# REINITIATION OF DNA SYNTHESIS IN SENESCENT HUMAN FIBROBLASTS UPON FUSION WITH CELLS OF UNLIMITED GROWTH POTENTIAL

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ABSTRACT

Postreplicative, "senescent" human fibroblasts were fused to HeLa or to SV-40 transformed human fibroblasts with Sendai virus. DNA synthesis was reinitiated in senescent nuclei in a high proportion of the heterodikaryons. The [$^3$H]thymidine labeling index of senescent fibroblast nuclei in heteropolykaryons was a function of the ratio of HeLa to senescent nuclei.

It is now well established that mass cultures and individual clones of human diploid fibroblast-like cells have finite replicative life spans (12). Three lines of evidence suggest that such in vitro clonal senescence may be related to in vivo aging: (*a*) the longevity of a culture is inversely related to the age of the donor (8, 11, 20); (*b*) the longevities of cultures from patients with certain genetically determined progeroid syndromes are sharply reduced (20, 6); and (*c*) cultures from a vascular site predisposed to arteriosclerosis may have comparatively limited life spans (18, 19). We have suggested that such cultures may cease replicating because constituent stem cells eventually undergo a type of terminal differentiation in vitro (21, 22), i.e. that the cessation of replication is attributable to the differential expression of normal genes, analogous to what presumably takes place in stem cell specialized cell transitions in vivo. An alternative view attributes the demise of such cultures to cumulative errors in macromolecular synthesis (24, 25); evidence both for (14, 16) and against this proposal has been published (13, 28, 26). If the terminal differentiation hypothesis were correct, we reasoned that DNA synthesis should be reinitiated upon heterokaryosis with actively replicating cells, since such a positive control of nuclear DNA and RNA synthesis had been clearly demonstrated by Harris and his colleagues in the case of three different types of terminally differentiated cells (nucleated erythrocytes, thoracic duct lymphocytes, and peritoneal macrophages) (10). We have recently described the results of such cell fusion experiments in which the actively replicating partners were cells from early passages of normal diploid fibroblast-like cells at population-doubling level (PDL) 20 or less (23). In those experiments, quite the opposite result was observed; not only did such young fibroblasts fail to rescue DNA synthesis in old nuclei, but their own DNA synthesis was extinguished. However, Harris's demonstrations of the positive control of DNA synthesis utilized cells of unlimited growth potential (HeLa and two cell lines of murine origin, A9 and Ehrlich ascites) as the actively replicating partners (9). We therefore predicted that in heterokaryons between old fibroblasts and SV40 transformed fibroblasts, or between old fibroblasts and HeLa cells, DNA synthesis would be reinitiated in the old nuclei (23). In the present communication, we show how these predictions have been confirmed.

## MATERIALS AND METHODS

Details of the methods of cell fusion, autoradiography, and propagation of Sendai virus and controls have been described elsewhere (23). Cell fusion was accomplished with Sendai virus suspended in chorioallantoic fluid which was placed over the "senescent" human fibroblasts previously plated on 22 $mm^2$ cover slips. The HeLa or SV40 transformed human fibroblasts were added in suspension (approximately 10,000 cells). Controls consisted of culturing the cell lines separately under physical conditions of fusion identical to those of the experimental (i.e. cocultivated) cultures; one-half was exposed to Sendai virus, the other half was exposed to media without serum. The [$^3$H]thymidine labeling indices of monokaryons exposed to the infected chorioallantoic fluid were virtually identical to those exposed to media without serum. The indices of the homodi- and polykaryons in the controls exposed to Sendai virus were similar to those enumerated in the experimental (i.e. cocultivated) cultures displayed in Fig. 2.

For identification of the heterokaryons, each partner was prelabeled with a radioisotope, the old human fibroblast with [$^3$H]methionine and the immortal cells with [$^{14}$C]thymidine. Double layer autoradiography (1) was utilized to exploit the isotopic prelabeling; the higher energy emissions from the $^{14}$C produced silver grains in the second layer of emulsion which was separated from the first by an intervening layer of celloidin (Fig. 1).

In order to detect DNA synthesis in heterokaryons after fusion, the cultures were pulsed with tritiated thymidine for three sequential 24-h periods, the first beginning immediately after the fusion procedure. The labeled thymidine was introduced in fresh medium at the

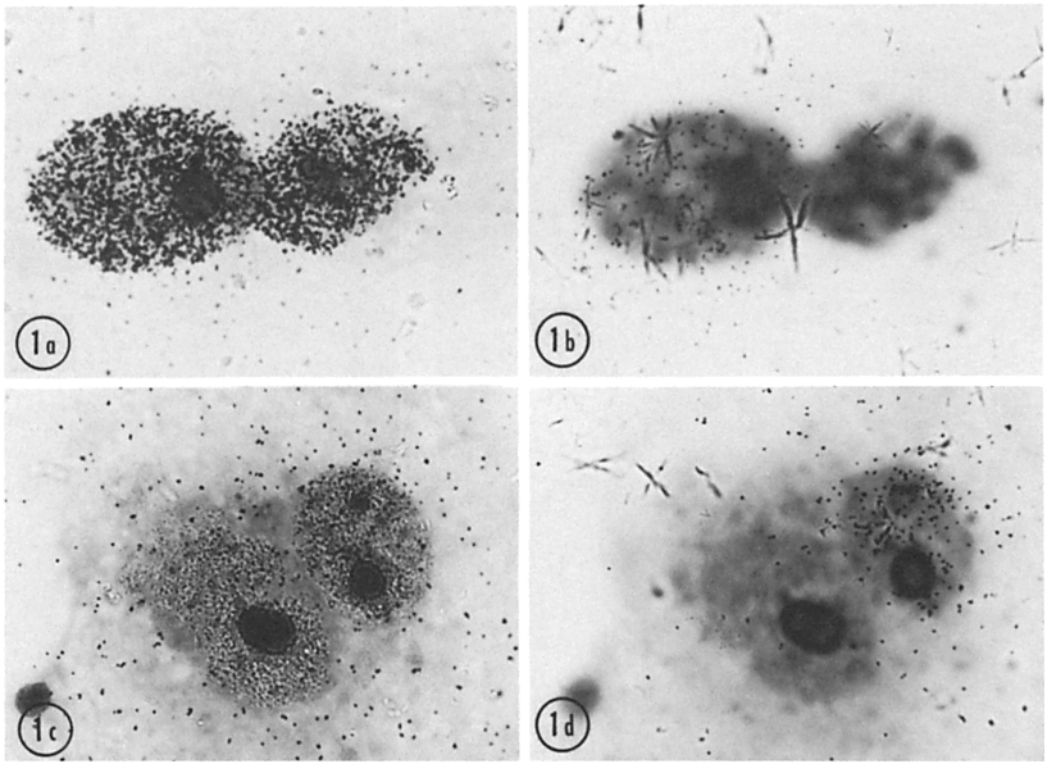


FIGURE 1 Heterodikaryons containing HeLa and senescent fibroblast nuclei. (*a*) Lower layer of emulsion showing silver grains over cytoplasm produced by [*methyl*-$^3$H]methionine prelabel (2 $\mu$Ci/ml, sp act 190 mCi/mmol, New England Nuclear, Boston, Mass.) and dense nuclear labeling produced by the [*methyl*-$^3$H]thymidine test label (0.5 $\mu$Ci/ml, sp act 6.7 Ci/mmol, New England Nuclear). (*b*) Upper layer of emulsion showing silver grains produced by the [*methyl*-$^{14}$C]thymidine prelabel (0.07 $\mu$Ci/ml, sp act 52.7 mCi/mmol, New England Nuclear) in the HeLa nucleus of the heterokaryon illustrated in (*a*); small fissures are seen on the top surface of celloidin. (*c*) Lower layer of emulsion showing an example in which the nuclei are not labeled with the test pulse of [$^3$H]thymidine. A slight increase in grain density due to the [$^{14}$C]thymidine prelabel is apparent over the HeLa nucleus. (*d*) Upper layer of emulsion showing silver grains produced by the [$^{14}$C]thymidine prelabel in the HeLa nucleus of the heterokaryon illustrated in (*c*). ($\times$ 950)

beginning of each pulse period. A 24-h pulse period was selected to ensure that a portion of the cells would have completed an entire round of DNA synthesis, thus producing a dense nuclear label which could be unambiguously identified. With the 24-h pulse used in these experiments, unscheduled or repair DNA synthesis could contribute to the observed labeling indices. However, in our experience as well as that of other investigators (3), a good correlation between the labeling index and the proliferative capacity of the culture has been observed, suggesting that grains produced by repair synthesis do not significantly contribute to the observed [$^3$H]thymidine nuclear labeling. Furthermore, in our previously reported experiments (23), such a degree of putative repair replication clearly could not be observed in old-old or old-young heterokaryons.

Previous experience had indicated no difference in labeling indices between senescent fibroblasts with and without [$^3$H]methionine prelabel. Our experience with [$^{14}$C]thymidine in low-passage proliferating human fibroblasts had indicated that approximately 5% of the [$^3$H]thymidine-labeled nuclei could not be detected because of background grains produced by the [$^{14}$C]thymidine prelabel (23). The high labeling indices observed in the HeLa and SV80 lines indicate that masking of DNA synthesis by the prelabel background was no more than 5% in these experiments. More than 99% of the HeLa and SV80 nuclei were successfully prelabeled with [$^{14}$C]thymidine.

The senescent human fibroblast line used in the first HeLa-old fibroblast fusion (experiment I) (Fig. 2 A, B) and in the SV80-old fibroblast fusion (experiment III) (Fig. 2 E, F) had been initiated from the upper arm skin of a premature infant with a normal male karyotype (line S-1) (23); that used in the second HeLa-old fusion (experiment II) (Fig. 2 C, D) was initiated from a postmortem biopsy from the forearm of a 44-yr old male. At the time of these experiments the former had undergone approximately 62 population doublings and the latter 45. The SV80 line, and SV40 transformed human fibroblast line, was obtained from the laboratory of Dr. George Todaro (27) and the HeLa cells (line M-HeLa) (5) from the laboratory of Dr. George Kenny. All cell lines were negative for mycoplasma under aerobic and anaerobic culture conditions (15).

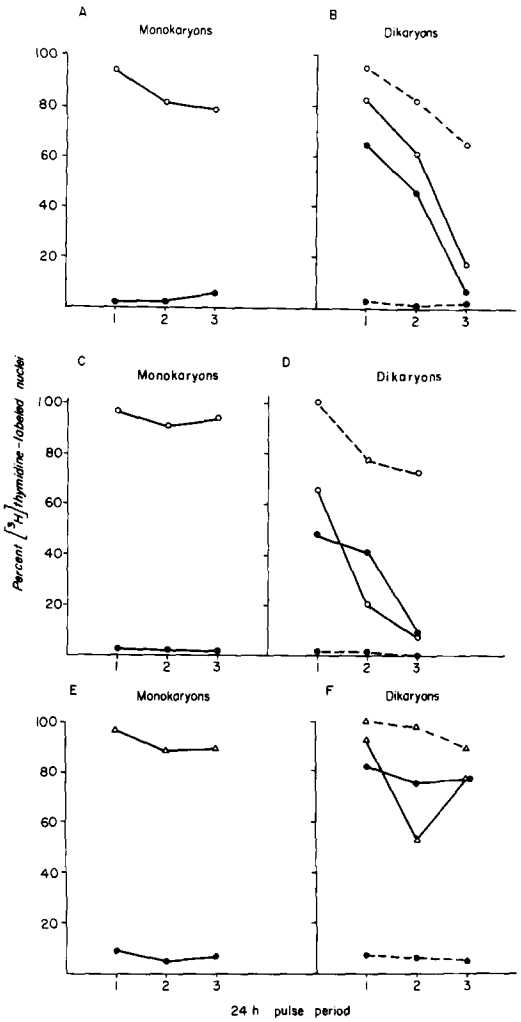


FIGURE 2 [$^3$H]Thymidine indices of monokaryons (*A*, *C*, and *E*) and dikaryons (*B*, *D*, and *F*) enumerated from experimental cultures involving crosses between two different strains of fibroblasts and HeLa cells (experiments II and III) (*A* and *B*, *C* and *D*) and between old fibroblasts and SV80 cells (experiment III) (*E* and *F*). Old fibroblast nuclei in monokaryons and heterodikaryons ●——●; old nuclei in homodikaryons ●----●; HeLa nuclei in monokaryons and heterodikaryons O——O; HeLa nuclei in homodikaryons O----O; SV80 nuclei in monokaryons and heterodikaryons Δ——Δ; SV80 nuclei in homodikaryons Δ----Δ.

## RESULTS

The results of crosses between senescent fibroblasts and HeLa cells and between senescent fibroblasts and SV80 cells are shown in Fig. 1. Each point represents the consecutive counts of 500–1,500 monokaryons and 38–196 homo- and heterodikaryons obtained by screening the preparations under oil immersion (Table I). In both crosses the [$^3$H]thymidine labeling indices of the old fibroblast nuclei in heterodikaryons were substantially higher than of those in old fibroblast monokaryons and homodikaryons (Fig. 2). In the HeLa-old fibroblast crosses, the indices of both HeLa and old nuclei in the heterokaryons declined significantly at the latter pulse periods in both experiments, while in the SV80-old fibroblast cross

TABLE I

*The Numbers of Homo- and Heterodikaryons Counted during Each Pulse Period and the Numbers of the Various Types of Nuclei in Such Dikaryons which Were Labeled with [³H]Thymidine during These Pulse Periods*

| Experiment no. | No. of dikaryons | | | No. of labeled old nuclei | | | No. of labeled HeLa nuclei | | | No. of labeled SV80 nuclei | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *day 1* | *day 2* | *day 3* | *day 1* | *day 2* | *day 3* | *day 1* | *day 2* | *day 3* | *day 1* | *day 2* | *day 3* |
| I | | | | | | | | | | | | |
| HeLa-old heterodikaryons | 67 | 70 | 81 | 43 | 32 | 6 | 55 | 43 | 14 | — | — | — |
| Old homodikaryons | 148 | 171 | 196 | 5 | 0 | 2 | — | — | — | — | — | — |
| HeLa homodikaryons | 32 | 45 | 49 | — | — | — | 61 | 74 | 34 | — | — | — |
| II | | | | | | | | | | | | |
| HeLa-old heterodikaryons | 101 | 82 | 47 | 49 | 33 | 4 | 66 | 17 | 4 | — | — | — |
| Old homodikaryons | 111 | 112 | 55 | 4 | 4 | 0 | — | — | — | — | — | — |
| HeLa homodikaryons | 106 | 119 | 167 | — | — | — | 202 | 189 | 244 | — | — | — |
| III | | | | | | | | | | | | |
| SV80-old heterodikaryons | 38 | 56 | 43 | 31 | 33 | 33 | — | — | — | 35 | 24 | 33 |
| Old homodikaryons | 112 | 121 | 124 | 16 | 15 | 13 | — | — | — | — | — | — |
| SV80 homodikaryons | 37 | 62 | 47 | — | — | — | — | — | — | 74 | 118 | 82 |

The human fibroblast line used in experiments I and III was initiated from the skin of the upper arm of a newborn male infant and the fibroblast line used in experiment II was obtained at autopsy from the forearm skin of a 44-yr old male. The [³H]thymidine labeling indices, as calculated from these data, appear in Fig. 3.

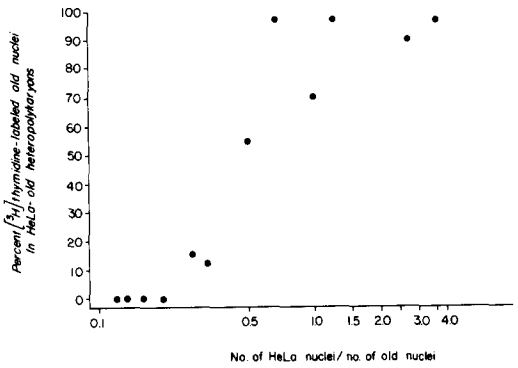


FIGURE 3 The [³H]thymidine labeling index plotted as a function of the ratio HeLa/old fibroblast nuclei in heteropolykaryons. The horizontal axis is the ratio HeLa/old nuclei plotted on a natural log scale. A HeLa-old heteropolykaryon was scored as labeled if one or more old nuclei were labeled.

they remained quite high. No mitotic figures were observed in the heterokaryons.

The [³H]thymidine labeling indices of heteropolykarons were also enumerated (data not shown). In both crosses, but most strikingly in the HeLa-old fibroblast cross, the indices of both HeLa and old nuclei were lower in such heteropolykaryons. Since old nuclei were generally preponderant, it occurred to us that there might be a dose-dependent relationship. In Fig. 3, we graph the ratio of HeLa to old nuclei against the ³H labeling indices of old nuclei in heteropolykaryons. This data is compiled from counts obtained from the 24-h labeling periods showing the highest ³H labeling indices in the heteropolykaryons. The labeling index observed at a 1:1 ratio approximates that observed in the dikaryon displayed in Fig. 1. In the SV80-old fibroblast cross there also appears to be a lower labeling index in polykaryons; however, the number of observation is insufficient to permit a comparable analysis.

## DISCUSSION

We believe that the results presented in this report indicate that with respect to at least one round of nuclear DNA synthesis, the phenotypes of the HeLa and SV80 cells are dominant over the phenotype of the senescent human fibroblasts. There is, however, another interpretation which must be considered. In these experiments, the possibility of selective fusion to a phenotypically young cohort of cells within the senescent population cannot be excluded. Selective fusion could be brought about by decreased viability of certain classes of fused cells or specific changes in the plasma membrane of the senescent phenotype so that they would fuse efficiently only with certain cell types. We favor the former interpretation for the following reasons: (*a*) there was no significant

cell loss observed during these experiments; (*b*) HeLa cells under these conditions appear to fuse with equal efficiency with low passage actively proliferating fibroblasts and with fibroblasts in senescent cultures; and (*c*) 10–20% of the old cells counted were fused to HeLa cells, whereas the [$^3$H]thymidine labeling index remained consistently less than 5%.

It is our belief that these observations support the terminal differentiation hypothesis of in vitro senescence of human fibroblasts because of the similarity to the results obtained by Harris and his associates in their heterokaryon studies with nonreplicating cells of known differentiated function. Our previous results indicated that early-passage proliferating fibroblasts were recessive to the senescent phenotype under the same experimental conditions (23). These two sets of observations suggest that the postmitotic fibroblasts may synthesize a specific repressor compound(s) which inhibits the initiation of DNA synthesis; certain cell lines of apparently unlimited replicative potential may inactivate this repressor or contain a pre-empting gene product (which could be of viral origin).

Chick embryo fibroblasts (9) and embryonic rat lung cells from subcultures of primary cultures (2), both of which were presumably normal diploid cells (although the chick fibroblasts were heavily irradiated), were shown to induce nuclear swelling of chick erythrocytes and, in the case of the rat cells, to reinitiate RNA synthesis in chick erythrocytes. However, to the best of our knowledge, there is no published demonstration that such fusions can induce DNA synthesis, although it seems quite possible that this would follow the reinitiation of RNA synthesis. Proof of the reinitiation of DNA replication in such systems would be of considerable interest; in the light of our previous results (23), they would suggest that differentiated cells are capable of inhibiting nuclear synthetic functions only in their own precursor cells at certain stages of differentiation. If this were the case, the postulated inhibitor compound(s) has a very specific site(s) of action within the cell.

Clearly, these results do not constitute proof of a terminal differentiation model as an explanation of clonal senescence, nor do they rule out loss of replicative function being caused by the accumulation of abnormal macromolecules. However, it seems reasonable to assume that if a cell contained massive quantities and varieties of abnormal proteins and numerous random mutations attributable to abnormal DNA polymerase(s) (as predicted by the error catastrophe theory) (25), a proportion of such abnormal macromolecules would have qualitatively aberrant biological activity so that DNA synthesis could not be efficiently complemented. On the other hand, there is no direct evidence to support this assumption and one could certainly argue about the expected time course, in heterokaryons, of such a dominant effect. Indeed, in the HeLa crosses (but not in the SV80 crosses), there is evidence that the senescent phenotype is dominant in the later pulse periods.

Finally, we emphasize that the observation period in these experiments was comparatively short and that only one aspect of cell cycle function was measured. It remains to be seen to what extent complete and sequential cell cycle function can be restored. Two published synkaryon experiments suggest that this is possible (7, 4). However, these particular experimental designs did not permit an unambiguous conclusion, since the cells which were hybridized may have been derived from residual, actively replicating clones. A modified protocol (17) may provide more definitive evidence.

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