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An Altered Repertoire of *fos/jun* (AP-1) at the Onset of Replicative Senescence

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With multiple divisions in culture, normal diploid cells suffer a loss of growth potential that leads to replicative senescence and a finite replicative capacity. Using quantitative RT–PCR, we have monitored mRNA expression levels of c-*fos*, c-*jun*, *Jun*B, c-*myc*, *p53*, H-*ras*, and histone H4 during the replicative senescence of human fibroblasts. The earliest and the largest changes in gene expression occurred in c-*fos* and *jun*B at mid-senescence prior to the first slowing in cell growth rates. The basal level of c-*fos* mRNA decreased to one-ninth that of the early-passage levels, while *jun*B declined to one-third and c-*jun* expression remained constant. The decline in the basal c-*fos* mRNA level in mid-senescence should lead to an increase in Jun/Jun AP-1 homodimers at the expense of Fos/Jun heterodimers and may trigger a cascade of further changes in c-*myc*, *p53*, and H-*ras* expression in late-passage senescent fibroblasts.

INTRODUCTION

The *in vitro* culture of normal diploid fibroblasts has served as a model system for studying cellular senescence [1, 2]. As originally observed by Hayflick and Moorhead [3], human diploid fibroblasts reach replicative senescence at a characteristic population doubling level (PDL) following repeated passage in culture. The notion that replicative senescence has significant parallels with *in vivo* aging originally came from studies showing that population doubling potential is inversely proportional to donor age [4–7] and that a species' maximum lifespan is correlated with the *in vitro* replicative potential of its cultured fibroblasts [8–9]. The accumulated evidence also indicates that *in vitro* senescing fibroblasts mimic many aspects of the functional decline that occurs during *in vivo* aging [1, 10, 11], including chromosome shortening at telomeres [12–14].

A link between replicative senescence and oncogene expression was first noted in cultures of rodent fibroblasts. Growth of primary rodent fibroblasts to the crisis state, when senescence and the loss of growth potential is reached, is often accompanied by the spontaneous outgrowth of immortalized and/or transformed cells. The sudden appearance of immortalized cells following crisis can result from the aberrant expression of oncogenes, since transfection of normal fibroblasts with various oncogenes can readily rescue cells from senescence and transform them [15–18]. In contrast, the anti-oncogene *p53* can reverse cell transformation and immortalization [19–22].

Although human fibroblasts do not spontaneously give rise to transformed cells *in vitro*, human fibroblasts infected with oncogenic viruses such as SV40 do generate rare transformants [23]. The large-T antigen in SV40 binds the protein products of the *p53* and retinoblastoma (*Rb*) anti-oncogenes [24]. Recent evidence suggests that *p53* and *Rb* play prominent roles in suppressing human cell transformation and immortalization [20–26]. Thus, in both rodent and human cells, rescue from senescence occurs, at least in part, through perturbations in oncogene expression, suggesting that changes in oncogene mRNA levels might be regulatory during replicative senescence.

To investigate whether oncogene expression changes during the normal process of replicative senescence, we examined the basal mRNA levels of various oncogenes from fibroblasts at differing PDLs. Since the inducibility of c-*fos* was recently reported to be suppressed in senescent cells [27], we were especially interested in analyzing the mRNA levels for senescence-specific changes in the *fos/jun* family of oncogenes, which encode the AP-1 transcription factors [28, 29]. Because *p53*, c-*myc*, and H-*ras* play important roles in growth regulation and cancer, the mRNA levels of these three oncogenes were also examined. While the mRNA levels of all the tested oncogenes underwent some fluctuations with increasing PDL, only c-*fos* and, to a lesser extent, *jun*B had marked changes in basal expression. These data point to the

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fos/jun family of oncogenes as possible participants in the regulation of replicative senescence.

MATERIALS AND METHODS

Cell culture and the determination of PDL. MRC-5 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DME) and Ham's nutrient F-12 medium (Sigma, No. 8900) supplemented with fetal calf serum (Hazelton Laboratories). Confluent MRC-5 cells were washed in balanced salt buffer (15 m*M* Hepes, 130 m*M* NaCl, 5 m*M* KCl, 1 m*M* sodium phosphate, and 0.3% glucose) and treated with balanced salt buffer containing 0.25% trypsin (Sigma). The trypsin was siphoned off after 1 min and the cells were incubated at room temperature for 2 to 4 min. Trypsinization was stopped by resuspending the cells in DME/F12 medium containing 10% fetal calf serum. Two aliquots of the cells from each replicate plate were counted in a hemocytometer and the mean cell count was used to calculate PDL. Using this measure of cell density, 3×10^5 cells from each flask were then placed into a new 25-cm^2 T flask along with 5 ml of medium. The T flasks with loosened caps were incubated in a humidified incubator in the presence of 3.5% CO_2. In our standard protocol each PDL determination represents the mean from two replicate cultures. The increase in PDL divided by the number of days to confluency was taken as a crude measure of the relative PDL growth rates for early- and late-passage cells.

Induction of the quiescent state. In order to compare early- and late-passage cells in the same growth state, diploid MRC-5 fibroblasts were forced into a quiescent state by density arrest for 7 days at PDLs of 25, 35, 45, 55, and 69. Fresh medium was added on the fourth day of density arrest, so that growth factors were not limiting. In contrast to density arrest, depriving cells of essential growth factors in low-serum media to force the cells into a quiescent state can cause perturbations in oncogene expression [30, 31] and can obscure some of the changes observed with replicative senescence [32, 33]. Moreover, the density-arrested state of confluent cultured cells is, in some ways, closer to the *in vivo* state found in intact tissues, where cell growth is often checked by adjacent cells or by the extracellular matrix. As support for the notion that density-arrested cells best approximate noncycling cells *in vivo*, we have found that c-*fos* mRNA is expressed at high levels in human liver and breast biopsy tissue (data not shown). Therefore, we have chosen to induce quiescence by allowing the cells to remain confluent at normal growth-factor levels for 7 days before harvesting.

Serum induction of serum-starved cells. The induction of oncogene mRNA was carried out as previously reported [34]. MRC-5 cells were grown to 70% confluency in the above medium with 10% serum, transferred to medium with 0.5% serum for 48 h, and finally transferred to medium with no serum for 30 min. To induce the serum-responsive oncogenes, medium with 15% serum was added and the cells were incubated at 37°C for various times before harvesting. The cells were washed, scraped from the plate, and collected in ice-cold phosphate-buffered saline.

RNA isolation and the RT–PCR assay for mRNA levels. Total cellular RNA was purified by guanidine–thiocyanate extraction [35]. As a check on the quality and quantity of isolated RNA, samples were denatured with glyoxal and dimethyl sulfoxide [36] and electrophoresed on 1% agarose gels. For quantitation, mRNA levels were assayed by RT–PCR analysis [37–40]. Briefly, cDNA was prepared using a constant amount of total cellular RNA, reverse transcriptase (RT), and random oligo primers. Incorporation of [^{3}H]dTTP was used to ensure that each RNA sample gave uniform amounts of cDNA product. The cDNA was amplified in the presence of specific gene primers and 5 μCi [^{32}P]dATP using three thermocyler temperatures (1.5 min at 94°C, 1.0 min at 63°C, and 1.5 min at 72°C) for 22 cycles. The final reaction products were electrophoresed on a 6% polyacrylamide gel, stained with ethidium bromide, and visualized under uv light. The PCR bands were excised with a razor blade and the incorporated label was measured by Cerenkov radiation. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or hypoxanthine phosphoribosyltransferase (*HPRT*) served as our major controls. Control experiments in which reverse transcriptase was left out showed that less than 1% of the signal was caused by the amplification of contaminating DNA.

Calibration of the RT–PCR assay. Quantitative performance of the RT–PCR assay was obtained through a series of control and calibration studies. In the first set of experiments, duplicate reactions of single primer pairs were subjected to 16 to 28 cycles on the thermocyler to determine the linear range of DNA amplification. Since all primer pairs were in the linear range below 25 cycles, we standardized our reactions at 22 cycles. In a second set of experiments, DNA input was decreased by serial dilution to define the linear range of cDNA input. Third, we performed multiplex PCR with *GAPDH* or *HPRT* added as internal controls. Because multiplex PCR had an error rate higher than that of single-primer-pair PCR (±30% versus ±10% for single-primer-pair PCR), we typically ran multiple tubes of single-primer-pair PCR and estimated the "tube effect" by calculating the variance from triplicate tubes widely spaced in the thermocycler. However, single-primer-pair PCR was periodically matched against multiplex PCR with internal *GAPDH* controls to ensure that no unexpected effects were occurring. Taken together, our data and those of others [37–41] indicate that the quantitative RT–PCR assay determines accurately the relative expression levels of multiple genes.

PCR primers. All PCR primers were synthesized by the University of Michigan's DNA core facility. Primers were selected that were close to 50% GC and 28 to 30 bases in length. Where possible, primer pairs were chosen from adjacent exon sequences to prevent the artifactual detection of RNA precursors or genomic DNA. All DNA sequences were obtained from GenBank or from published sources. The sequence of each primer pair, the PCR product size, and the location in cDNA are shown in Table 1.

RESULTS

The basal levels of c-fos and junB decline in midsenescent MRC-5 fibroblasts. To examine the basal expression of various oncogenes during replicative senescence, we have cultured human fetal lung MRC-5 fibroblasts [42]. When passaged under our standard culture conditions, MRC-5 cells stop dividing by a PDL of 70 [33, 43]. To minimize the effects of changes in growth rates on oncogene expression, we forced MRC-5 cells into quiescence at PDLs of 25, 35, 45, 55, and 69 by keeping the cells in a density-arrested state for 7 days. Under these conditions, cell proliferation was strongly inhibited by the end of the stationary incubation.

Total RNA was isolated and the expression of various mRNA levels was analyzed by RT–PCR. The sensitive RT–PCR assay allowed us to determine mRNA levels from many genes simultaneously using the same RNA samples. Primers were designed to probe the cDNA for six oncogenes: *p53*, c-*myc*, H-*ras*, c-*fos*, c-*jun*, and *jun*B. The housekeeping genes *GAPDH* or *HPRT* served as ubiquitously expressed controls, which changed little during senescence. Histone H4 served as a cell-cycle-regulated control.

Amplification of all cDNA sequences was carried out for 22 cycles in a reaction mix that included [^{32}P]dATP

TABLE 1

PCR Primers, Product Size, and Location

Gene	Primer sequence	Product size and position
c-*fos*	(F) 5′-GAATAAGATGGCTGCAGCCAAATGCCGCAA-3′ (R) 5′-CAGTCAGATCAAGGGAAGCCACAGACATCT-3′	236 bp spanning exons 3 and 4
p53	(F) 5′-TACAAGCAGTCACAGCACATGACGGAGGTT-3′ (R) 5′-AGTTGTAGTGGATGGTGGTACAGTCAGAG-3′	220 bp at +487
c-*jun*	(F) 5′-GGAAACGACCTTCTATGACGATGCCCTCAA-3′ (R) 5′-GAACCCCTCCTGCTCATCTGTCACGTTCTT-3′	325 bp at +15
*jun*B	(F) 5′-CCAGTCCTTCCACCTCGACGTTTACAAG-3′ (R) 5′-GACTAAGTGCGTGTTTCTTTTCCACAGTAC-3′	257 bp at 3′ end of cDNA
c-*myc*	(F) 5′-CTGGCAAAAGGTCAGAGTCTGGATCACCTT-3′ (R) 5′-TGTCTCAGGACTCTGACACTGTCCAACTTG-3′	187 bp in exon 2
H-*ras*	(F) 5′-ACCATCCAGCTGATCCAGAACCATTTTGTG-3′ (R) 5′-CTGTACTGGTGGATGTCCTCAAAAGACTTG-3′	233 bp spanning exons 1 and 2
Histone H4	(F) 5′-GAGGAAAGGGCGGAAAAGGCTTAGGCAAAG-3′ (R) 5′-CAAAAAGGGCCTTTGGGATCGAAACGTGCA-3′	347 bp at +10
HPRT	(F) 5′-ACATCTGGAGTCCTATTGACATCGCCAGTA-3′ (R) 5′-GACTGCTTCTTACTTTTCTAACACACGGTG-3′	395 bp at +74 into exon 9
GAPDH	(F) 5′-AAGAAGATGCGGCTGACTGTCGAGCCACAT-3′ (R) 5′-TCTCATGGTTCACACCCATGACGAACATG-3	457 bp spanning exons 1 to 6

to allow quantitation. Figure 1 shows the ethidium bromide-stained PCR products of all the tested genes following polyacrylamide gel electrophoresis. Each PCR band was then cut out and counted by Cerenkov radiation to generate a graph of the relative mRNA expression rates as a function of PDL for each of the genes (Fig. 2).

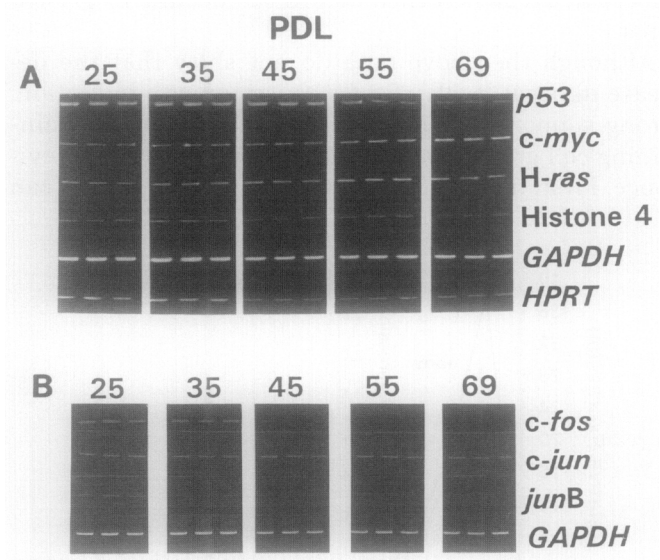

FIG. 1. Oncogene mRNA expression in quiescent MRC-5 cells at increasing PDL. MRC-5 fibroblasts were made quiescent at 25, 35, 45, 55, or 69 PDL by incubation for 7 days in a confluent state. Total cellular RNA was isolated from two to four 150-mm plates and cDNA was prepared. Aliquots of the cDNA were then used as a template for PCR amplification using oncogene-specific primer pairs to amplify segments of the respective cDNAs. [^{32}P]dATP was added to the PCR reaction to allow accurate quantitation of the amplified DNA. The reaction products were electrophoresed on 6% polyacrylamide gels and visualized under uv light in the presence of ethidium bromide. Assays for each gene at each stage were done in triplicate.

In Fig. 2A, the level of *p53* mRNA declined 50% between PDLs 45 and 55, while H-*ras* mRNA (Fig. 2B) increased 60% between the same PDLs. Expression of c-*myc* mRNA was up-regulated 50% by PDL 69 (Fig. 2A), but other experiments (not shown) indicated that c-*myc* mRNA can fluctuate near the end of the replicative lifespan. Figures 2B and 2C show that mRNA expression of *HPRT* and *GAPDH* changed little. Low basal levels of histone H4 mRNA expression were detected by the sensitive RT–PCR technique and were found to decline 50% by the end of the lifespan (Fig. 2C).

The greatest changes that we observed were in the *fos/jun* family of transcription factors. Figure 2D demonstrates that the basal level of c-*fos* mRNA decreased by PDL 45 to one-ninth the level at PDL 25, while *jun*B mRNA decreased to one-third. In contrast, c-*jun* mRNA declined temporarily, but regained its previous level of expression. This dramatic decline in basal c-*fos* expression is unlikely to be related to cell-cycle effects, because cell-cycle-regulated histone H4, c-*jun*, c-*myc*, *p53*, and H-*ras* were all relatively constant between PDLs of 25 and 45 (Fig. 2) and the rate of population doubling remained constant through PDL 45 [33, 43]. Since the c-*fos* protein product combines with the c-*jun* product to form a heterodimer that binds AP-1 enhancer elements [28, 29], the Fig. 2 data suggest that there is a pronounced alteration in the repertoire of the Fos/Jun family of transcription factors in midsenescence.

The decline in basal levels of c-fos mRNA is not concurrent with a marked decline in peak serum-inducible levels. Since c-*fos* mRNA has been reported to be

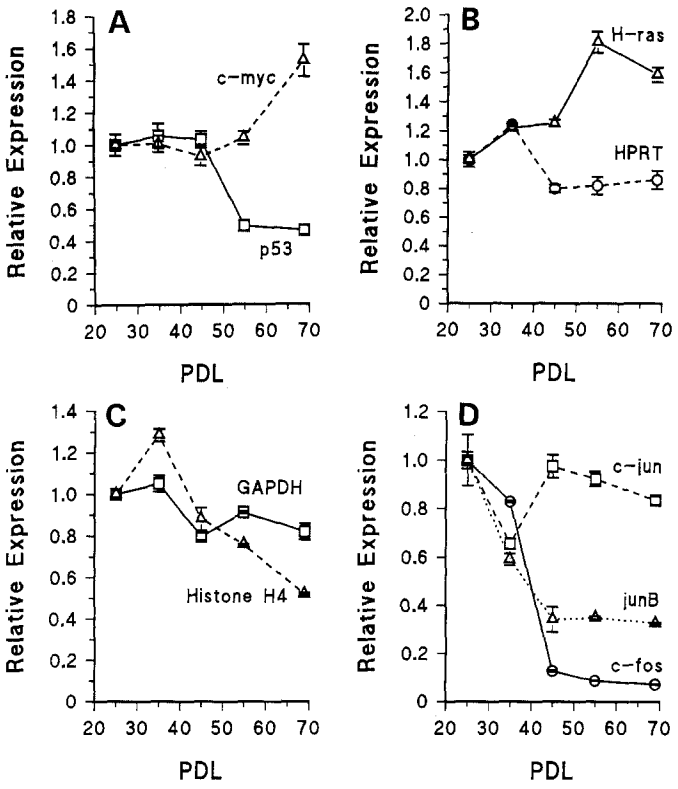

FIG. 2. Relative oncogene mRNA expression during replicative senescence. Each band in Fig. 1 was excised with a razor blade and counted by Cerenkov radiation. Each data point gives the mean and SEM (n = 3). (A) *p53* (squares) versus c-*myc* (triangles). (B) H-*ras* (triangles) versus *HPRT* (circles). (C) *GAPDH* (squares) versus histone H4 (triangles). (D) c-*fos* (circles), c-*jun* (squares), and *junB* (triangles) plotted together.

poorly induced in senescent cells [27], the early decline in basal c-*fos* mRNA could be part of the same phenomenon. To test this, we serum starved MRC-5 cells at PDLs of 28 and 63 and monitored c-*fos* mRNA induction following serum stimulation. Figure 3 demonstrates that serum stimulation strongly induced c-*fos* at 15 min in both early- and late-passage cells, although the peak level was 40% less in late-passage cells. In three other serum-induction experiments with late-passage cells (PDLs of 65 to 69), we found a marked reduction in inducible c-*fos* mRNA in only one culture, which appeared to be the most senescent with a large proportion of the cells in a postmitotic state (data not shown). Other investigators have also observed strong c-*fos* induction in late-passage fibroblasts [44, 45]. Apparently, the decline in c-*fos* inducibility is a late-passage trait that is separate from the decline in basal expression levels at midsenescence.

By 16 h after the induction of serum-starved cells, basal levels of c-*fos* mRNA returned to relative values near those observed in density-inhibited cells (Fig. 3, insert). These data indicate that even in serum-stimulated cells, marked differences in basal c-*fos* mRNA levels of early- and late-passage cells slowly return after the induction of c-*fos* mRNA wanes.

The kinetics of induction of p53, c-myc, and H-ras in early- versus late-passage MRC-5 cells. MRC-5 cells at PDLs of 28 and 63 were serum starved for 48 h as before. Fifteen percent serum was added and the mRNA expression levels of *p53*, c-*myc*, and H-*ras* were assayed at various times of serum induction using RT–PCR (Fig. 4). These results suggest that there are small but significant changes in the kinetics of *p53* and H-*ras* induction in early- versus late-passage cells. However, the meaning of these small perturbations is unclear, because low serum alone can perturb oncogene expression and obscure some of the changes observed with replicative senescence [30–33].

DISCUSSION

We have demonstrated that basal c-*fos* and *junB* mRNA levels decline rapidly from early (PDL = 25)- to mid (PDL = 45)-passage fibroblasts. Since we have previously shown that MRC-5 cells have a constant rate of population doubling up to a PDL of 45 [33, 43], the marked decline in basal c-*fos* mRNA precedes the slowdown in MRC-5 growth rates. Because c-*jun* mRNA levels remain relatively constant during senescence, a decrease in c-*fos* expression further suggests a switch from Fos/Jun AP-1 heterodimers to Jun/Jun AP-1 homodimers at midsenescence before the decline in growth rates.

Although the above data do not show that the decrease in basal levels of c-*fos* causes growth inhibition, strong support for an active role of Fos/Jun in maintaining cell growth rates comes from three lines of evidence. First, high constitutive levels of c-*fos* mRNA can

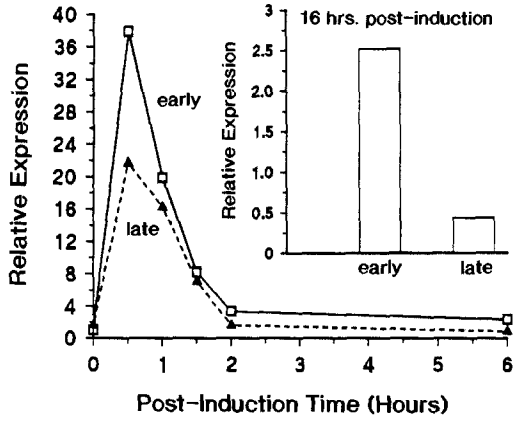

FIG. 3. Serum induction of c-*fos* mRNA in early- and late-passage MRC-5 cells. Serum-starved early (PDL 28)- or late (PDL 63)-passage cells were induced with 15% serum for 0.5, 1.0, 1.5, 2.0, 6.0, or 16 h. The cells were harvested and c-*fos* mRNA was quantitated by RT–PCR as before. The insert shows basal levels of c-*fos* mRNA at 16 h in an expanded scale.

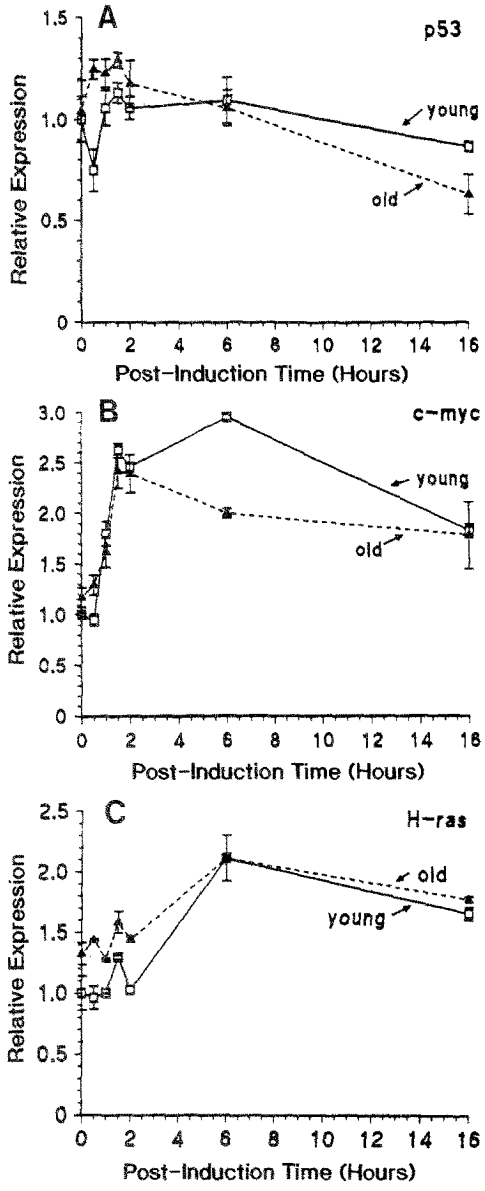

FIG. 4. Serum induction of *p53*, c-*myc*, and H-*ras* mRNA in early- and late-passage MRC-5 cells. Serum-starved early (PDL 28)- or late (PDL 63)-passage cells were induced with 15% serum for 0.5, 1.0, 1.5, 2.0, 6.0, or 16 h. The cells were harvested and *p53*, c-*myc*, and H-*ras* mRNAs were quantitated by RT–PCR as before.

immortalize chicken embryonic fibroblasts [46], indicating that c-*fos* can rescue chicken cells from senescence. Second, either antisense c-*fos* mRNA [47] or antibodies to Fos protein [48] markedly inhibit [^{3}H]thymidine incorporation and cell proliferation in asynchronously growing cells, indicating that the presence of the Fos protein is required for continued cell proliferation. Third, recent experiments demonstrate that inhibiting AP-1 binding blocks DNA synthesis and that the transcription of AP-1-regulated genes is markedly reduced in late-passage fibroblasts as AP-1 binding activity falls [49]. Moreover, the same study also showed that late-passage cells produce predominantly Jun/Jun homodimers instead of the Fos/Jun heterodimers made in early-passage cells. These data and our observation that basal c-*fos* mRNA levels continue to fall between PDLs 45 and 69 (Fig. 2D) point to the decline in basal c-*fos* mRNA levels and the associated change in AP-1 dimer composition as possible factors in the observed growth inhibition. Of course, given the genetic evidence that several complementation groups are involved in immortalization [1, 2, 50], other genes besides those of the *fos/jun* family are likely to play major roles in controlling growth inhibition.

Recent data indicate that the balance of growth and antigrowth oncogenes may be of considerable importance [51, 52]. Following the decline in c-*fos* and *jun*B mRNA levels and the associated changes in AP-1 composition, compensatory changes in other oncogenes may occur. This could account for the apparent fluctuations in *p53*, c-*myc*, and H-*ras* expression at higher PDLs (Fig. 2). For example, *p53* has an upstream AP-1 binding site [53], so that its down-regulation following c-*fos* and *jun*B modulation may represent a coordinate AP-1-mediated effect. Indeed, given the large number of AP-1 binding sites in the genome, alteration in AP-1 dimer composition could have pleiotropic effects on gene expression [54, 55]. Moreover, near the end of the replicative lifespan, the anti-oncogene *Rb* protein dephosphorylates and becomes more active [56]. Since the c-*myc* product apparently cooperates with *Rb* through direct protein binding [57], c-*myc* activity may be altered in response to the decreased phosphorylation of *Rb*. While critical oncogenes are probably missing from this analysis, these data suggest that an oncogene cascade may occur during replicative senescence in which early oncogene changes trigger later changes in other oncogenes.

The normal end state for these changes in oncogene expression may be the senescent, postmitotic cell. However, in those rare cells in which an oncogene is mutated, the balance may become perturbed such that the compensating increases in growth-promoting activities of genes such as *p53*, c-*myc*, and H-*ras* in late-passage cells serve to promote immortalization and transformation. If so, this might partially explain the large increases in cancer incidence with age [58–60].

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