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Transcription factor AP-1 activity is required for initiation of DNA synthesis and is lost during cellular aging

(Fos/Jun/cellular senescence/signal transduction)

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ABSTRACT **Activation of the AP-1 complex of transcription factors is one of the earliest nuclear responses to mitogenic stimuli. We demonstrate directly that AP-1 activity is required for human cells to proliferate in response to serum. We also find that activity of the AP-1 complex is selectively reduced in old human fibroblasts prior to their entering a fully senescent state. Levels of Fos protein induced through diverse signal transduction pathways, the amount of AP-1 DNA binding activity *in vitro*, and the activity of an AP-1-dependent reporter gene *in vivo* are substantially decreased as fibroblasts age. Moreover, the composition of the AP-1 complex changes, so that old cells produce predominantly Jun–Jun homodimers instead of Fos–Jun heterodimers. Changes in AP-1 activity may be due in part to changes in posttranslational modification of Fos protein that impair its ability to form active DNA-binding heterodimers with Jun. These data suggest that changes in AP-1 activity may contribute to the inability of senescent cells to proliferate in response to mitogens.**

Human diploid fibroblasts (HDFs) undergo a finite number of population doublings *in vitro* (1), which is inversely proportional to the age of the donor. This observation and the direct correlation seen between the maximum life span of animal species and the replicative life span of their fibroblasts cultured *in vitro* (2) suggest that mechanisms underlying HDF senescence *in vitro* may play a role in the decline of many functions seen during aging *in vivo* (for a review, see ref. 3). Senescent HDFs are unable to proliferate in response to normally mitogenic extracellular stimuli and appear to be blocked within the G_1 phase of the cell cycle (4, 5). Because growth factor receptors generally appear to be functional in senescent HDFs (6), the block to proliferation apparently lies between the stimulation of membrane receptors and the initiation of S phase.

Expression of several cellular genes including c-*fos* (7–9), c-Ha-*ras* (10), and c-*myc* (11, 12) is required for cells to progress through the G_1 phase of the cell cycle and enter into DNA synthesis. But expression of these and other cell-cycle-regulated genes is not appreciably reduced in senescent cells (13), with one reported exception, that of c-*fos* (14). c-*fos* is a particularly attractive candidate as a control point for cell proliferation since it encodes a transcription factor with the potential to regulate events later in progression through G_1. Fos is a member of a family of transcription factors that contain highly conserved positively charged DNA-binding domains and leucine-zipper motifs. Fos proteins act in heterodimeric complexes with members of the Jun family of protooncogenes by binding to specific regulatory elements termed AP-1 sites (for review, see ref. 15). We show here that AP-1-binding factors constitute a control point for G_1 progression by selectively inactivating AP-1-binding factors in living cells. Moreover, we show that as cells begin to age they show a striking decline in AP-1 activity. We suggest that AP-1 activity may be one of the major molecular defects responsible for the decline in the proliferative response to mitogens seen during cellular senescence.

MATERIALS AND METHODS

Cell Culture and Emulsion Autoradiography. Human Hs68 fibroblasts (American Type Culture Collection CRL 1635) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM glutamine and 10% (vol/vol) fetal bovine serum (FBS). Young, old, and senescent fibroblasts were tested in parallel with other experiments for their ability to synthesize DNA by emulsion autoradiography using Kodak nuclear track emulsion as described (9).

Microinjection and Immunocytochemistry. Microinjection and visualization of cells was as described (9). Synthetic double-stranded oligodeoxynucleotides used in all assays contained a single copy of a wild-type AP-1 site (5′-TCGACGGTATCGATAAGCTATGACTCATCCGGGG-GATC-3′) or a mutant site (5′-TCGACGGTATCGATAAGC-TATAAGTTATCCGGGGGATC-3′). Oligonucleotides were injected at 0.3 mg/ml with nonspecific rabbit IgG at 0.5 mg/ml. Fixation and staining with rabbit anti-Fos and anti-β-galactosidase was as described (9). Micrographs in Figs. 3 and 4 were taken using identical exposure and printing conditions to allow direct comparison.

DNA Affinity Precipitation Assays. Radiolabeled cells were harvested in phosphate-buffered saline (PBS) containing 0.1% Nonidet P-40 (1 ml per 10-cm plate). Nuclei were pelleted 15 sec at 4°C at 12,000 × *g*, and buffer was aspirated. Proteins were extracted in 4 vol of high-salt buffer (20 mM Hepes, pH 7.9/400 mM KCl/10 mM NaF/0.5 mM MgSO4/1 mM EDTA/1 mM dithiothreitol/1 mM aprotinin/1 mM leupeptin/1 mM pepstatin) for 30 min on ice. Nuclei were pelleted 10 min at 4°C as above; the supernatants were recovered, and DNA affinity precipitation assays were performed as described (16) with minor modifications. Extracts containing equal amounts of incorporated ^{35}S were incubated 30 min at 20°C with either 0.5 μg of biotinylated wild-type oligonucleotides or 0.5 μg of mutant oligonucleotides described above. Binding products were recovered with strepavidin-agarose beads, washed, boiled in sample buffer, and electrophoresed through 12.5% denaturing polyacrylamide gels. Gels were fixed, impregnated with 1 M salicylate, and fluorographed at −80°C for 48 hr.

Abbreviations: HDF, human diploid fibroblast; MPD, mean population doubling; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate.

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Metabolic Labeling and Immunoprecipitation. Young, old, and senescent cells were grown to 75% confluence and deprived of serum for 48 hr prior to refeeding with methionine-free DMEM containing [^{35}S]methionine at 0.3 mCi/ml (1 Ci = 37 GBq). After stimulation, cells were washed with PBS and harvested on ice in RIPA buffer (20 mM Tris·HCl, pH 7.5/100 mM NaCl/5 mM KCl/1 mM EDTA/0.25% deoxycholate/0.25% Nonidet P-40/0.25% Tween 20). Lysates were sonicated, pelleted at 4°C for 2 min, and preadsorbed with protein A-Sepharose for 30 min at 4°C. Samples of each lysate containing identical amounts of ^{35}S were incubated with excess Fos or Jun antibody for 2 hr at 4°C with rocking. Antibody–antigen complexes were recovered on protein A-Sepharose beads, washed five times in ice-cold RIPA buffer, and boiled in sample buffer prior to one- or two-dimensional gel electrophoresis (17).

RESULTS

Microinjection of Excess AP-1-Binding Sites Blocks DNA Synthesis in Response to Growth Factors. Introduction of double-stranded oligonucleotides containing sequences corresponding to regulatory elements in the c-*fos* gene blocks c-*fos* induction by extracellular stimuli, presumably by titration of the endogenous transcription factors required for these responses (18, 19). Thus, microinjection of oligonucleotides containing a consensus AP-1 binding site should effectively compete for proteins required for transcriptional activation of genes containing these sites, neutralizing cellular AP-1 activity. To test whether AP-1 activity was required to traverse G_1, AP-1 oligonucleotides (0.3 mg/ml) and nonspecific rabbit IgG (0.5 mg/ml) were mixed and microinjected into quiescent serum-deprived young HDFs. Cells were refed with medium containing 10% FBS and [^{3}H]thymidine (2 μCi/ml). Thirty-six hours after refeeding, cells were fixed, stained for IgG to identify injected cells (9, 20), and coated with nuclear track emulsion to identify cells that had initiated DNA synthesis. Injection of nonspecific antibodies has no detectable effects on DNA synthesis (20). Approximately 80% of uninjected cells incorporated [^{3}H]thymidine in 36 hr (Fig. 1*A*), whereas none of the cells injected with a consensus AP-1 oligonucleotide (stained cells in Fig. 1*B*) initiated DNA synthesis during this period. This was not due to nonspecific effects of oligonucleotide microinjection, since cells injected with the same concentration of a mutant oligonucleotide identical at 35 of 38 base pairs were not prevented from synthesizing DNA in response to serum stimulation (Fig. 1*C*). In three experiments, an average of 82% of the uninjected cells (123 of 150 cells), 4% of the cells injected with the AP-1 oligonucleotide (7 of 172 cells), and 70% of the cells injected with a control oligonucleotide (102 of 146 cells) incorporated [^{3}H]thymidine. These results suggest that proteins that bind specifically to this AP-1 site are required for initiation of DNA synthesis.

***In Vitro* AP-1-Binding Activity Is Markedly Reduced in Old Fibroblasts.** To characterize the proteins that bound the AP-1 oligonucleotides used in the microinjection experiments and to determine whether there were changes in these proteins as the cells aged *in vitro*, we performed DNA affinity precipitation assays (16) using [^{35}S]methionine-labeled nuclear protein extracts from serum-stimulated young [mean population doublings (MPD) = 42] and old (MPD = 77) fibroblasts (Table 1). As shown in Fig. 2, the wild-type AP-1 probe bound large amounts of Fos and Jun proteins in young cells; this is a specific interaction, because the proteins were not recovered with the mutant probe. In contrast, the wild-type probe recovered virtually no detectable Fos protein from the old cells, and the quantity of Jun bound was reduced by about half. This suggests that there is a significant change in the amount and composition of cellular AP-1 binding proteins in old cells. Because the DNA affinity precipitation assays are performed at high oligonucleotide concentration, this assay detects relatively low-affinity protein–DNA interactions and, therefore, probably underestimates the reduction in effective AP-1 binding activity in the old cells. Consistent with this

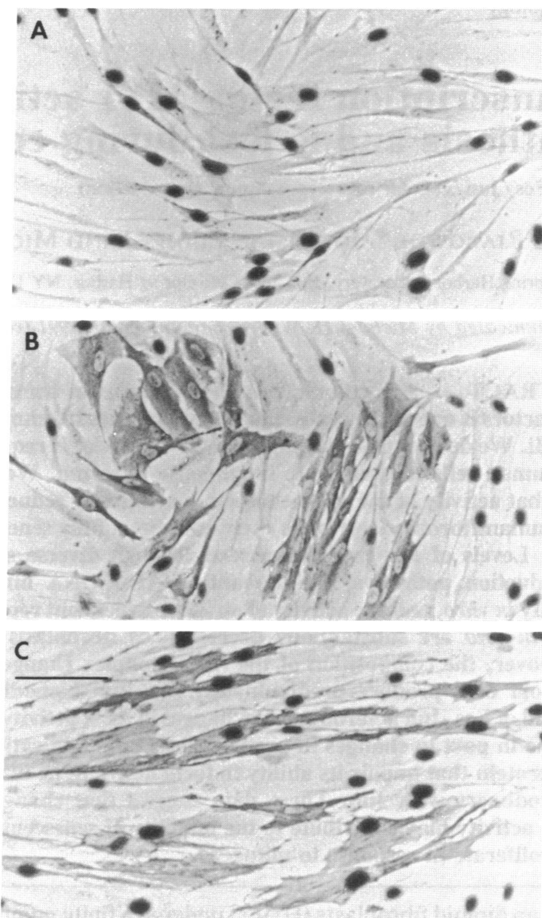

FIG. 1. Microinjection of wild-type and mutant AP-1 oligodeoxynucleotides. Young (MPD = 38) Hs68 fibroblasts injected with wild-type (*B*) or mutant (*C*) AP-1 oligonucleotides were tested for their ability to synthesize DNA after serum-stimulation by [^{3}H]thymidine incorporation followed by emulsion autoradiography. (*A*) Uninjected cells. Cytoplasmic staining marks injected cells. (Bar = 50 μm.)

Table 1. Proportion of cells incorporating [^{3}H]thymidine at various passages

Cells	Passage	% labeled nuclei	
		− serum	+ serum
Young	38	7	87
	40	11	80
	42	8	82
Old	76	6	24
	77	8	21
	78	5	19
Senescent	80	2	5

Cells deprived of serum for 48 hr were grown in medium containing [^{3}H]thymidine (2 μCi/ml) in the presence or absence of 10% FBS for 36 hr. Cells were fixed, coated with nuclear track emulsion, and developed 4 days later, and fields of 200 cells were visually scored for incorporation of [^{3}H]thymidine (9). Cells for all experiments were subcultured within 1 day of reaching confluence at ratios of 1:1 to 1:8. Young fibroblasts were typically from 30 to 45 MPDs and old fibroblasts were from 75 to 78 MPDs. Senescent fibroblasts required 20 days to achieve 75% confluence after a final 1:2 subculturing.

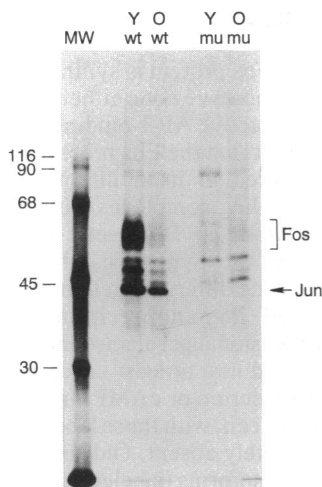

FIG. 2. DNA affinity precipitation assay of AP-1 binding proteins in young and old fibroblasts. Young (lanes Y, MPD = 42) and old (lanes O, MPD = 77) Hs68 fibroblasts were deprived of serum for 48 hr, refed with medium containing 10% FBS and [^{35}S]methionine (0.5 mCi/ml), and incubated for 90 min. Nuclear extracts were prepared and incubated with 0.5 μg of biotinylated wild-type (lanes wt) or mutant (lanes mu) AP-1 oligonucleotides. Bound proteins were recovered with streptavidin-agarose beads and analyzed by electrophoresis on a 12.5% polyacrylamide gel followed by fluorography.

conclusion, we observe an ≈10-fold reduction in serum-stimulated AP-1 activity as measured in mobility-shift assays (data not shown).

AP-1 Transcriptional Activity in Intact Cells Decreases During Aging. The reduction in AP-1 DNA-binding activity that accompanies cellular aging suggests that transcription of AP-1-dependent genes is reduced in old cells. To test this hypothesis, we microinjected individual young (MPD = 46) and old (MPD = 76) cells with a *lacZ* reporter gene (0.5 mg/ml) under the control of four tandem AP-1 sites. Six hours after injection, cells were refed with medium containing or lacking phorbol 12-myristate 13-acetate (PMA; 100 ng/ml), incubated for 2 hr, and fixed for indirect immunofluorescence staining. As shown in Fig. 3, expression of β-galactosidase was low in unstimulated young fibroblasts and undetectable in unstimulated old fibroblasts. PMA induced high levels of expression in young fibroblasts and considerably lower levels in old fibroblasts. Although we are unable to measure quantitatively the fluorescence intensities in these cells, the differences between the young and old cells, relative to the consistent background staining in all fields, were highly reproducible in several experiments. Thus, we conclude that transcription of AP-1-dependent genes is impaired in old cells.

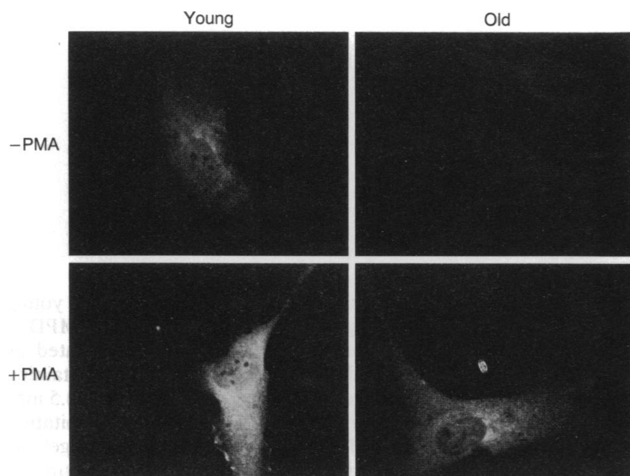

FIG. 3. *In vivo* AP-1 activity in young (*Left*) and old (*Right*) fibroblasts. Hs68 cells were microinjected with a plasmid containing the *Escherichia coli lacZ* gene downstream of four tandemly repeated AP-1 sites. Cells were left untreated (*Upper*) or were treated with PMA (100 ng/ml) (*Lower*). The cells were fixed and stained for β-galactosidase, mounted, and photographed. (×1360.)

Expression of Fos Protein Is Reduced in Aging Human Fibroblasts. Since the DNA precipitation assays suggested that reduced AP-1 activity in old fibroblasts may be due to decreased binding of Fos, we asked whether Fos protein levels were reduced in old cells. Young (MPD = 42) and old (MPD = 76) serum-deprived fibroblasts were refed with complete medium, fixed 90 min later, and stained for the presence of Fos. Young and old fibroblasts contained very low levels of protein reactive with Fos antibody prior to serum stimulation (Fig. 4 *A* and *B*). After stimulation, Fos protein rose to much higher levels in young compared to old fibroblasts (compare Fig. 4 *C* and *D*). Similar results were seen in two other human fibroblast lines, HF and A2 (data not shown). Thus, the amount of protein reactive with our Fos antiserum is reduced in old cells.

To corroborate this observation, we performed immunoprecipitation analysis using young, old, and senescent cells (Table 1). Serum-deprived fibroblasts were stimulated with various agents in the presence of [^{35}S]methionine and harvested 90 min later for immunoprecipitation analysis. Fig. 5 shows that young cells expressed much higher levels of Fos than old or senescent fibroblasts in response to cAMP, PMA, serum, or platelet-derived growth factor. Fos induction was impaired in old cells in response to all of the stimuli tested, even though they act through several different cis-acting elements in the c-*fos* promoter. Furthermore, these results also indicate that Fos synthesis was decreased to similar extents in old and senescent cells, despite a 4- to 5-fold higher proliferation rate in the former cultures. Thus, Fos synthesis levels fall as proliferation potential declines, not after entry into a fully senescent state.

We next did a time-course experiment to measure the levels of Fos and Jun expression in serum-stimulated cells and the extent to which the proteins formed heterodimers. [^{35}S]Methionine was added at various times after stimulation, 1 hr prior to harvesting. Lysates were prepared under nondenaturing conditions and diluted to contain equal amounts of incorporated ^{35}S. Half of each lysate was immunoprecipi-

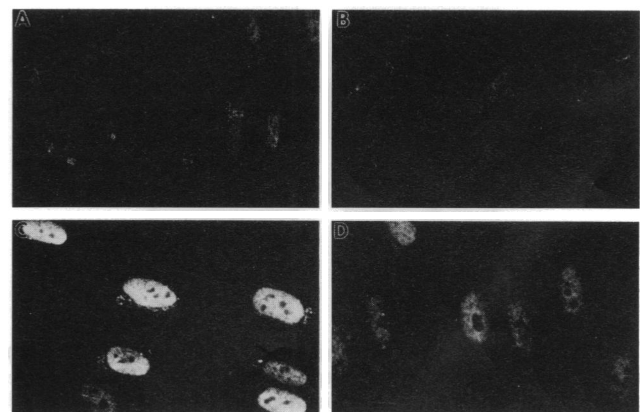

FIG. 4. Inducible expression of Fos protein in young and old fibroblasts. Fibroblasts grown on glass coverslips for 3 days were deprived of serum for 48 hr, refed with medium containing 10% FBS, fixed 90 min later, and processed for indirect immunofluorescence using rabbit anti-Fos as primary antibody. (*A*) Unstimulated young cells. (*B*) Unstimulated old cells. (*C*) Stimulated young cells. (*D*) Stimulated old cells. (×1440.)

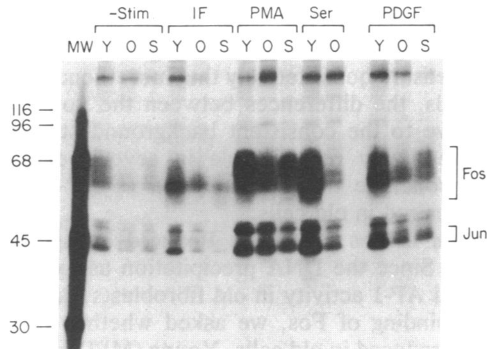

FIG. 5. Immunoprecipitation of Fos from stimulated and unstimulated young, old, and senescent fibroblasts. Young (lanes Y, MPD = 40), old (lanes O, MPD = 76), and senescent (lanes S, MPD = 80) fibroblasts deprived of serum for 48 hr and at a density of 75% of confluence were refed with medium containing [^{35}S]methionine (0.3 mCi/ml) plus 0.5 mM isobutylmethylxanthine and 10 μM forskolin, which increases intracellular levels of cAMP (lanes IF); PMA (100 ng/ml); 10% FBS (lanes Ser); or platelet-derived growth factor (lanes PDGF, 10 ng/ml). Unstimulated cells are in lanes −Stim. Immunoprecipitations were done with an affinity-purified polyclonal antibody under nondenaturing conditions. Similar results were obtained using a monoclonal antibody (TF-161) that recognizes Fos. The sample containing Fos immunoprecipitated from serum-stimulated senescent cells was lost during preparation. In subsequent experiments levels of Fos in old and senescent serum-stimulated cells were very similar. Bands identified as Fos and Jun were specifically lost when control immunoprecipitations were done in the presence of excess unlabeled bacterially expressed Fos fusion protein (data not shown). MW, molecular size standards (kDa).

tated with Fos antibodies (Fig. 6*A*) and half was immunoprecipitated with Jun antibodies (Fig. 6*B*). Old fibroblasts synthesized much lower amounts of Fos protein than young fibroblasts when quiescent (Fig. 6*A*, lanes 1 and 2) or during the first (lanes 3 and 4) or second (lanes 5 and 6) hour of serum stimulation. In young cells, a fraction of the newly synthesized Fos protein was recovered in complexes with Jun protein within 1 hr of stimulation (Fig. 6*B*, lane 3) when using antibodies directly recognizing Jun. In contrast, Jun antibodies did not coprecipitate detectable amounts of Fos protein

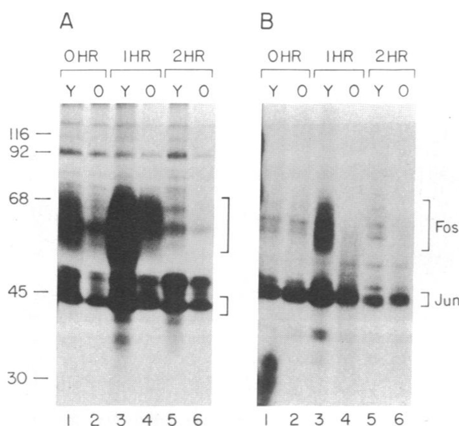

FIG. 6. Time course of Fos and Jun expression. Young (lanes Y) and old (lanes O) fibroblasts deprived of serum for 48 hr were pulse-labeled for 1 hr in [^{35}S]methionine medium lacking (lanes 1 and 2) or containing (lanes 3–6) 10% FBS. Cells were labeled before addition of serum (0 hr) or during the first (1 hr) or second (2 hr) hour of serum stimulation. Lysates containing equal amounts of incorporated [^{35}S]methionine were immunoprecipitated with Fos antibodies (*A*) or with Jun antibodies (*B*), electrophoresed, and fluorographed as in Fig. 5. Bands labeled Fos and Jun were not recovered when Jun antibodies were preincubated with unlabeled bacterially expressed Jun protein (data not shown).

from old fibroblasts (Fig. 6*B*, lane 4). This is largely due to reduction in total Fos protein concentration in the old cells. But, although some Fos protein is synthesized by these cells (Fig. 6*A*, lane 4), we observe none either in complex with Jun (Fig. 6*B*, lane 4) or in active AP-1-binding complexes (Fig. 2). Therefore, we suggest that the Fos protein synthesized in old cells may be impaired in its ability to form active heterodimeric DNA-binding complexes with Jun.

Fos Protein Is Modified Differently in Young and Old Fibroblasts. To test the hypothesis that the Fos protein synthesized in old cells differs from that produced by young cells, we analyzed immunoprecipitated Fos by high-resolution two-dimensional gel electrophoresis. Fig. 7 shows that Fos was resolved into at least seven isoforms in young cells stimulated with serum or cAMP. In old cells only some of the isoforms were seen, with those labeled 1–4 being barely detectable or completely absent. Old serum-stimulated cells contained two other isoforms (labeled 8 and 9) at more acidic isoelectric points, and old cAMP-stimulated cells showed only one major isoform (labeled 5) and barely detectable amounts of isoforms labeled 1 and 4. Thus, in addition to expressing lower amounts of Fos in response to stimulation, the Fos protein expressed by old fibroblasts is modified to produce a smaller number of more acidic (and presumably more highly phosphorylated) isoforms. These modified forms of Fos may differ in their ability to form heterodimers with Jun or to bind DNA.

DISCUSSION

This study shows that AP-1 activity is required in primary human fibroblasts for the initiation of DNA synthesis in response to serum. Since Fos is a major constituent of the proteins that bind specifically to the oligonucleotide microinjected (Fig. 2), the previously described requirement for Fos during mitogen-induced growth (7–9) is likely due to partici-

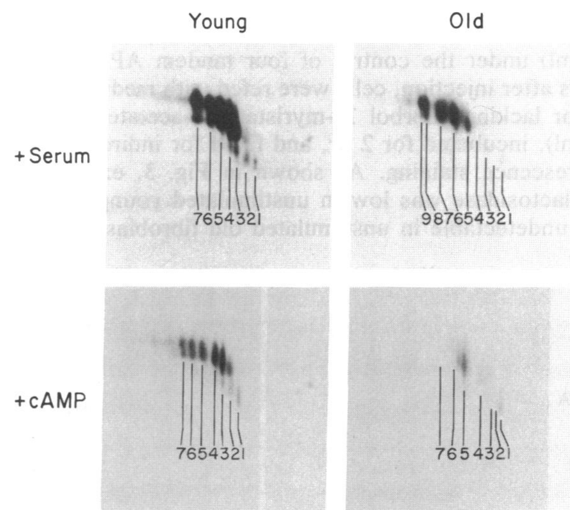

FIG. 7. Old fibroblasts lack isoforms of Fos seen in young fibroblasts. Lysates from young (MPD = 42) (*Left*) and old (MPD = 78) (*Right*) fibroblasts were prepared and immunoprecipitated as described in Fig. 5. (*Upper*) Cells were refed with medium containing 10% FBS. (*Lower*) Cells were refed with medium containing 0.5 mM isobutylmethylxanthine and 10 μM forskolin. Immunoprecipitation products were resolved on high-resolution two-dimensional gels as described (17). All immunoprecipitations contained equal amounts of [^{35}S]methionine. Total immunoprecipitation products were electrophoresed on high-resolution two-dimensional gels except for young cells stimulated with serum in which only 25% of the immunoprecipitation product was electrophoresed to allow resolution of Fos isoforms. Left to right corresponds to acidic to basic and top to bottom corresponds to lower to higher electrophoretic mobility in the denaturing second dimension. Isoforms are labeled 1–9.

pation of Fos in a DNA-bound transcription complex. We also determined that AP-1 activity was decreased significantly in old and senescent fibroblasts. This observation contrasts with generally modest (up to 2-fold) differences seen in other cellular properties during aging, including amounts of Oct-1 binding activity (data not shown), levels of DNA methylation (21), translocation of protein kinase C (31), size and number of extrachromosomal DNA elements (23), length of telomere sequences (24), rate of DNA replication (25), and the expression of 11 growth-related genes excluding c-*fos* (13).

Old cells that displayed decreased AP-1 activity *in vitro* and *in vivo* were not fully senescent. They were still capable of a limited number of cell divisions, but at a severely reduced growth rate reflected by a 3.5-fold decrease in the number of cells incorporating [^{3}H]thymidine over a 36-hr period (Table 1). The fact that AP-1 activity decreased during the onset of proliferative decline in these cells, rather than when cells became fully senescent, suggests a critical connection between decreased AP-1 activity and the ability of fibroblasts to proliferate in culture.

Decreased AP-1 activity could be due to reduced expression of components of the AP-1 complex or to production of dominant inhibitors that block the DNA-binding or transcriptional activity of the complex. Our data are most consistent with reduced synthesis coupled with altered posttranslational processing of the Fos protein. Reduced synthesis of Fos protein is likely due to a reduction in c-*fos* gene transcription as reported for another primary human cell line (14). Although our data do not directly address the mechanism of this reduction, reduced Fos protein synthesis in response to several stimuli that act through diverse regulatory elements in the c-*fos* promoter (15) suggests that a generalized repression of c-*fos* transcription, perhaps due to expression of a dominant inhibitor, rather than selective loss of inducing signals, is responsible for reduced Fos synthesis. This model is consistent with observations suggesting dominance of the senescent phenotype (22, 26).

One such inhibitor might be the product of the retinoblastoma (Rb) gene. Rb, which is a growth suppressor gene (27), inhibits the expression of reporter genes driven by a c-*fos* promoter (28) and is not phosphorylated in senescent IMR-90 fibroblasts in response to serum addition (29). In contrast, we did not see changes in the expression or phosphorylation state of Rb or p53 immunoprecipitated from young and old Hs68 fibroblasts, even though these cells showed large differences in their ability to express Fos. However, in subsequent experiments we have seen decreased phosphorylation of Rb in fully senescent Hs68 cells (data not shown) as reported for IMR-90 fibroblasts, but only several MPDs later than initial decreases in Fos expression. Since phosphorylation of Rb begins at the onset of DNA synthesis, well after the early to mid-G_1 requirement for Fos for entry into S phase (9), decreased phosphorylation of Rb may be secondary to loss of AP-1 activity and subsequent inability of cells to traverse G_1.

In addition to the decrease in overall AP-1 activity, the selective reduction in Fos synthesis means that there is a significant change in the composition of residual AP-1 complexes in aging and senescent cells. This change in composition is potentially very important in the light of recent reports that AP-1 proteins interact with other transcription factors and that Fos–Jun heterodimers can differ in activity from Jun–Jun homodimers (30).

These data provide a clear example of a change in a specific transcriptional activity during *in vitro* aging in fibroblasts. They also suggest, but do not prove, that changes in AP-1 activity might be responsible for the inability of normal diploid fibroblasts to replicate indefinitely. One way to address this question would be to establish cell lines that express higher levels of Fos in a regulated manner and then determine if these lines would proliferate longer than naive parental lines. However, since AP-1 activity is due to the protein products of at least two gene families, and our data indicate that Fos is posttranslationally modified to different extents during *in vitro* aging, evaluation of such experiments may not be straightforward. An approach that may prove more productive is to identify more of the regulatory proteins that control Fos expression.

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