# Molecular and Cell Biology of Replicative Senescence

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Normal eukaryotic cells have only a limited capacity for cell division. This property has been termed the finite replicative life span of cells, and the process that limits cell division has been termed cellular or replicative senescence.

The finite replicative life span of cells was formally described about 30 years ago (Hayflick 1965). Since then, the phenomenon has been studied by a number of investigators, primarily by studying the proliferation of cells in culture. Several important features of replicative senescence have emerged (for review, see Stanulis-Praeger 1987; Goldstein 1990; McCormick and Campisi 1991; Peacocke and Campisi 1991).

First, replicative senescence is particularly stringent in human cells, which virtually never spontaneously immortalize. This is in sharp contrast to cells derived from several rodent species, which spontaneously immortalize at low but detectable frequencies. Nonetheless, when cell cultures are monitored carefully, it is clear that cells derived from very many, if not all, species and organs divide only a limited number of times.

Second, senescence is a stable state and is not apoptotic or programmed cell death. Senescent cells arrest growth with a $G_1$ DNA content but remain viable and metabolically active for long periods of time. Moreover, many genes (but not all genes) remain mitogen-inducible in senescent cells, despite the fact that the cells have lost the ability to respond to mitogens by initiating DNA replication.

Third, both the finite replicative life span phenotype and the final senescent state are dominant phenotypes in somatic cell fusion experiments. Moreover, at least four genetic complementation groups for immortality have been identified. Thus, replicative senescence appears to be a genetically dominant trait and to be controlled by multiple genetic loci.

Fourth, in addition to the cessation of cell proliferation, replicative senescence entails selected changes in differentiated functions. Thus, by the criteria of a stable and irreversible growth arrest and altered cell function, senescent cells resemble terminally differentiated cells. In contrast to terminal differentiation, however, replicative senescence occurs solely as a consequence of completing a finite number of cell divisions.

Despite substantial progress in understanding the prevalence, genetics, and molecular correlates of cell senescence, very little is known about the primary regulatory mechanisms that establish a finite replicative life span and maintain the senescent state. In addition, the biological significance of replicative senescence is incompletely understood. In this paper, we review our results, and those of other workers, which support the concepts that replicative senescence reflects processes which occur during the aging of organisms and that it constitutes a tumor suppressive mechanism. We also summarize our recent findings which suggest that senescent cells express one or more dominant transcription factors that may act to repress the proliferation of senescent cells.

## REPLICATIVE SENESCENCE AND AGING

The biological significance of replicative senescence has been viewed from two, not mutually exclusive, perspectives. One of these holds that replicative senescence occurs throughout the life of multicellular organisms and that aged organisms accumulate senescent cells. Implicit in this view is the more controversial hypothesis that senescent cells cause or contribute to age-related pathology. These ideas have remained highly speculative and controversial primarily because it is not possible to identify senescent cells in vivo. Replicative senescence is generally studied in culture, where cells can be stimulated to proliferate in a controlled fashion for the many population doublings required for most or all cells in a culture to senesce, as judged by their failure to synthesize DNA in response to physiological stimuli. However, measurements of DNA synthesis do not distinguish senescent cells from other nonreplicating cells, such as quiescent or terminally differentiated cells, in complex tissues.

Support for the idea that replicative senescence in culture reflects processes that occur during the aging of organisms comes from three lines of evidence. First, cell cultures established from short-lived species senesce more rapidly than cultures established from long-lived species (Goldstein 1974; Hayflick 1976; Röhme 1981). In addition, cells from human donors afflicted with hereditary premature aging syndromes senesce much more quickly in culture than cells from age-matched controls (Goldstein 1969; Martin et al. 1970). These findings suggest that the replicative life span of cells and the chronological life span of organisms are related and perhaps controlled by a common set of genes. Finally, cell cultures established from old individuals tend to senesce more rapidly than cultures

from younger individuals (Martin et al. 1970; Schneider and Mitsui 1976). This suggests that cells in renewable tissues undergo replicative senescence in vivo and accumulate in aged tissues. Taken together, these findings—although providing strong correlative evidence for the relationship between replicative senescence and aging—suffer from a lack of direct evidence for such a link.

## A BIOMARKER FOR REPLICATIVE SENESCENCE

We recently found that senescent human cells of diverse tissue origins express an unusual β-galactosidase which has a pH optimum of about 6 (G.P. Dimri et al., in prep.). The pH optimum of this activity, which we refer to as the senescence-associated β-galactosidase (SA-β-gal), differed from that of the nearly ubiquitous lysosomal β-galactosidase (pH optimum of 4) and that of the bacterial β-galactosidase that is commonly used as a reporter enzyme (pH optimum ~7.5). The origin and function of this activity are unknown. However, it provides an ideal marker for testing the idea that senescent cells exist and accumulate with age in vivo: It lends itself to simple histochemical staining, and its detection does not rely on measurements of DNA synthesis.

First, we found that this activity was expressed by senescent human fibroblasts and keratinocytes in culture (Fig. 1), as well as by senescent human umbilical vein endothelial cells, adult mammary and surface ovarian epithelial cells, and neonatal melanocytes. However, SA-β-gal was not expressed by presenescent, quiescent, or terminally differentiated cells in culture (Fig. 1). It also was not expressed by a wide variety of cultured immortal or tumor cells (Fig. 1 and data not shown) but could be induced in two immortal human cell lines by normal human chromosomes 1 and 4, which have been shown to reverse the immortal phenotype in these cells (Fig. 1) (Ning et al. 1991; Hensler et al. 1994). Thus, this marker was tightly linked to the senescent phenotype of a variety of human cells in culture.

A pertinent feature of this marker was its expression in human skin samples obtained from different-aged donors. Although we found age-independent staining in the hair follicle and eccrine glands of the skin, there was a striking age-dependent staining pattern in the dermis and epidermis. Skin sections from relatively young donors (< 39 years of age) showed minimal to no SA-β-gal staining in the dermis and epidermis (Fig. 2). In contrast, skin sections from relatively old donors (> 69 years of age) showed moderate to strong SA-β-gal staining in either the dermis, epidermis, or both (Fig. 2). The cell type giving rise to this staining in the dermis was identified as fibroblasts, and the positive-staining cells in the epidermis were identified as basal (undifferentiated) keratinocytes. Thus, SA-β-gal activity serves as a biomarker for replicative senescence in culture and in vivo and provides the first in situ evidence that senescent cells exist, and accumulate with age, in vivo.

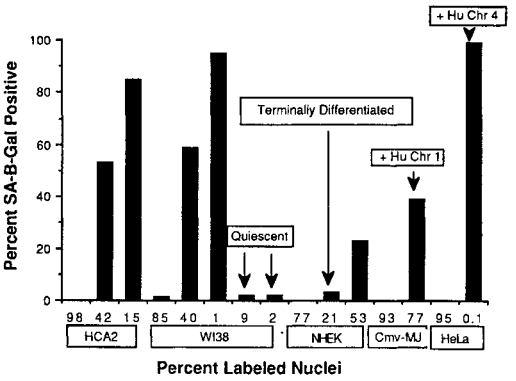


**Figure 1.** SA-β-gal and proliferative capacity in cultured human cells. HCA2 and WI38 are normal neonatal foreskin and fetal lung human fibroblasts, respectively. Cells at early, mid-, and late passage were labeled with [$^3$H]thymidine for 72 hr (Seshadri and Campisi 1990), fixed for 3–5 min at room temperature in 2% formaldehyde-0.2% glutaraldehyde, and stained for SA-β-gal using X-gal as a substrate and citric acid/sodium phosphate buffer, pH 6.0 (G.P. Dimri et al., in prep.). After autoradiography, 100–500 cells were counted at 200× magnification and the percentages of radiolabeled nuclei (% LN) and SA-β-gal-positive cells were determined. WI38 cells were made quiescent by depriving them of serum for 3 (9% LN) or 7 (2% LN) days, radiolabeled and stained for SA-β-gal. Neonatal human epidermal keratinocytes (NHEK) were obtained from Clonetics (San Diego, California), passaged in serum-free medium according to the supplier's instructions, and radiolabeled and stained for SA-β-gal at early (77% LN) and mid- (53% LN) passage. Early-passage NHEK were induced to terminally differentiate by addition of 2 mM $CaCl_2$ to the medium; 4 days later, they were radiolabeled and stained for SA-β-gal. CMV-MJ are immortal human fibroblasts that are induced to senesce within several doublings after normal human chromosome 1 is introduced by microcell fusion (Hensler et al. 1994). The parent and fusion cultures were radiolabeled and stained for SA-β-gal 8–10 doublings after fusion. HeLa are human cervical carcinoma cells that senesce upon introduction of human chromosome 4 (Ning et al. 1991). The parent culture and hybrid clones containing 60 cells or less were radiolabeled and stained for SA-β-gal.

## REPLICATIVE SENESCENCE AND TUMOR SUPPRESSION

A second view of replicative senescence holds that it constitutes a tumor suppressive mechanism. The evidence for this view is more solid than the evidence that senescence is related to aging, because it derives from both correlations as well as mechanistic studies on etiology and progression of malignant tumors.

First, both naturally occurring and experimentally induced tumors often contain cells that have escaped the limits imposed by replicative senescence. That is, tumors often contain immortal cells or cells that have an extended replicative life span (Ponten 1976; Sager 1989). Moreover, immortality greatly increases a cell's susceptibility to progression toward a more malignant phenotype because it permits the extensive cell divi-

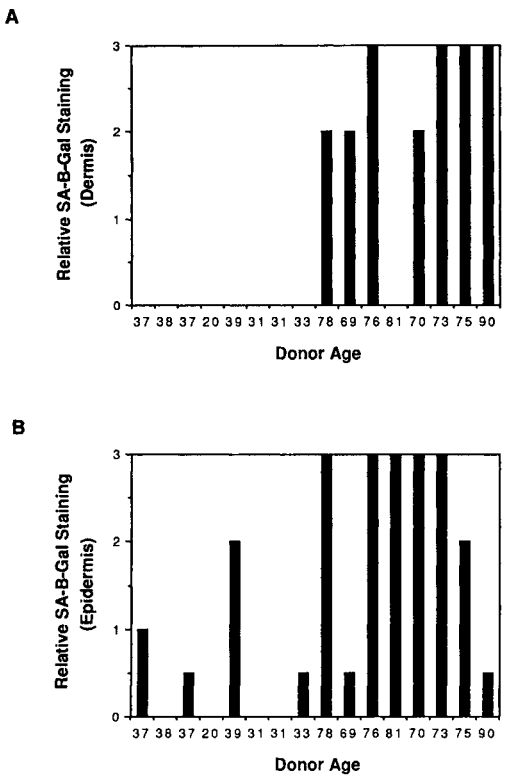


**Figure 2.** SA-$\beta$-gal staining in human skin samples from young and old donors. Human skin specimens were frozen in liquid nitrogen, thin (4 $\mu$m) sections were cut, and the sections were fixed and stained for SA-$\beta$-gal as described for cultured cells in Fig. 1. The sections were evaluated in a blinded fashion for the prevalence and intensity of SA-$\beta$-gal staining in the dermis and epidermis and were then assigned an overall staining value between 0 and 3.

sion that is often necessary for cells to acquire successive mutations.

Second, it is evident that some oncogenes, including those carried by certain human viruses, act at least in part by immortalizing or extending the replicative life span of cells (Weinberg 1985; Galloway and McDougall 1989; Shay and Wright 1991). Thus, genetic lesions, or the strategies of certain oncogenic viruses, that lead to tumorigenesis may and do involve mechanisms that permit cells to escape replicative senescence.

Finally, among the cellular genes that are known to be critical for establishing and/or maintaining the senescent phenotype are two well-recognized tumor suppressor genes: the retinoblastoma susceptibility gene (Rb) and the p53 gene.

## ROLE OF P53 AND RB IN REPLICATIVE SENESCENCE

Thus far, the only genes that are known to reliably immortalize or extend the replicative life span of human cells are the oncogenes of certain DNA tumor viruses. These genes include the large T antigen of the SV40 virus, and the combination of the E1A and E1B genes of adenoviruses and E6 and E7 genes of the papillomaviruses (Shay and Wright 1991). T antigen is known to bind and inactivate both the Rb and p53 proteins, whereas E1A and E7 are known to inactivate Rb, and E1B and E6 are known to inactivate p53. Taken together, these findings suggest that the actions of both Rb and p53 are critical for replicative senescence. They further suggest that inactivation of both Rb and p53 is essential in order for cells to circumvent the normal proliferative constraints imposed by replicative senescence.

Support for the importance of Rb and p53 in replicative senescence derives primarily from studies of life span extension or immortalization by SV40 T antigen. T antigen extends the replicative life span of human cell cultures; at the end of their replicative life span, T-antigen-expressing cultures enter a state termed crisis, in which cell proliferation and cell death occur, and from which rare immortal variants may emerge (Shay and Wright 1989).

Human fibroblasts grown in the presence of antisense oligonucleotides (oligos) that inhibited the expression of Rb and p53 behave very similarly to cells expressing SV40 T antigen (Fig. 3) (Hara et al. 1991). That is, the Rb and p53 antisense oligos extended replicative life span to the same extent as T-antigen expression. Of interest were the effects of the Rb and p53 antisense oligos alone. The Rb antisense oligo, when used alone, extended proliferation about half as well as T antigen. In contrast, the p53 antisense oligo had little effect on replicative life span when used alone, but potentiated the effect of the Rb antisense oligo (Fig. 3). Thus, inactivation of Rb and p53 could account for all the life span extension caused by T antigen.

In addition, we found that p53 and Rb were important for maintaining the growth-arrested senescent state. We constructed expression vectors bearing either a wild-type SV40 T antigen or T antigens that were mutated in either the p53 or Rb binding domains. These expression vectors were introduced into senes-

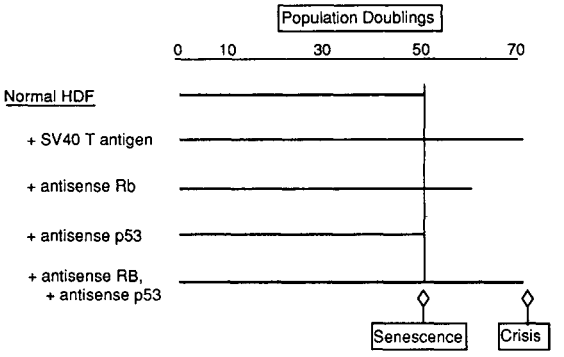


**Figure 3.** Rb and p53 inactivation accounts for the life span extension caused by SV40 T antigen. The replicative life span of TIG-1 normal human fibroblasts, grown in the absence or presence of T antigen or antisense oligonucleotides that inhibited expression of Rb or p53, was determined as described by Hara et al. (1991).

**Table 1.** Stimulation of DNA Synthesis by Wild-type, Rb, and p53 Binding-Deficient SV40 T Antigens

| Condition | Plasmid | % Labeled nuclei |
|---|---|---|
| Presenescent, quiescent | CMV-T | 80 (70–100) |
| | CMV-T[K1] | 59 (50–75) |
| | CMV-T[p] | 54 (52–56) |
| | CMV-$\beta$gal or none | 6 (1–10) |
| Senescent | CMV-T | 78 (65–78) |
| | CMV-T[K1] | 9 (4–15) |
| | CMV-T[p] | 24 (21–29) |
| | CMV-$\beta$gal or none | 2 (0–5) |
| | CMV-T[K1] + CMV-T[p] | 65 (102–152) |

Expression vectors (CMV, human cytomegalovirus early region promoter) for wild-type SV40 T antigen (CMV-T) or T antigens mutated in the p53 (CMV-T[p]) or Rb (CMV-T[K1]) binding domains were microinjected into the nucleus of quiescent (serum-deprived) or senescent human diploid fibroblasts. An expression vector for the *E. coli* $\beta$-galactosidase was coinjected and served to identify injected cells by histochemical staining at pH 7.5 (Dimri et al. 1994). The injected cells were given [$^3$H]thymidine (10 $\mu$Ci/ml) for 48 hr, processed for autoradiography, and 100–500 cells were counted under bright field microscopy to determine the percentage of injected cells that synthesized DNA (% labeled nuclei). The numbers in parentheses show the range of values over 2–5 independent experiments, except the last entry (CMV-T[K1] + CMV-T[p]) shows the number of injected cells with labeled nuclei/total number of injected cells in a single experiment.

cent cells by microinjection, and their ability to induce DNA synthesis was monitored by the incorporation of [$^3$H]thymidine and autoradiography (Table 1). Only the wild-type T was capable of inducing a large fraction of senescent cells to initiate DNA synthesis. The Rb-binding-defective T was a very poor inducer, showing only 10–15% of wild-type activity. The p53-binding-defective T was a weak inducer, showing about 30% of wild-type activity. Both mutants showed 65–70% of wild-type activity in quiescent presenescent cells. The results suggest that Rb and p53 are important for maintaining the growth-arrested state of senescent cells.

Taken together, the antisense and T-antigen mutant experiments support the idea that the Rb and p53 tumor suppressor genes are critical for establishing and maintaining replicative senescence. Rb and p53 are, of course, among the genes that frequently suffer a loss-of-function mutation in tumors. Thus, cell senescence and tumorigenesis may be viewed as alternate processes, each strongly influenced by certain tumor suppressor genes (Fig. 4).

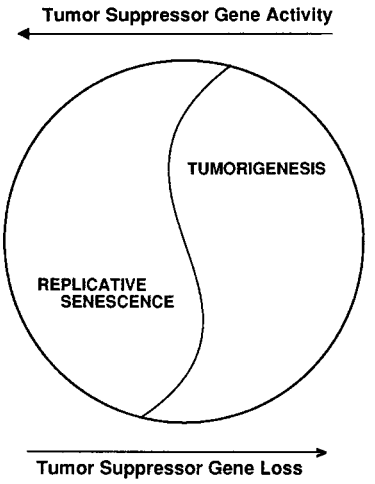


**Figure 4.** Replicative senescence and tumorigenesis may be alternative processes, each dependent (albeit in opposing ways) on the function of tumor suppressor genes such as *Rb* and *p53*.

## SELECTIVE REPRESSION OF GROWTH REGULATORY GENES

The growth arrest associated with replicative senescence is exceedingly stringent. It is refractory to all known physiological mitogens, and of course ensures essentially complete resistance to tumorigenic transformation. Even SV40 T antigen, which induces senescent cells to initiate DNA synthesis (Table 1), fails to induce a complete cell cycle: SV40-infected senescent cells initiate and complete DNA replication but fail to undergo mitosis (Gorman and Cristofalo 1985).

The immediate cause for the failure of senescent cells to proliferate may be the selective repression of a subset of genes whose expression is essential for cell proliferation (use here interchangeably with growth). These genes include two early response genes and several genes whose expression is induced just prior to the start of S phase (Fig. 5).

The first growth-regulatory gene that was shown to be repressed in senescent cells was the c-*fos* proto-oncogene (Seshadri and Campisi 1990). This gene is an immediate-early growth response gene, and it encodes one component of the AP-1 transcription factor. The expression of c-*fos* is selectively repressed in senescent human fibroblasts at the level of transcription. Other immediate-early response genes, such as the c-*myc* proto-oncogene, ornithine decarboxylase, JE, and KC, are expressed similarly in presenescent and senescent cells (Rittling et al. 1986; Seshadri and

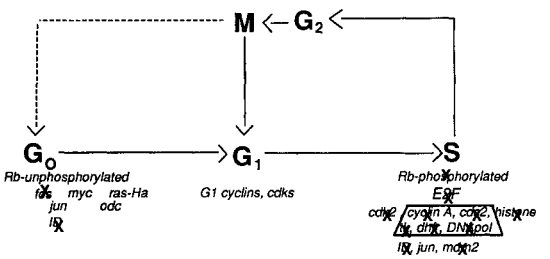


**Figure 5.** Growth-related gene expression in senescent human fibroblasts. Shown are a subset of the genes that are induced by mitogen in early-passage cells, and the genes whose induction fails in senescent cells (marked by an X). Of several immediate-early genes, only two (*fos* and *Id*) are repressed in senescent cells. In contrast, several late $G_1$ genes are repressed in senescent cells, but many of these may be the result of the repression of E2F activity.

Campisi 1990). Thus, senescent cells may fail to proliferate due, at least in part, to repression of the c-*fos* proto-oncogene. However, attempts to reactivate DNA synthesis by providing senescent cells with an expressible c-*fos* gene have not met with success (Table 2).

Recently, we found a second immediate-early gene whose expression is necessary for growth and whose expression was repressed in senescent human fibroblasts. This was the Id gene, which encodes a helix-loop-helix (HLH) protein that inhibits the activity of basic helix-loop-helix (bHLH) transcription factors (Sun et al. 1991). Human fibroblasts expressed three Id genes: *Id-1H* and *Id-1H'*, which are derived by alternate splicing of a single gene, and *Id-2H*. All three Id genes were induced as immediate-early genes upon growth factor stimulation of quiescent fibroblasts and showed a second peak of expression in mid- to late $G_1$. All three Id genes were repressed in senescent human fibroblasts (Hara et al. 1994). Thus, a second reason why senescent cells fail to proliferate may be the repression of Id genes. However, Id expression vectors, whether alone or in combination with a c-*fos* expression vector, were incapable of stimulating senescent cells to initiate DNA replication (Table 2).

As noted earlier, several late $G_1$ genes are also repressed in senescent human fibroblasts. These include the replication-dependent histones, cdc2, cyclin A, dihydrofolate reductase, thymidine kinase, and DNA polymerase $\alpha$ (see Dimri et al. 1994). Many of these genes are positively regulated by a common transcription factor, E2F. E2F acts as a heterodimer, and two E2F components, E2F-1 and DP-1, have been shown to be particularly synergistic for *trans*-activating activity. We found that DP-1 was expressed similarly in presenescent and senescent cells. In contrast, E2F-1 was repressed in senescent cells, and senescent cells were markedly deficient in E2F-binding activity (Dimri et al. 1994). Thus, senescent cells may fail to express several late $G_1$ genes due to repression of E2F-1, which leads to a deficiency in E2F activity (Fig. 5). However, an E2F-1 expression vector did not stimulate senescent cells to initiate DNA synthesis (Dimri et al. 1994), nor did it synergize with c-*fos* or Id expression vectors to do so (not shown).

**Table 2.** Restoration of Early-response Gene Expression Does Not Reactivate DNA Synthesis in Senescent Human Fibroblasts

| Plasmid injected | % Labeled nuclei |
|---|---|
| None | 8 |
| CMV-T | 86 |
| CMV-fos | 8 |
| CMV-Id-1H | 19 |
| CMV-Id-2H | 9 |
| CMV-Id-1H, c-fos | 20 |

Expression vectors for wild-type SV40 T antigen, c-*fos*, or human *Id* genes were microinjected into senescent human fibroblasts, and the cells were monitored for ability to initiate DNA replication, as described in Table 1.

Taken together, these findings suggest that the immediate cause for the growth arrest associated with senescence is the selective repression of growth regulatory genes. These genes include the c-*fos* and Id immediate-early genes, and the E2F-1 gene, whose repression in turn may be responsible for the failure of senescent cells to express several late $G_1$ genes. Although any one of these gene repressions can explain the growth arrest, it is clear that the expression of none of these genes, alone or in combination, is sufficient to reverse the growth arrest. It is entirely possible that other, yet-to-be discovered, growth regulatory genes are also repressed in senescent cells. On the other hand, as noted earlier, the senescent phenotype is dominant in somatic cell hybrids. This finding predicts that senescence should not be reversed by restoring deficiencies in gene expression. Rather, the dominance predicts that senescent cells express one or more inhibitors of cell proliferation whose inhibitory activity is dominant over the growth stimulatory activities of c-*fos*, Id, and E2F.

## DOMINANT GROWTH SUPPRESSORS

What is the nature of dominant growth suppressors expressed by senescent cells? First, senescent cells may express very high levels of growth inhibitors present in quiescent cells. This is clearly the case for p21/sdi-1, the Cdk2 inhibitor, which was first identified as a transcript that is present at higher levels in senescent cells relative to quiescent cells (Noda et al. 1994). In addition, our preliminary evidence suggests that senescent cells express at least two novel transcription factors that may act to suppress growth.

We suggest that one of these factors is a bHLH protein. This hypothesis derives from our search for cellular genes that are capable of complementing the T-antigen mutants in Rb and p53 binding. In these studies, we found that the cellular *mdm2* gene complemented the p53-binding-deficient T antigen. This was not a surprising result because it is known that *mdm2* encodes a negative regulator of p53 activity

(Momand et al. 1992). What was surprising was our finding that the *Id-1H* gene complemented the Rb-binding-deficient T antigen. Thus, the Rb-binding-deficient T was unable to stimulate senescent cells to initiate DNA synthesis (~10% of wild-type T activity), although it was capable of stimulating quiescent cells (~70% of wild-type activity). This finding alone suggests that the activity of Rb, or an Rb-related protein, differs between senescent and presenescent cells. When the Rb-binding-deficient T was introduced into senescent cells together with a variety of cellular genes, only the *Id-1H* gene restored this mutant to near wild-type activity. Our working hypothesis is that senescent cells express a bHLH protein that cooperates with Rb, or an Rb family member, to suppress growth. We propose that this bHLH protein may act in a dominant fashion and that Id acts to inactivate it. Thus, senescent cells may differ from presenescent cells both in the expression of this putative growth suppressor and in the repression of its potential inactivator.

We found a second potential senescence-associated negative growth regulator in the course of our studies on E2F activity in presenescent and senescent cells. As noted earlier, senescent human fibroblasts were markedly deficient in E2F-binding activity, but they expressed relatively high levels of a novel protein/DNA complex. This complex was not an E2F-specific complex (Dimri et al. 1994) and its binding site was outside the E2F-binding site in the oligonucleotide probe. Our preliminary data suggest that this binding site, when cloned upstream of a heterologous promoter, acts as a transcriptional silencer sequence in senescent cells, but not in presenescent cells. Thus, this complex, which we term senescence factor or SNF, may act to suppress transcription in senescent cells.

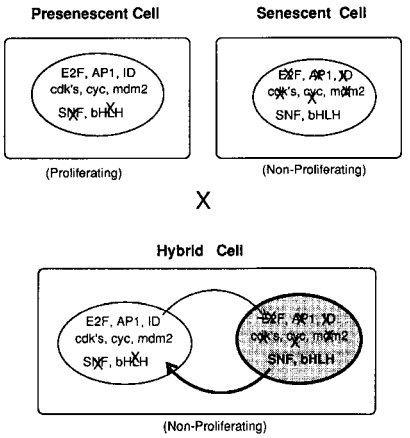


**Figure 6.** Dominance of the senescent state. When proliferating presenescent and senescent cells are fused, the resulting hybrid fails to initiate DNA replication. This is the case despite the fact that the presenescent cell can provide the senescent cell with activities that it lacks. Senescent cells are believed to express dominant acting growth suppressors. We propose that two of these suppressors may be a bHLH protein and SNF.

Taken together, our results suggest that it is not surprising that restoration of AP-1, E2F, or Id expression to senescent cells is not sufficient to induce DNA replication because senescent cells express dominant growth inhibitors, of which a bHLH factor and SNF are candidates (Fig. 6).

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