression increases in serum-stimulated senescent cells as efficiently as in young cells (12, 14). As already mentioned (see above), the PCNA product is part of the DNA-synthesizing machinery itself; thymidine kinase is also associated with the DNA-synthesizing apparatus (although dispensable), and yet, it is clearly expressed in senescent cells (12). The first question raised by this and other reports is why some growth-regulated genes are expressed in senescent cells and others are not. c-fos is an early growth-regulated gene (30, 33) but so is c-myc (34), and yet the latter is expressed in senescent cells (12) and the former is not (14). Thymidine kinase and PCNA are both connected with DNA synthesis, but again they behave differently in senescent cells. It is not just a question of growth factor receptors, which are present normally in senescent cells (35, 36); indeed, c-fos, c-myc, and PCNA are all inducible by platelet-derived growth factor only but behave differently in senescent cells. What's more, the fact that the expression of some early genes is impaired while the expression of some late genes is not suggests that some pathways through G0/G1 proceed independently and in parallel.

Even more important is the finding, in the present report, that although PCNA mRNA is not detectable in senescent WI-38 cells, the PCNA gene is transcribed and hnRNA transcripts are detectable.

A translational defect has been reported in senescent IMR-90 (37) and in senescent WI-38 cells (14) for ornithine decarboxylase; a transcriptional repression has been invoked for c-fos by Seshadri and Campisi (14). For PCNA, it seems as if the defect lies between transcription and processing, pointing out how variable and complex are the mechanisms of altered gene expression in senescent cells. This remains, though, the

-- C-D. Chang, P. Phillips, K. E. Lipson, V. J. Cristofalo, and R. Baserga, unpublished data.
first report of the absence of an mRNA coding for a protein of the DNA replication complex, absence due to a post-transcriptional regulation. Two important future questions are: 1) whether the c-fos product affects the processing of PCNA hnRNA; and 2) whether the post-transcriptional, pre-translational block of PCNA extends also to other genes of the DNA-synthesizing machinery, such as DNA polymerase-α, RNA primase, and others.

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