Expression of c-*fos* and AP-1 Activity in Senescent Human Fibroblasts Is Not Sufficient for DNA Synthesis

David W. Rose,* Gregory McCabe,‡ James R. Feramisco,* and Mark Adler‡

*Departments of Medicine and Pharmacology, University of California, San Diego Cancer Center, La Jolla, California 92093; and ‡Sam and Rose Stein Institute for Research on Aging, University of California, San Diego, La Jolla, California 92093

Abstract. Human fibroblasts have a limited replicative life span when maintained in culture after which they become unresponsive to treatment with mitogens, a phenomenon most commonly called senescence. Experiments indicating that serum does not induce expression of the c-*fos* proto-oncogene in senescent fibroblasts raised the issue of a potential central role for c-*fos* in the phenotype of sustained growth arrest. This was directly tested by microinjection of oncogenic c-Ha-*ras* protein into senescent fibroblasts. While *ras* injection was found to induce marked nuclear c-*fos* expression and functional AP-1 transcription activity, this did not lead to DNA synthesis. These results suggest that the senescence phenotype cannot be solely attributed to the absence of c-*fos* expression and that the proliferative block in these cells is either independent of AP-1 transcriptional activity, downstream of it, or involves multiple molecular mechanisms.

ESSENTIALLY all eukaryotic cells demonstrate a finite capacity for cell division when serially passaged in vitro (Hayflick and Moorhead, 1961). This phenomenon, referred to as cellular senescence, is characterized by maintenance of viability and metabolism in the presence of sustained cell cycle arrest (reviewed in Goldstein, 1990; McCormick and Campisi, 1991). Because cultured fibroblasts taken from young individuals are capable of a greater number of population doublings than cells from older subjects, cellular senescence is considered to reflect and contribute to the aging process of the organism.

Recent studies have addressed the molecular basis for the block to proliferation in senescent cells. Comparison of the expression of cell cycle dependent genes in early passage and senescent fibroblasts reveals that growth arrest in the latter occurs near the G1/S boundary of the cell cycle just before the onset of DNA synthesis (Rittling et al., 1986). Senescent fibroblasts exhibit a highly attenuated growth response following addition of serum or a variety of purified growth factors despite the presence of normal numbers of growth factor receptors (Goldstein and Shmookler Reis, 1985).

In presenescent fibroblasts rendered quiescent by serum deprivation, stimulation by mitogenic growth factors results in the rapid expression of the proto-oncogenes c-*fos* and c-*myc*, and an increased expression of c-*ras*, before the onset of proliferation (Greenberg and Ziff, 1984; Muller et al., 1984; Lu et al., 1989). Expression of all three genes has been shown to be essential for the initiation of DNA synthesis (Holt et al., 1986; Heikkila et al., 1987; Mulcahy et al., 1985; Riabowol et al., 1988). Similar studies conducted using senescent human fibroblasts have shown that while c-*myc* and c-*ras* are expressed in response to mitogen treatment (Rittling et al., 1986), c-*fos* appears repressed as a result of a specific transcriptional block (Seshadri and Campisi, 1990). On the basis of this observation, it has been suggested that an inherent mechanistic difference exists between the responses of presenescent and senescent cells to treatment with serum, and that the lack of c-*fos* transcription may account for the failure of the latter to proliferate (Goldstein, 1990; McCormick and Campisi, 1991). Evidence for a central role for c-*fos* in the senescent phenotype is thus indirect and derived from the observation of a deficiency in expression of the proto-oncogene in senescent cells. A more direct test of this hypothesis is therefore important.

Here, we describe an experimental approach which allows a direct assessment of the potential role of c-*fos* in the proliferation of senescent fibroblasts. Through direct microinjection of human Ha-*ras* oncogene protein (T-24 *ras*) into senescent human fibroblasts, we find that c-*fos* is rapidly expressed. Moreover, by coinjection of a reporter gene construct containing an AP-1 regulated promoter, we determined that the c-*fos* protein expressed in these cells was likely to be active as the senescent cells injected with the *ras* protein efficiently expressed the AP-1 regulated reporter gene. The injected cells, however, failed to progress through S-phase of the cell cycle as detected by a lack of DNA synthesis. These results suggest that expression of c-*fos*, and active AP-1 transcription factor, is not sufficient to relieve the cell cycle block in senescent human fibroblasts.

The mammalian *ras* proto-oncogene family consists of three members, c-Ha-*ras*, c-Ki-*ras*, and c-N-*ras*, each of

Address correspondence to Dr. Feramisco at Departments of Medicine and Pharmacology, University of California, San Diego, La Jolla, CA 92093.

which can acquire oncogenic characteristics by point mutations at discrete locations in their coding sequences (Tabin et al., 1982), or by overexpression (Wigler et al., 1984). Microinjection of oncogenic *ras* protein into quiescent fibroblasts has been shown to result in changes in cell morphology (Feramisco et al., 1984), induction of c-*fos* expression (Stacey et al., 1987), and initiation of DNA synthesis. In contrast, senescent human fibroblasts injected with oncogenic c-Ha-*ras* expression vectors (Lumpkin et al., 1986) fail to synthesize DNA. Transformation of cycling or quiescent fibroblasts by microinjected Ha-*ras* proteins proceeds temporally, with stimulation of membrane ruffling and pinocytosis within 30–90 min postinjection, induction of c-*fos* expression within 1–2 h, and increased DNA synthesis within 24 h. We sought to determine which identifiable components of the oncogenic *ras* proliferative program were active in senescent human fibroblasts.

Materials and Methods

Cell Culture

Human fetal lung fibroblasts (IMR-90 cells) were maintained at 37°C in a humidified incubator under a 5% CO_2 atmosphere in DME/high glucose (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts, Inc., Calabasas, CA) and penicillin/streptomycin. Senescent cells were generated from late passage human fetal lung fibroblasts, IMR-90 cells, which became nonproliferative after 47–55 population doublings during serial passage in vitro (Adler et al., 1991). Typically 5–7% of these cells were capable of DNA synthesis as compared to ~80% of early passage cells defined by <20 population doublings. An additional operational criterion for senescence was that the cells could not grow to confluence 4 wk after their final passage. Cells were grown initially on plastic dishes and were then prepared for microinjection by trypsinization and replating on sterile glass acid-washed coverslips at subconfluent density. Before injection, early passage and senescent fibroblasts were plated separately at equal density and rendered quiescent by incubation in DME supplemented with 0.5% serum for at least 36 h. Under these conditions, the proliferating fraction in both early and late passage cultures was <7%.

Microinjection

The following samples and concentrations were used in microinjection experiments: oncogenic T-24 *ras* protein, at a final concentration of ~1.0 mg/ml, resulting in the introduction of 10^5–10^6 molecules per cytoplasmically injected cell. The *ras* protein was purified from an *Escherichia coli* expression vector system (Gross et al., 1985) to near homogeneity as in Feramisco et al. (1984). Inert carrier IgG (Sigma Chemical Co., St. Louis, MO) was coinjected with all samples at a concentration of 2 mg/ml to allow the definitive identification of all microinjected cells. The coinjected IgG used was either murine or rabbit depending upon the particular experiment. Control injections of carrier IgG alone were carried out in all cases and were found not to affect any of the assays described below. In experiments involving the use of expression vectors, the purified 4XTRE/lacZ plasmid or the 5XCRE/lacZ plasmid (Meinkoth et al., 1990) were used at a final concentration of 0.5 mg/ml and nuclear microinjections were performed. In these cases, smaller amounts of protein were injected into the nuclei, however, some spillover into the cytoplasm was routinely observed.

Samples were microinjected into IMR-90 cells at room temperature using an Eppendorf microinjector (model 5242, Brinkman Instruments Inc., Westbury, NY) attached to an Eppendorf micromanipulator (model 5170 or 5171) and an inverted microscope (Carl Zeiss, Oberkochen, Germany). Samples were loaded into Eppendorf Femtotips using Microloader pipette tips. Injections were performed with a typical needle pressure of ~120 hPa for cytoplasmic injections and about 75 hPa for nuclear injections.

Staining and Fluorescence Microscopy

To examine morphological effects of oncogenic *ras* protein microinjection, early passage and senescent IMR-90 cells were injected with either murine IgG alone or with IgG and T-24 *ras*. After 3 h of incubation at 37°C, the cells were fixed for 30 min in 95% ethanol, 5% acetic acid and then stained to visualize the distribution of coinjected IgG. Microinjected murine IgG was detected using HRP-conjugated rabbit anti-mouse IgG (Amersham Corp., Arlington Heights, IL) followed by 3,3′ diaminobenzidine tetrahydrochloride as substrate. Coverslips were permanently mounted on glass microscope slides for analysis.

To quantitate DNA synthesis in injected cells, a solution of 5-bromo-2′-deoxyuridine (BrdU)[1] (Amersham Corp., cell proliferation kit) was added to the culture medium for 24 h after microinjection. After labeling, coverslips were washed in PBS, and the cells were then fixed in 3.7% formaldehyde in PBS and incubated with a monoclonal rat antibody against BrdU (Accurate Chemical and Scientific Corp., Westbury, NY). The antibody was diluted in a solution of 0.5% NP-40 in PBS containing 1,000 units/ml of DNAse I (Sigma Chemical Co.), 10 mM $MgCl_2$, and 5 mg/ml BSA. Staining was visualized using rhodamine-conjugated donkey anti-rat IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Microinjected cells were detected by staining with a fluorescein isothiocyanate-conjugated horse anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). Expression of c-*fos* protein was detected by staining with a polyclonal antibody to human c-*fos* (Oncogene Science, Inc., Manhasset, NY) followed by sequential use of biotinylated horse anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and streptavidin-conjugated Texas red (Amersham Corp.). The anti-*fos* antibody is specific for c-*fos* and other *fos* family members.

In experiments measuring transcriptional activity from microinjected reporter plasmids, nuclei of IMR-90 cells were injected with mixtures of carrier IgG and the appropriate expression vector either in the absence or presence of T-24 *ras* protein. To test for the presence of AP-1 (*fos* dependent) transcriptional activity, the 4XTRE/lacZ construct (Meinkoth et al., 1990) was used. As a control, the 5XCRE/lacZ expression vector was microinjected in parallel. 6 h after injection, cells were fixed and stained overnight with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) to detect β galactosidase expression. To insure the activity of the vector, duplicate injections of the 5XCRE/lacZ plasmid were performed, and the cells were treated with 1 mM 8-bromo-cAMP and 1 mM isobutylmethylxanthine for 3 h before staining. In all cases, injected cells were detected by staining with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Cappel Laboratories). All injection experiments described were performed a minimum of three separate times. Cells were viewed and results analyzed on an Axiophot epifluorescence microscope (Carl Zeiss) equipped with fluorescein and rhodamine fluorescence filters. Photomicrographs were taken on Technical Plan 100 film (phase contrast) or T-Max 400 film for fluorescence (Eastman Kodak Co., Rochester, NY).

Results

ras-Induced Morphological Changes

Microinjection of T-24 *ras* protein into senescent fibroblasts resulted in membrane-associated morphological changes within a short period of time, which became obvious with immunohistochemical staining within 2–4 h (Fig. 1). Increased membrane ruffling, closely associated with pinocytosis, has been shown previously to be an integral part of the *ras*-induced proliferative process (Bar-Sagi and Feramisco, 1986). Similar cell surface ruffling and fluid-phase pinocytosis, although less distinct and sustained, occur before the mitogenic effect of serum or purified growth factors upon cells maintained in serum-free medium (Myrdal and Auersperg, 1986). The majority of *ras* protein-injected cells exhibited grossly observable cytoplasmic vacuolation. Senescent cells which were injected with carrier IgG alone for comparison purposes did not exhibit observable effects from the microinjection process (Fig. 1). These cell surface changes seen in senescent fibroblasts injected with T-24 *ras* protein provided evidence that at least some of the early

1. *Abbreviations used in this paper*: BrdU, 5-bromo-2′-deoxyuridine; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

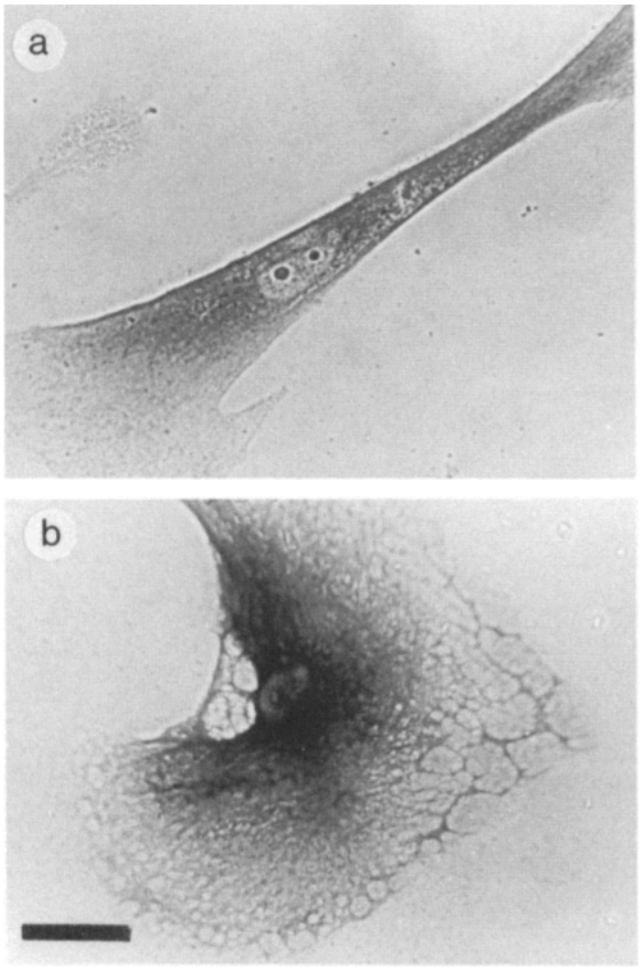


Figure 1. Morphological changes occur in senescent human fibroblasts as a result of oncogenic H-*ras* microinjection. Subconfluent IMR-90 cells were cultured on glass coverslips and microinjected with either T-24 *ras* protein (*b*) (containing carrier IgG for detection purposes) or with IgG alone (*a*). 3 h after injection, the cells were fixed and stained to visualize the distribution of injected IgG. Phase contrast, scale bar, 25 μm.

components of the proliferative process were intact, and that the block in proliferation was therefore either independent of these phenotypic changes or downstream of them. In addition, the morphological changes demonstrated the biological activity of the *ras* protein in this cell line. This observation raised the question of whether *ras*-mediated induction of c-*fos* expression, observed in quiescent presenescent fibroblasts, also occurs in senescent cells.

ras-Induced fos Protein Expression

To evaluate the expression of c-*fos* after the injection of oncogenic *ras* protein, senescent fibroblasts were injected with *ras* protein and subsequently were stained simultaneously for the presence of coinjected carrier IgG and for the presence of c-*fos* protein in the nucleus of injected cells. The use of two different fluorescent probes allowed simultaneous detection of injected cells and c-*fos* expression in those individual cells (Fig. 2). In addition, a parallel set of microinjected cells were processed for the incorporation of the

Table I. c-fos *Expression and DNA Synthesis as a Result of Oncogenic H-*ras *Microinjection in IMR-90 Cells*

Protein injected	c-*fos* expression		BrdU incorporation	
	Presenescent	Senescent	Presenescent	Senescent
	(% of cells injected)			
T-24 *ras*	71 (92/129)*	62 (95/154)	77 (85/111)	5 (7/127)
IgG alone	10‡ (14/136)	10 (12/117)	3 (4/122)	2 (2/99)

* Numbers in parenthesis indicate total numbers of cells scored positive for c-*fos* expression or DNA synthesis relative to total number of cells injected. ‡ microinjection of control protein resulted in weak nuclear c-*fos* staining in a small percentage of cells. This staining was not as intense as that seen when *ras* protein was injected.

thymidine analog BrdU to identify cells which entered S phase of the cell cycle. The results demonstrated (Table I) that the majority of the serum-starved early passage fibroblasts synthesized DNA after *ras* injection whereas only a rare cell in senescent populations was found to incorporate BrdU. Unexpectedly, injected *ras* protein induced a strong positive signal for c-*fos* in the majority of nuclei in both presenescent and senescent fibroblasts (Fig. 2). Because the majority of *ras*-injected senescent cells expressed c-*fos* protein while few cells synthesized DNA under the same conditions, it appears clear that expression of c-*fos* in these cells was not sufficient to stimulate DNA synthesis.

AP-1 Dependent Gene Expression in Senescent Cells

Because members of the c-*fos* family form complexes with members of the c-*jun* family, constituting the family of transacting transcription factors, AP-1, it was of importance to attempt to determine if senescent cells injected with the *ras* oncogene protein contained functional AP-1 transcription factor. This may be of particular importance in senescent cells, because AP-1 is known to play a role in the transcriptional regulation of a variety of cellular genes, many of which are subject to control by signals for growth or transformation (Lee et al., 1987*a,b*; Piette and Yaniv, 1987; Angel et al., 1987; Franza et al., 1988). To test this possibility, reporter constructs containing the *E. coli* lac-Z gene fused to promoters containing either AP-1 (TRE), or cAMP-responsive (CRE), regulatory sequences (Meinkoth et al., 1990; 1991) were assayed for expression of β-galactosidase in the senescent cells after coinjection with the *ras* protein or control protein. Previous studies demonstrated that microinjection of the *ras* protein into cell lines stably transfected with these reporter genes induces β-galactosidase expression from promoters containing AP-1 binding sites but not CRE sequences (D. Rose, manuscript in preparation). While it has not been directly proven that the injected *ras* protein activates transcription of the AP-1 regulated reporter gene via a functional AP-1 protein complex containing *fos* and *jun*, it is likely that this is indeed the case (Chiu et al., 1988). Thus, these experiments would test whether or not senescent cells were capable of showing enhanced transcription of an AP-1 reporter construct after oncogenic *ras* injection. Both presenescent and senescent cells coinjected with *ras* protein and the TRE-lac Z plasmid exhibited efficient β galactosidase expression (Fig. 3). The cells expressed detectable β-galactosidase as early as 3 h postinjection, and required active *ras* protein in addition to the construct for this expression. In

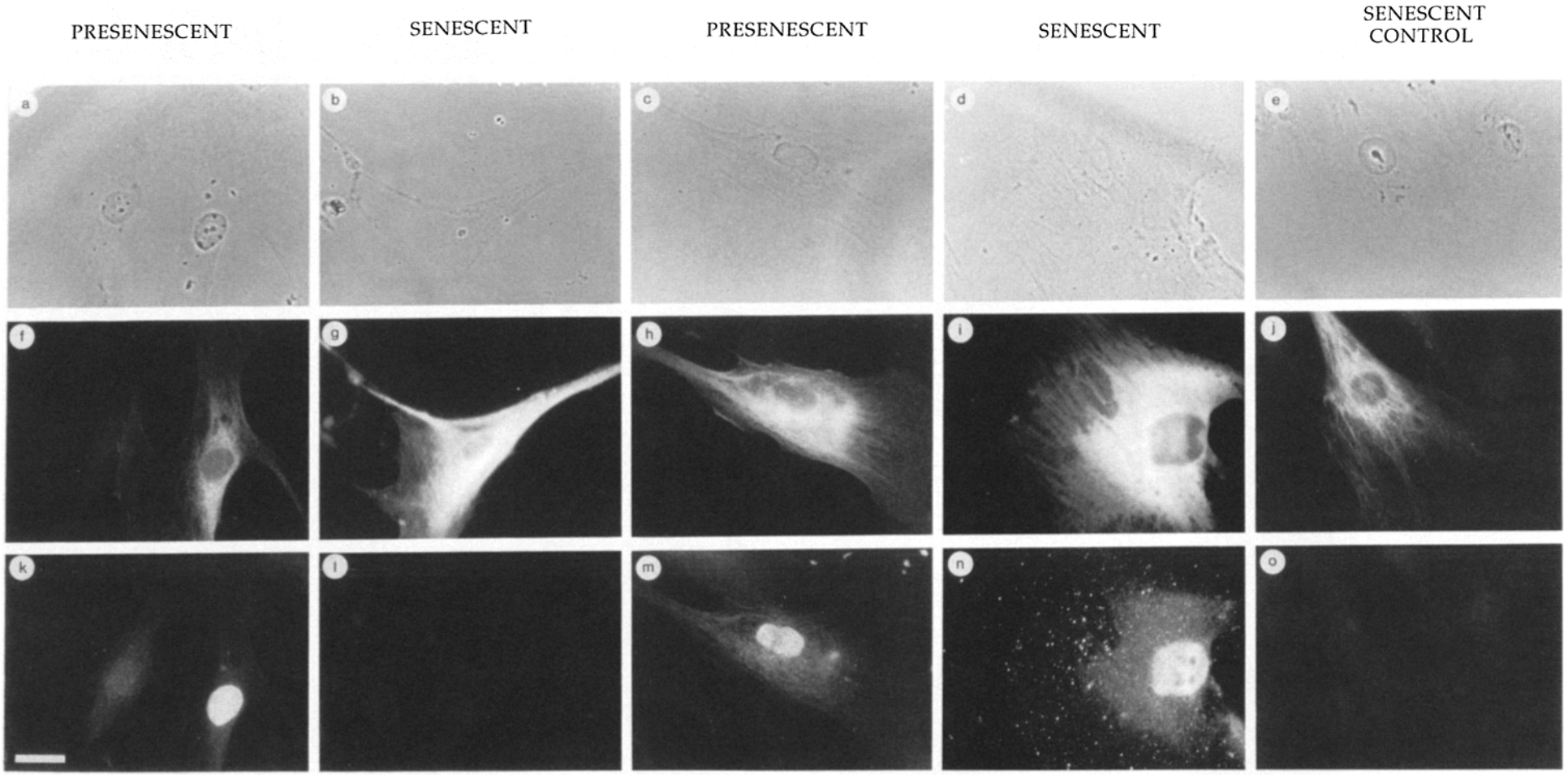


Figure 2. Expression of c-*fos* in *ras*-injected senescent fibroblasts in the absence of DNA synthesis. Microinjection was carried out as in Fig. 1. Shown are phase contrast (*a–e*) and the corresponding fluorescence photomicrographs which demonstrate staining for microinjected protein (*f–j*), incorporation of BrdU (*k*, *l*) or expression of c-*fos* (*m–o*). Microinjection of *ras* induces DNA synthesis in presenescent fibroblasts (*a*, *f*, *k*) but not in senescent fibroblasts (*b*, *g*, *l*). Microinjection of *ras* results in c-*fos* expression regardless of whether the cells are presenescent (*c*, *h*, *m*) or senescent (*d*, *i*, *n*). Senescent cells do not express c-*fos* in response to carrier IgG injection (*e*, *j*, *o*). Scale bar, 25 μm.

Table II. Beta Galactosidase Expression as a Result of Coinjection of Oncogenic H-ras and pTRE/lacZ Plasmid in IMR-90 Cells

Sample injected	β-galactosidase expression (% of cells injected)	
	Presenescent	Senescent
pTRE/lacZ	6 (15/268)*	5 (14/283)
pTRE/lacZ and T-24 *ras*	78 (204/263)	70 (217/312)
pCRE/lacZ	4 (12/276)	4 (9/248)
pCRE/lacZ‡	78 (258/331)	82 (207/253)
pCRE/lacZ and T-24 *ras*	5 (12/267)	5 (13/247)

* Numbers in parenthesis indicate total numbers of cells expressing β-galactosidase relative to the total number of cells injected.
‡ after microinjection, cells were treated for 6 h with 8-bromo-cAMP and isobutylmethyxanthine.

contrast, neither type of cell expressed the marker enzyme when the CRE-lacZ reporter construct was coinjected with *ras* protein. The activity of the CRE-lacZ plasmid was confirmed by duplicate microinjections followed by treatment of the cells with 8-bromo-cAMP and isobutylmethylxanthine (Table II). These results suggested that while senescent cells injected with the *ras* protein show enhanced AP-1 dependent gene expression, not all types of transcription factors (i.e., cAMP response element binding protein) are thus activated. The results document the presence in senescent cells of active AP-1 transcriptional activity specific for a TRE-containing regulatory element in response to oncogenic *ras* protein microinjection. Thus, while this activity has recently been shown to be decreased in mitogen-stimulated human fibroblasts as the cells age (Riabowol et al., 1992), injection of *ras* protein appears to generate a sufficient amount of active AP-1 to give rise to reporter activity.

Discussion

Evidence for a causative role of c-*fos* in the senescent phenotype has been, to date, the observation of a deficit of its expression in senescent cells. Considering the potential importance of this possibility, we sought a direct experimental approach to test the role of c-*fos* by attempting to induce the expression of the protein in an intact cellular context. Using an assay which permits examination of effects upon individual cells, we have investigated the role of c-*fos* in senescent cell growth control. Expression of the c-*fos* proto-oncogene is an essential prerequisite for the initiation of DNA synthesis in quiescent fibroblasts stimulated with serum. Senescent human fibroblasts do not express c-*fos*, however, in response to serum treatment (Seshadri and Campisi, 1990), despite the observation that these cells appear to express and regulate a number of other genes in a manner typical of fibroblasts. If the observed repression of c-*fos* expression were the primary defect in senescent cells, one would anticipate that its expression would restore the proliferative potential of the cells, particularly in the presence of a strong replicative signal such as oncogenic *ras*. The present results, expression of c-*fos* protein in the absence of DNA synthesis, appear to indicate that this is not the case. Recent results obtained by others (Surmacz et al., submitted for publication) lead to a similar conclusion. Moreover, the present results appear to indicate that while senescent cells are capable of enhanced expression of indicator constructs regulated by AP-1 promoter sequences when they are coinjected with oncogenic *ras* protein, this increased activity, presumably a downstream effect of increased c-*fos* expression (Schonthal et al., 1988) is not sufficient to alleviate the block in the cell cycle of senescent cells. Thus, c-*fos* expression is likely to be necessary but not sufficient for the proliferation of these cells (Surmacz et al., submitted for publication).

These results support the view that senescent cells do not fail to proliferate because of an inherent inability to transduce these early stimulatory signals for growth. Senescent cells possess apparently normal growth factor receptors (Goldstein and Shmookler Reis, 1985). The generation of transcriptionally active AP-1 following injection of oncogenic *ras* protein into senescent cells indicates that at least some of the intracellular signaling pathways are operative. It is still possible that the arrest to growth in senescent cells is due to the selective repression of one or several normal responses to signals for growth. Microinjection of oncogenic *ras* protein apparently provides a positive signal of sufficient intensity to overcome the repression of c-*fos*. This result is of interest in and of itself in view of the general lack of induction of c-*fos* expression by serum. One possible explanation for this is that injected *ras* oncogene proteins, which cause an exaggerated ruffling response in comparison to the response seen for serum treatment, overcome a putative block in the normal signaling process associated with the growth factor receptors in the plasma membrane. As it appears that purified mitogens may also induce c-*fos* expression to some extent in senescent cells (Paulsson et al., 1986), this possibility seems plausible.

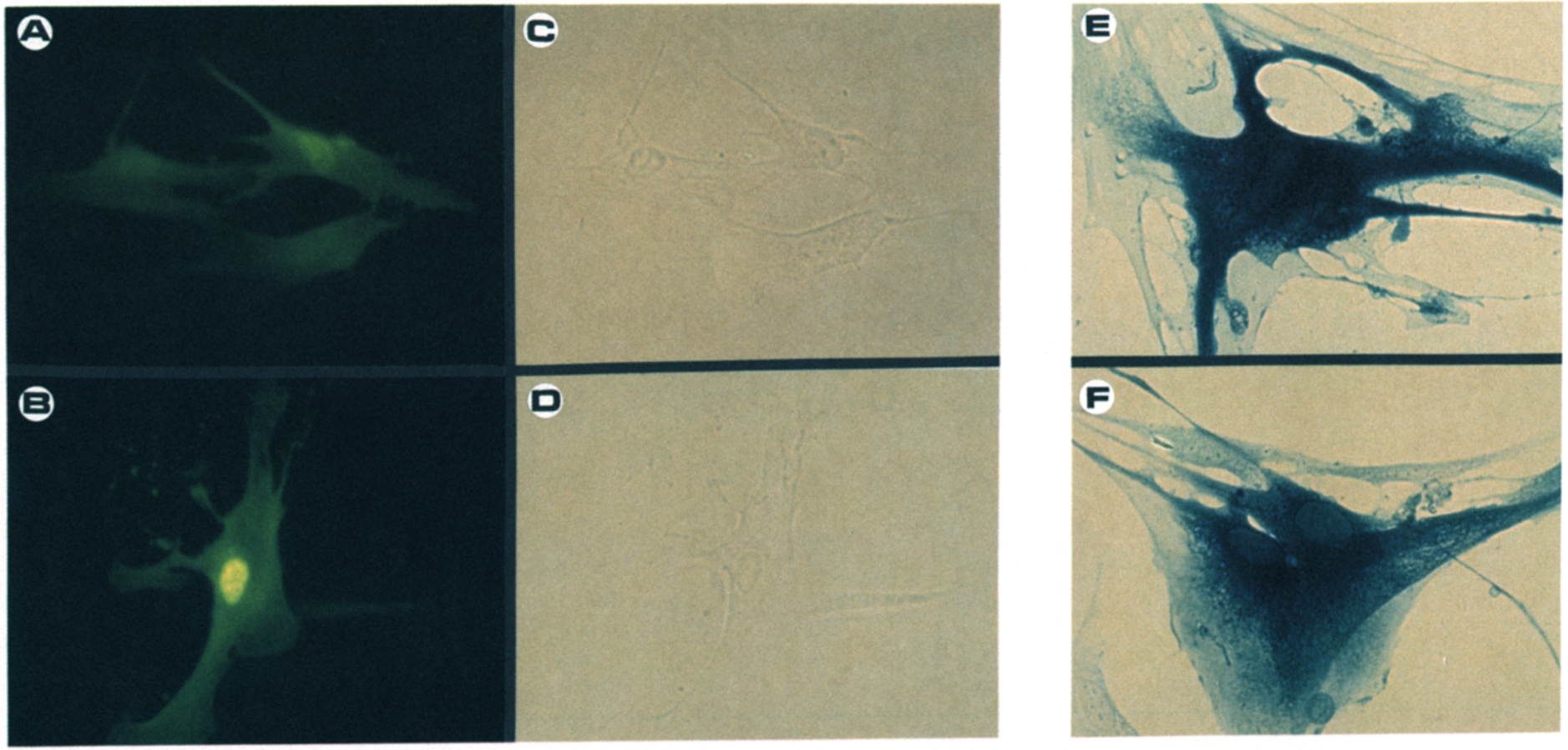
Another potential underlying mechanism in senescence involves the regulation of the retinoblastoma susceptibility gene product (Rb). The Rb protein is thought to function in a growth-inhibitory manner in normal cells. It is found in a hypophosphorylated state in quiescent cells, and it is thought that traversion of the G1/S cell cycle boundary, allowing DNA synthesis to occur, is dependent upon the hyperphosphorylation of the protein (Mihara et al., 1989). In senescent cells, this phosphorylation does not occur (Stein et al., 1990). Additionally, SV40 T antigen, which preferentially binds to and is thought to inactivate the hypophosphorylated form of the Rb protein, also induces DNA synthesis when introduced into senescent cells (Wright et al., 1989; Radna et al., 1989) and induces c-*fos* expression (Surmacz et al. submitted for publication). In the 5′ promoter region of the c-*fos* gene, a negative-acting Rb response element has recently been identified (Robbins et al., 1990). It is not currently known whether the phosphorylation state of the Rb protein has any effect upon its ability to influence the expression of the c-*fos* gene, or whether such a mechanism is operative during senescence. We are currently in the process of evaluating the regulation of the Rb protein in response to microinjection of oncogenic *ras* protein. It is clear, however, from results presented here that if in fact the phosphorylation state of the Rb protein is a determining factor in the lack of progression from G1 to S phase in senescent cells, it appears not to be the mechanism responsible for repression of c-*fos* expression. Because microinjection of *ras* protein affects c-*fos* protein expression but not DNA synthesis, the two

FLUORESCEIN
(anti-IgG)

PHASE CONTRAST

PRESENESCENT

SENESCENT



WITHOUT
T-24 *ras*

WITH T-24 *ras*

events appear to be independently regulated. Events downstream or parallel to c-*fos* expression deserve particular attention in future efforts to explain the molecular nature of the senescent state.

We acknowledge A. Schonthal for helpful discussions and suggestions. We would like to thank A. Thorburn and J. Meinkoth for critical reading of the manuscript.

Supported by grants from the National Cancer Institute and the Council for Tobacco Research. D. Rose is the recipient of support from a Public Health Service postdoctoral training grant.

Received for publication 19 May 1992 and in revised form 3 August 1992.

References

Adler, M. J., C. Coronel, E. Shelton, J. E. Seegmiller, and N. N. Dewji. 1991. Increased gene expression of Alzheimer disease β-amyloid in senescent cultured fibroblast. *Proc. Natl. Acad. Sci. USA.* 88:16–20.

Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulator trans-acting factor. *Cell.* 49:729–739.

Bar-Sagi, D., and J. R. Feramisco. 1986. Induction of membrane ruffling: Fluid phase pinocytosis in quiescent fibroblasts by ras proteins. *Science (Wash. DC).* 233:1061–1068.

Chiu, R., W. J. Boyle, J. Meek, T. Smeal, T. Hunter, and M. Karin. 1988. The c-fos protein interacts with c-jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell.* 54:541–552.

Feramisco, J. R., M. Gross, T. Kamata, M. Rosenberg, and R. W. Sweet. 1984. Microinjection of the oncogene form of the human H-ras (T-24) protein results in rapid proliferation of quiescent cells. *Cell.* 38:109–117.

Franza, B. R. Jr., F. J. Rauscher III, S. F. Josephs, and T. Curran. 1988. The fos complex and fos related antigens recognize sequence elements that contain AP-1 binding sites. *Science (Wash. DC).* 239:1150–1153.

Goldstein, S. 1990. Replicative senescence: the human fibroblast comes of age. *Science (Wash. DC).* 249:1129–1133.

Goldstein, S., and R. J. Shmookler Reis. 1985. Methylation patterns in the gene for the alpha subunit of chorionic gonadotropin are inherited with variable fidelity in clonal lineages of human fibroblasts. *Nucleic Acids Res.* 13: 7055–7060.

Greenberg, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature (Lond.).* 211:433–438.

Gross, M., R. W. Sweet, G. Sathe, S. Yolcoyama, O. Fasano, M. Goldfarb, M. Wigler, and M. Rosenberg. 1985. Purification and characterization of human H-ras proteins expressed in Escherichia coli. *Mol. Cell. Biol.* 5: 1015–1024.

Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 25:585–621.

Heikkila, R., G. Schwab, E. Wickstrom, S. K. Loke, D. H. Pluznik, R. Watt, and L. M. Neckers. 1987. A c-myc antisense oligonucleotide inhibits entry into S phase but not progress from G0 to G1. *Nature (Lond.).* 328:445–449.

Holt, J. T., T. Venkat-Gopal, A. D. Moulten, and A. W. Nienhaus. 1986. Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation. *Proc. Natl. Acad. Sci. USA.* 83:4794–4798.

Lee, W., A. Haslinger, M. Karin, and R. Tjian. 1987*a*. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionine gene and SV40. *Nature (Lond.).* 325:368–372.

Lee, W., P. Mitchell, and R. Tjian. 1987*b*. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell.* 49:741–752.

Lu, K., R. A. Levine, and J. Campisi. 1989. c-ras-Ha gene expression is regulated by insulin-like growth factor and by epidermal growth factor in murine fibroblasts. *Mol. Cell Biol.* 9:3411–3417.

Lumpkin, C. K., J. E. Knepper, J. S. Butel, J. S. Smith, and O. M. Pereira-Smith. 1986. Mitogenic effects of the proto-oncogene and oncogene forms of c-H-ras DNA in human diploid fibroblasts. *Mol. Cell. Biol.* 6:2990–2993.

McCormick, A., and J. Campisi. 1991. Cellular aging and senescence. *Curr. Opinion Cell Biol.* 3:230–234.

Meinkoth, J., A. S. Alberts, and J. R. Feramisco. 1990. Construction of mammalian cell lines with indicator genes driven by regulated promoters. *CIBA Found. Symp.* 150:47–56.

Meinkoth, J., M. R. Montminy, J. S. Fink, and J. R. Feramisco. 1991. Induction of a cyclic AMP-responsive gene in living cells requires the nuclear factor CREB. *Mol. Cell. Biol.* 11:1759–1764.

Mihara, K., X. R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A. Tang, and Y. K. T. Fung. 1989. Cell cycle dependent regulation of phosphorylation of the human retinoblastoma gene product. *Science (Wash. DC).* 246:1300–1303.

Mulcahy, L. S., M. R. Smith, and D. W. Stacy. 1985. Requirement for ras protooncogene function during serum-stimulated growth of NIH 3T3 cells. *Nature (Lond.).* 312:241–243.

Muller, R., R. Bravo, J. Burckhardt, and T. Curran. 1984. Induction of c-fos gene and protein by growth factors precedes activation of c-myc. *Nature (Lond.).* 312:716–720.

Myrdal, S. E., and N. Auersperg. 1986. An agent or agents produced by virus-transformed cells cause unregulated ruffling in untransformed cells. *J. Cell Biol.* 102:1224–1231.

Paulsson, Y., M. Bywater, S. Pfeifer-Ohlsson, R. Ohlsson, S. Nilsson, C. H. Heldin, B. Westermark, and C. Betsholtz. 1986. Growth factors induce early pre-replicative changes in senescent human fibroblasts. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2157–2162.

Piette, J., and M. Yaniv. 1987. Two different factors bind to the α-domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-fos enhancers. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1331–1337.

Radna, R. L., Y. Caton, K. K. Jha, P. Kaplan, G. Li, F. Traganos, and H. L. Ozer. 1989. Growth of immortal simian virus 40 tsA-transformed human fibroblasts is temperature dependent. *Mol. Cell. Biol.* 9:3093–3096.

Riabowol, K. T., R. J. Vosatka, E. B. Ziff, N. J. C. Lamb, and J. R. Feramisco. 1988. Microinjection of fos-specific antibodies blocks DNA synthesis in fibroblast cells. *Mol. Cell. Biol.* 8:1670–1676.

Riabowol, K., J. Schiff, and M. Gilman. 1992. Transcription factor AP-1 activity is required for initiation of DNA synthesis and is lost during cellular aging. *Proc. Natl. Acad. Sci. USA.* 89:157–161.

Rittling, S. R., K. M. Brooks, V. J. Cristofalo, and R. Baserga. 1986. Expression of cell cycle-dependent genes in young and senescent WI-38 fibroblasts. *Proc. Natl. Acad. Sci. USA.* 86:3316–3320.

Robbins, P. D., J. M. Horowitz, and R. C. Mulligan. 1990. Negative regulation of human c-fos expression by the retinoblastoma gene product. *Nature (Lond.).* 346:668–671.

Schönthal, A., P. Herrlich, H. J. Rahmsdorf, and H. Ponta. 1988. Requirements for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. *Cell.* 54:325–334.

Seshadri, T., and J. Campisi. 1990. Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts. *Science (Wash. DC).* 247:205–209.

Stacey, D. W., T. Watson, H. F. Kung, and T. Curran. 1987. Microinjection of transforming ras protein induces c-fos expression. *Mol. Cell. Biol.* 1: 523–527.

Stein, G. H., M. Beeson, and L. Gordon. 1990. Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts. *Science (Wash. DC).* 249:666–669.

Tabin, C. J., S. M. Bradley, C. I. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dahr, D. R. Lowy, and E. H. Chang. 1982. Mechanism of activation of a human oncogene. *Nature (Lond.).* 300: 143–149.

Wigler, M., O. Fasano, E. Taparowsky, S. Powers, T. Kataoka, D. Birnbaum, K. Shimizu, and M. Goldfarb. 1984. Structure and activation of ras genes. *Cancer Cells.* 2:419–425.

Wright, W. E., O. M. Pereira-Smith, and J. W. Shay. 1989. Reversible cellular senescence: Implications for immortalization of normal human diploid fibroblasts. *Mol. Cell. Biol.* 9:3088–3092.

Figure 3. Microinjection of oncogenic H-*ras* results in functional AP-1 activity in presenescent and senescent human fibroblasts. Nuclei of presenescent (*a*, *c*, *e*) or senescent (*b*, *d*, *f*) IMR-90 cells were injected with a mixture of carrier IgG and 4X TRE/lacZ expression vector (Meinkoth, et al., 1990) either in the absence (*a–d*) or presence (*e*, *f*) of added T-24 *ras* protein. 6 h later, all cells were fixed and stained with the chromogenic substrate Xgal to detect β galactosidase expression and also stained for injected protein. Fluorescence photomicrographs demonstrate which of the cells in *c* and *d* have been microinjected; the corresponding phase contrast pictures demonstrate the *ras*-dependence of β galactosidase expression (the lack of blue staining in *c* and *d*) and the presence of AP-1 transcriptional activity in cells which have been injected with *ras* protein, regardless of the age of the cells.