MOLECULAR AND CELLULAR BIOLOGY, July 1994, p. 4991–4999
0270-7306/94/$04.00+0

Vol. 14, No. 7

Loss of Serum Response Element-Binding Activity and Hyperphosphorylation of Serum Response Factor during Cellular Aging

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Received 21 January 1994/Returned for modification 2 March 1994/Accepted 28 April 1994

Human diploid fibroblasts undergo a limited number of population doublings in vitro and are used widely as a model of cellular aging. Despite growing evidence that cellular aging occurs as a consequence of altered gene expression, little is known about the activity of transcription factors in aging cells. Here, we report a dramatic reduction in the ability of proteins extracted from the nuclei of near-senescent fibroblasts to bind the serum response element which is necessary for serum-induced transcription of the c-*fos* gene. In contrast, the activities of proteins binding to the RNA polymerase core element, TATA, as well as to the cyclic AMP response element were maintained during cellular aging. While no major differences in the expression of the serum response factor (SRF) that binds the serum response element were seen between early-passage and late-passage cells, hyperphosphorylation of SRF was observed in near-senescent cells. Furthermore, removal of phosphatase inhibitors during the isolation of endogenous nuclear proteins restored the ability of SRF isolated from old cells to bind the SRE. These data, therefore, indicate that hyperphosphorylation of SRF plays a role in altering the ability of this protein to bind to DNA and regulate gene expression in senescent cells.

Primary diploid fibroblasts undergo a limited number of population doublings in vitro that is inversely proportional to the age of the donor and is directly proportional to the life span of the species from which they were explanted (16, 17, 27, 41). These facts, together with the observation that fibroblasts isolated from individuals with premature aging syndromes undergo fewer doublings than cells from normal control individuals (4), have resulted in the widespread use of the human diploid fibroblast as an in vitro model of cellular aging.

Results from a variety of experiments suggest that the loss of human diploid fibroblast proliferative potential with increased in vitro age occurs as a consequence of a genetic program, perhaps as a mechanism for limiting the growth of cancer cells (for a review, see references 11 and 28). To understand better the nature of such an aging program, the expression of a large number of genes has been examined in young (low-passage) and old (high-passage) human fibroblasts (39, 46, 47). For the majority of cellular genes examined, changes in expression have been found to be minimal. For example, the expression of genes encoding actin, ornithine decarboxylase, c-Myc, $p105^{Rb}$, and Jun, among others (34, 35, 39, 46, 47, 56), decreases by ≤50% as cells become senescent. In contrast, considerable changes in expression levels have been observed for a subset of growth-related genes such as those encoding insulin-like growth factor (IGF)-binding protein 3, cyclin D1, cdc2, cdk2, cyclin A, the A chain of platelet-derived growth factor, cyclin B, and Fos (2, 10, 23, 31, 34, 38, 48, 54–56).

Although the expression of many genes has been examined during cellular aging, few studies have examined the basic mechanism(s) responsible for altering expression levels. The major aim of our study was to investigate the mechanisms underlying the decreased expression of the c-*fos* gene when fibroblasts approach the end of their in vitro lifespans. The c-*fos* gene was chosen as a model in our studies for a variety of reasons. First, it is an immediate-early gene encoding a transcription factor that regulates the expression of several other genes (1). Second, Fos has been observed to be required for DNA synthesis and cell growth (19, 22, 30, 32, 37). Third, of the several gene products that have been found to be necessary for progression through the G_1 phase of the cell cycle, Fos is among the few whose expression cannot be induced by mitogenic stimuli in senescent cells (33, 34, 46), and overexpression of Fos in senescent cells is capable of inducing limited DNA synthesis (33). Finally, the DNA sequence elements upstream of c-*fos* have been found in the promoters of other growth-related genes, and the factors that bind to such sequence elements have been well characterized. Thus, the biochemical mechanisms underlying the decreased expression of a growth-regulatory gene such as c-*fos* has the potential to play a role in the regulation of other genes that are differentially expressed during cellular senescence.

A variety of stimuli regulate transcription through the region of DNA located 5′ of the c-*fos* gene which consists of three major sequence elements in addition to a TATA box. These elements include the cyclic AMP (cAMP) response element (CRE), which is regulated by cAMP-dependent protein kinase, and a *sis*-inducible element (SIE) that responds to platelet-derived growth factor-dependent pathways (3, 36, 43, 53). However, the element that appears most important in regulating the c-*fos* gene is the serum response element (SRE), which binds a variety of proteins. The SRE is a 20-bp sequence of dyad symmetry located 300 bp upstream from the c-*fos* transcriptional initiation site and is the major determinant of the transcriptional activation of c-*fos* in response to serum and purified growth factors (7, 8, 13–15, 50). This element is both necessary and sufficient to confer the normal transient kinetics of transcription seen for the intact c-*fos* promoter as deter-

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mined by promoter mutation studies (21, 40). Mutation of the SRE also strongly inhibits serum inducibility of transcription in vivo and nuclear regulatory factor binding in vitro (13). Since we had observed previously that the induction of c-*fos* expression in late-passage cells was most impaired in response to serum (versus phorbol esters and platelet-derived growth factor [34]), altered SRE activity might underlie the decreased expression within senescent cells of c-*fos* and possibly other normally serum-responsive genes.

Among the best characterized of the factors which bind specifically to the SRE is the serum response factor (SRF). SRF is a 67-kDa nuclear phosphoprotein that binds as a homodimer to the SRE and interacts with other proteins (for a review, see reference 51). One protein with which DNA-bound SRF interacts is $p62^{TCF}$, resulting in the formation of a ternary complex (42, 44). Although ternary complex formation is important for the growth factor-mediated induction of c-*fos*, subtle mutations of the SRE that impair the ability of $p62^{TCF}$ to form a ternary complex do not affect the ability of the SRE to interact with SRF. This result has revealed the existence of $p62^{TCF}$-dependent and -independent pathways in the transcriptional activation of the c-*fos* gene (12). A component of $p62^{TCF}$ (Elk1) has been isolated and found to be regulated through the growth factor-inducible mitogen-activated protein kinase cascade (18, 25). For both $p62^{TCF}$ and Elk1, the availability of active SRF appears to be necessary for the ability of the SRE to confer serum responsiveness to the c-*fos* promoter. Taken together, these data suggest a central role for SRF in the regulation of c-*fos* transcription (51).

In our analysis of c-*fos* promoter activity during cellular senescence, we have compared the abilities of proteins extracted from the nuclei of young and old human diploid fibroblasts to bind the SRE, using electrophoretic mobility shift assays. Our results demonstrate a dramatic decrease in the formation of SRF-specific complexes as cells age in vitro. Immunoprecipitation assays, immunofluorescence, and immunoblotting using anti-SRF (α-SRF) antibodies show that similar levels of SRF protein are present in nuclear extracts prepared from young and old cells. However, phosphorylation of SRF appears to be markedly increased in near-senescent cells. Furthermore, we also find that nuclear proteins isolated from old cells in the absence of the phosphatase inhibitor sodium fluoride are capable of binding to the SRE. Thus, hyperphosphorylation of SRF appears to inhibit the binding of SRF to the SRE, and this lack of binding may underlie the lack of expression of the c-*fos* gene during cellular senescence.

MATERIALS AND METHODS

Cell culture and preparation of nuclear extracts. Human Hs68 fibroblasts (American Type Culture Collection CRL no. 1635) were grown in Dulbecco's modified Eagle's medium supplemented with 1 mM glutamine and 10% (vol/vol) fetal bovine serum. The growth states of young and old cells were assessed by their ability to incorporate [^{3}H]thymidine into their nuclei. Early-passage cells were defined as those that had undergone 35 to 40 mean population doublings (MPD) and incorporated [^{3}H]thymidine into 85 to 90% of cells versus late-passage cells which had undergone 82 to 86 MPD and incorporated [^{3}H]thymidine into fewer than 10% of cells over 24 h. Subconfluent cells continuously growing in Dulbecco's modified Eagle's medium containing 10% fetal calf serum were used for nuclear protein extraction for mobility shift assays and immunoblots. Cell lysates for SRF immunoprecipitations were prepared from cells deprived of serum for 48 h, restimulated with media containing $^{32}P_i$ or [^{35}S]methionine and 10% fetal bovine serum, and harvested 1 or 6 h after stimulation for ^{32}P- and ^{35}S-labelled proteins, respectively (34, 52).

Extraction of nuclear proteins was performed by a modification of the method of Dignam et al. (6). Briefly, cells were rinsed with cold phosphate-buffered saline (PBS), scraped, and triturated in 0.05% Nonidet P-40 in PBS. The released nuclei were pelleted and extracted with a buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.8), 500 mM KCl, 5 mM NaF, 0.5 mM $MgSO_4$, 1 mM dithiothreitol, and protease inhibitors (aprotinin, pepstatin, and leupeptin) at a concentration of 0.1 mg/ml each (34).

Synthesis and labelling of probes. Single-stranded oligonucleotides were synthesized chemically using the phosphoramidite method. To generate double-stranded probes, equal amounts of two complementary single-stranded oligonucleotides were heated for 1 min at 90°C in annealing buffer (10 mM Tris [pH 7.5], 20 mM NaCl, 50 mM $MgCl_2$) and were slowly cooled to room temperature. These probes were designed to contain 5′ protruding termini that allowed labelling by use of the Klenow fragment of DNA polymerase. Labelling was done by incubating 50 ng of double-stranded oligonucleotides with 500 μM each dATP, dGTP, and dTTP, 20 μCi of [^{32}P]dCTP (3,000 Ci/mmol; Amersham), Klenow buffer (0.5 M Tris [pH 7.6], 0.1 M $MgCl_2$), and 5 U of Klenow enzyme. After 30 min of incubation at 37°C, unlabelled dCTP (5 mM) was added and the reaction was allowed to continue at 37°C for a further 30 min. The labelled probe was then gel purified. The following oligonucleotides were used for mobility shift assays: CRE wt, 5′-TCGACGGTATCGATAAGCTA**TGACGTCA**TCCGGG GGATC-3′; CRE mu, 5′-TCGACGGTATCGATAAGCTAT **AAGATTA**TCCGGGGGATC-3′; SRE wt/AP-1 wt, 5′-TTAC ACAGGATGT**CCATATTAGG**ACATC**TGCGTCA**GCAGG TTTCCA-3′; SRE mu/AP-1 mu, 5′-TTACACAGGATGT**G TATGCTAAC**ACATC**TAAGTTA**GCAGGTTTCCA-3′; SRE wt/AP-1 mu, 5′-TTACACAGGATGT**CCATATTAGG**ACATC **TAAGTTA**GCAGGTTTCCA-3′; TATA wt, 5′-GCAGAGCA **TATAA**GGTGAGGTAGGA-3′; TATA mu, 5′-GCAGAGCG **GCTCC**GGTGAGGTAGGA-3′. DNA fragments containing the SRE were 38 bp in length and contained (i) the wild-type SRE and a mutant AP-1 sequence (SRE wt/AP-1 mu), (ii) the wild-type sequences of both the SRE and AP-1, (SRE wt/AP-1 wt), and (iii) mutant sequences of both the SRE and AP-1 (SRE mu/AP-1 mu). Boldface letters indicate previously determined wild-type or mutant transcription factor binding sites.

Mobility shift assays. ^{32}P-labelled double-stranded oligonucleotides (2 ng; 30,000 cpm) were incubated with 4 μg of nuclear extracts in a buffer containing 20 mM Tris (pH 7.6), 4% Ficoll, 50 mM KCl, 1 mM EDTA, 0.2 mM dithiothreitol, 10 μM $ZnCl_2$, 1 μg of partially denatured and sonicated salmon sperm DNA, and 30 μg of bovine serum albumin in a total volume of 10 μl for 30 min. Unlabelled competitor DNA was included in some reaction mixtures, at 100-fold excess over labelled probe. In some reaction mixtures, 1 μl of control or experimental antibodies was added to 10 μl (total volume) of mobility shift assay mixtures. The reaction mixtures were electrophoresed through 4% nondenaturing polyacrylamide gels, and DNA-protein complexes were visualized by autoradiography after 12 h of exposure for all figures, as described elsewhere (34).

Western blotting (immunoblotting) and immunofluorescence. Nuclear proteins were isolated as described above, electrophoresed on polyacrylamide gels, and transferred onto nitrocellulose for 2 h at 25 V in a transfer buffer containing 120 mM Tris base, 40 mM glycine, and 20% methanol. Membranes were blocked in PBS containing 5% nonfat milk and 0.1% Tween 20 for 2 h at 37°C and incubated successively in 1:500

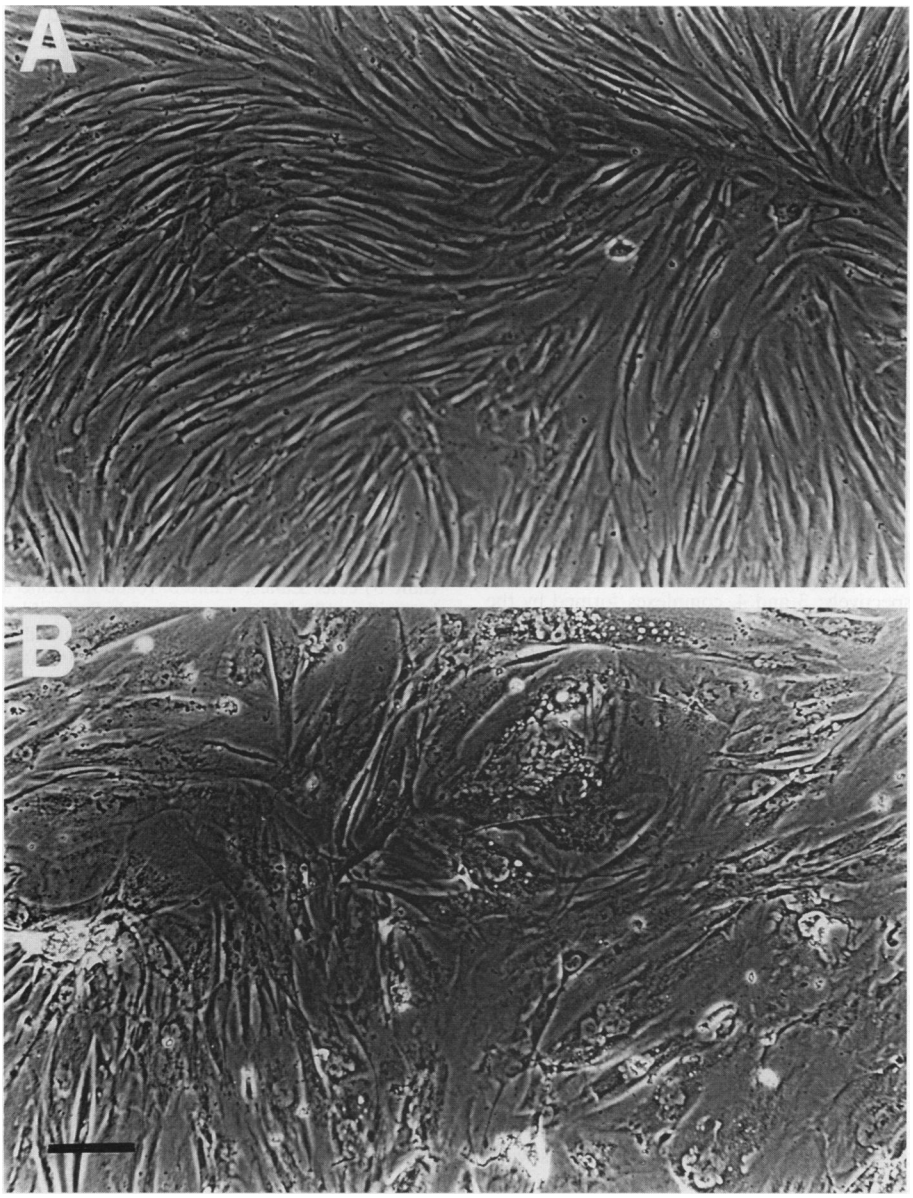

FIG. 1. Morphologies of young, proliferation-competent (A) and old, near-senescent (B) human diploid fibroblasts. Micrographs were taken as described elsewhere (34).

dilutions of α-SRF antibody (29), biotinylated sheep α-rabbit immunoglobulin G, and strepavidin-alkaline phosphatase for 1 h at 37°C. Antibodies were diluted in PBS containing 1% nonfat milk and 0.1% Tween 20 in PBS. Three washes of 10 min each were done with the same solution, minus antibody, between incubations. Nitroblue tetrazolium and bromochloroindoylphosphate were used as substrates under conditions recommended by the supplier (Gibco-BRL).

Staining of young and old cells with α-SRF antibodies for indirect immunofluorescence was done exactly as described elsewhere (37).

Antibodies used in this study. Antibodies were the kind gifts of M. Greenberg and R. Misra (α-SRF [29]). Polyclonal antibodies against the TATA-binding protein (TBP) were raised against intact bacterially expressed TBP (49), and α-CREB was a gift from L. Berkowitz and M. Gilman.

RESULTS

Specific complex formation with the c-*fos* SRE decreases in senescing fibroblasts. Mobility shift assays were performed to identify specific complexes that formed on the SRE as well as on the AP-1-like sequence located 3′ of the SRE and to determine changes in transcription factor binding activities on this element when cells approach the end of their in vitro life spans (9, 50). Nuclear proteins extracted from early (35 to 40 MPD)- and late (82 to 86 MPD)-passage human diploid fibroblasts were used in the mobility shift assays. Senescent cells were obtained by passaging young proliferation-competent human diploid fibroblasts until cell populations took more than 2 weeks to attain one population doubling. Typical morphologies of young compared with old fibroblasts are shown in Fig. 1.

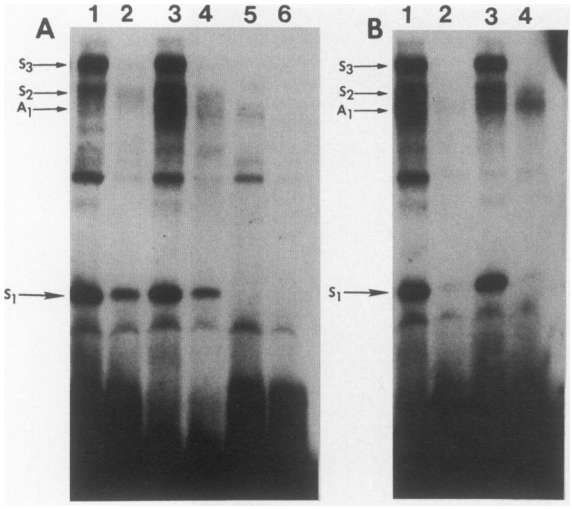

FIG. 2. (A) Binding activities of proteins in nuclear extracts from young and old cells to the c-*fos* SRE. Lanes: 1 and 2, complexes formed by the SRE wt/AP-1 mu oligonucleotide with proteins extracted from young and old cells, respectively; 3 and 4, complexes formed by the SRE and the juxtaposed AP-1 site (SRE wt/AP-1 wt) with proteins extracted from young and old cells, respectively; 5 and 6, complexes formed on the mutant probe (SRE mu/AP-1 mu) with proteins extracted from young and old cells, respectively. S_1, S_2, and S_3 identify complexes specific for the SRE, and A_1 identifies an AP-1-specific complex. (B) Lanes: 1, complexes formed as described for lane 3 of panel A; 2, assay with ^{32}P-labelled SRE wt/AP-1 wt probe and 100-fold unlabelled SRE wt/AP-1 wt probe as competitor; 3, assay with ^{32}P-labelled SRE wt/AP-1 wt probe and unlabelled mutant (SRE mu/AP-1 mu) as competitor; 4, assay with ^{32}P-labelled SRE wt/AP-1 wt and unlabelled SRE wt/AP-1 mu as competitor. On the basis of the results of competitions, S_1, S_2, and S_3 are complexes specific for the c-*fos* SRE. A_1 is specific for the AP-1 site located 3′ of the SRE.

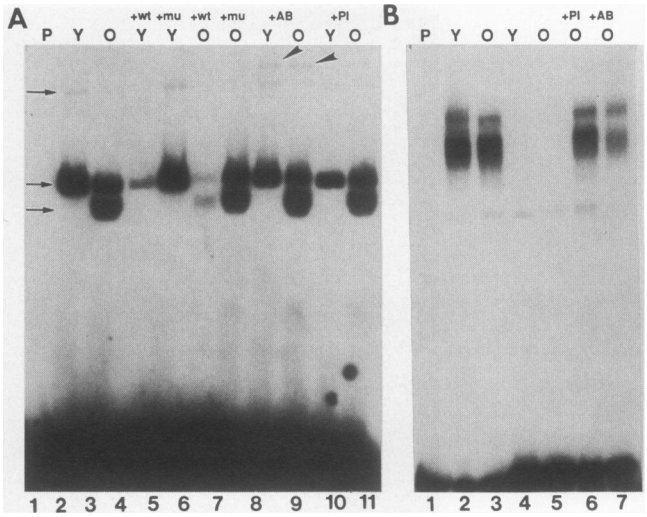

FIG. 3. (A) DNA-protein complexes formed on a ^{32}P-labelled TATA element in nuclear extracts from young (Y) (lane 2) and old (O) (lane 3) cells. Lanes: 4 and 5, reactions done as described for lane 2, but with a 100-fold excess of unlabelled wild-type (lane 4) or mutant (lane 5) competitor oligonucleotide included; 6 and 7, reactions done as described for lane 3, but with a 100-fold excess of unlabelled wild-type (lane 6) or mutant (lane 7) competitor oligonucleotide included; 8 and 9, reactions done with nuclear extracts preincubated with α-TBP antibodies; 10 and 11, reactions done with nuclear extracts preincubated with nonspecific antisera. The arrows indicate shifts specific for wild-type TATA box oligonucleotides, and the arrowheads in lanes 8 and 9 indicate complexes with lower mobilities seen with α-TBP but not with control antisera. Lane 1 is a negative control containing labelled probe (P) with no nuclear extract. (B) Binding of proteins from young (lane 2)- and old (lane 3)-cell nuclear extracts to the CRE. Binding to mutant CRE oligonucleotides (lanes 4 and 5) was not seen. Formation of complexes between nuclear proteins isolated from old cells and the wild-type CRE was inhibited when nuclear extracts were preincubated with α-CREB antibodies (lane 7) but not with preimmune serum (lane 6). AB, antibody; PI, preimmune.

Several shifted complexes were formed with wild-type probes when they were incubated with nuclear extracts from young, proliferation-competent cells (indicated by arrows in Fig. 2A, lane 1). These complexes are specific since they did not form on the SRE mu/AP-1 mu probe using extracts from young (lane 5) or old (lane 6) cells. To define further the specificity of shifted complexes, extracts from young cells were incubated with labelled SRE wt/AP-1 wt oligonucleotide in the absence of competitor DNA (Fig. 3B, lane 1) or with unlabelled SRE wt/AP-1 wt (lane 2), SRE mu/AP-1 mu (lane 3), or SRE wt/AP-1 mu (lane 4) competitors. Incubation with unlabelled SRE wt/AP-1 mu oligonucleotide specifically inhibited formation of the complexes identified as S_1, S_2, and S_3 but left the complex identified as A_1 intact (Fig. 2B, lane 4). Conversely, incubation of labelled SRE wt/AP-1 wt probe with unlabelled SRE mu/AP-1 mu oligonucleotide eliminated a nonspecific complex but left the complexes identified as A_1, S_1, S_2, and S_3 (lane 3) unaffected. Thus, S_1, S_2, and S_3 appear to be SRE-specific complexes and A_1 appears to be specific for the AP-1-like site.

We then performed mobility shift assays to compare the abilities of proteins extracted from young and old cells to form complexes with the SRE. When labelled SRE wt/AP-1 mu or SRE wt/AP-1 wt oligonucleotides were incubated with equal concentrations of protein extracted from the nuclei of young and old cells (Fig. 2A), the only band able to form using old cell extracts was the one corresponding to S_1, while a band with an intensity similar to that of S_1 but with much lower mobility (designated S_3) was totally absent in binding reaction mixtures using old cell extracts (compare lanes 1 and 3 to 2 and 4 of Fig. 2A). The fact that neither amino- nor carboxyl-terminal α-SRF antibodies interact with complex S_1 strongly suggests that this is a non-SRF-containing complex. Formation of this complex in both young and old cells varies from extract to extract. Similarly, formation of the band corresponding to AP-1 binding (A_1) in old-cell extracts varied slightly from extract to extract but was always greatly reduced from that seen in young-cell extracts, in agreement with previous reports (34, 35).

Additional experiments showed that extraction of nuclear proteins under conditions of different nonionic detergents, protease inhibitors, and reducing agents produced results similar to those obtained under our standard extraction conditions (data not shown). These results suggest that the lack of binding to the SRE in extracts from old cells is not likely due to interaction of SRF with inhibitors of binding, to nonspecific proteolysis, or to aberrant redox states of SRF.

TATA element and CRE binding activities are maintained in old cells. To rule out the possibility that the activity of all DNA-binding proteins was being lost during extraction procedures through some mechanism not specific for SRF, gel shift analyses were performed using probes containing consensus sites for the TBP (45) and for the CREB (3). TATA was chosen because of its presence in the promoters of a majority of known eukaryotic genes. As shown in Fig. 3A, proteins

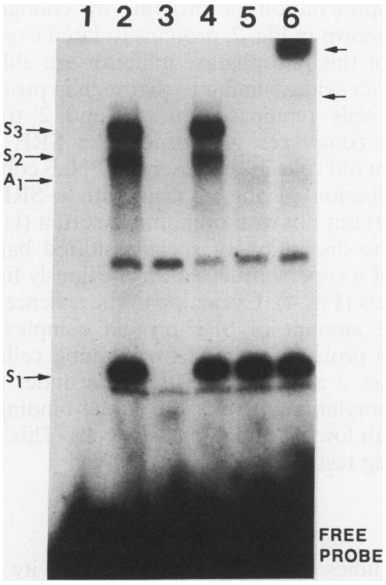

FIG. 4. Identification of complexes containing SRF. Labelled SRE wt/AP-1 wt oligonucleotide was incubated with no extract (lane 1), with extract from young fibroblasts in the absence of antibodies (lane 2), or in the presence of control (lane 4), amino-terminal α-SRF (lane 5), or carboxyl-terminal α-SRF (lane 6) antibodies. Lane 3 shows a binding reaction done with labelled SRE mu/AP1 mu oligonucleotide. S_1, S_2, and S_3 represent SRE-specific complexes. Arrows (lane 6) identify two supershifted complexes seen with carboxyl-terminal α-SRF antibodies.

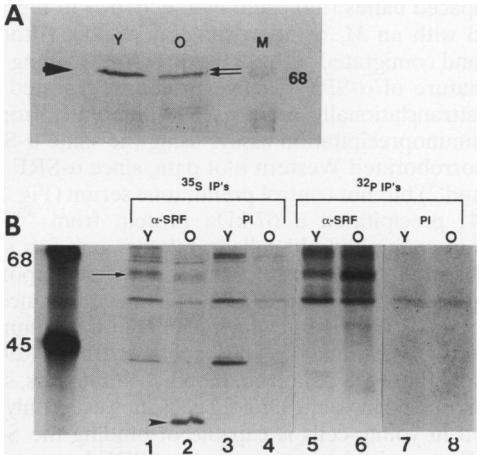

FIG. 5. (A) A Western blot of SRF in young (Y) and old (O) cells. Total nuclear extracts containing 100 μg each of protein were electrophoresed through a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with α-SRF antibodies. The rightward arrow identifies a band with a relative mobility of 67 kDa in young fibroblasts, identical to that of SRF. The leftward arrows identify two α-SRF-reactive bands with similar mobilities seen in near-senescent fibroblasts. (B) Immunoprecipitation (IP) of ^{35}S-labelled (lanes 1 to 4) or ^{32}P-labelled (lanes 5 to 8) SRF from young (lanes 1, 3, 5, and 7) and old (lanes 2, 4, 6, and 8) cells using α-SRF antibodies (lanes 1, 2, 5, and 6). Rabbit preimmune serum served as a control in lanes 3, 4, 7, and 8. The arrow identifies SRF, and the arrowhead indicates the 35-kDa protein found in α-SRF immunoprecipitates from old cells only.

extracted from the nuclei of young and old fibroblasts efficiently formed complexes with the TATA probe (lanes 2 and 3). Formation of these complexes was inhibited by inclusion of a 100-fold excess of unlabelled wild-type probe (lanes 4 and 6) but not by a 100-fold excess of mutant probe (lanes 5 and 7). Although binding was maintained with the TATA element in the old-cell nuclear extracts, we observed differences in the relative amounts of the complexes formed (identified by the arrows in Fig. 3A). A faster-migrating complex seen with nuclear extracts from old cells has very low abundance when extracts from young cells are used and varies somewhat between experiments in extracts from both young and old cells. Conversely, the more slowly migrating complex is formed poorly when extracts from old cells are used. Since multiple protein complexes are known to be formed sequentially on the TATA box in vitro (5), our results suggest that while these complexes may be formed differently in young and old cells, binding activity to the TATA box is nevertheless maintained in proteins isolated from the nuclei of old cells. Preincubation of binding mixtures with an antibody raised against bacterially expressed TBP (48) resulted in the appearance of a weak supershifted complex (lanes 8 and 9) that was not seen with control antisera (lanes 10 and 11). However, this result did not conclusively demonstrate that the complexes formed contained TBP, since the supershift observed was very weak and was not accompanied by a noticeable decrease in the intensity of the complexes.

To study the binding characteristics of proteins isolated from young and old cells to another gene-specific element, we performed mobility shift assays using probes containing the CRE. As shown in Fig. 3B, wild-type (lanes 2 and 3) but not mutant (lanes 4 and 5) oligonucleotides formed complexes to similar extents with proteins isolated from young (lane 2) and old (lane 3) cells. Complexes formed upon the CRE also appear to contain the CREB, since preincubation of cell lysates with α-CREB antibodies (lane 7) but not with preimmune serum (lane 6) inhibited formation of the complexes. Thus, binding of SRF to the SRE was lost as cells aged in vitro, despite the maintenance of TATA- and CRE-binding activities.

Similar levels of SRF are expressed in young and old cells, but phosphorylation of SRF is augmented in old cells. Our results indicated that some of the complexes formed over the SRE wt/AP-1 wt probe failed to form when nuclear extracts from old cells were used. To determine which of these complexes contained the SRF, mobility shift assays were performed in the presence of affinity-purified and previously characterized α-SRF antibodies directed against the amino or carboxyl termini of SRF (29) or with control antibodies. As shown in Fig. 4, addition of control antibodies did not change any of the complexes seen in the absence of added antibodies (compare lanes 2 and 4). In contrast, addition of α-SRF antibodies specifically blocked the formation of the S_2 and S_3 complexes and, in the case of the carboxyl-terminal α-SRF antibody, resulted in the formation of two supershifted complexes (identified by the leftward arrows in Fig. 4). These results confirm that the SRE-specific complexes (S_2 and S_3) that are lost completely in nuclear extracts from old fibroblasts (Fig. 2A, lane 5) are those containing SRF.

To determine if the loss of SRF binding in old cells was due to decreased levels of synthesis, we analyzed the expression of SRF by Western blotting and immunoprecipitation assays. After probing a membrane onto which total nuclear proteins were transferred with α-SRF antibodies, we observed only a slight difference in the intensity of a 67-kDa band recognized by the α-SRF antibodies in extracts isolated from young and old cells (Fig. 5A). We also noted that in old cells, the α-SRF antibodies recognized antigens that appeared to consist of two

closely spaced bands (indicated by the arrows in Fig. 5A) that migrated with an M_r of approximately 67,000. Although the upper band comigrated with SRF expressed by young cells, the diffuse nature of α-SRF-reactive proteins suggested that old cells posttranslationally modify SRF differently from young cells. Immunoprecipitation assays using the same α-SRF antibodies corroborated Western blot data, since α-SRF (Fig. 5B, lanes 1 and 2) but not control preimmune serum (Fig. 5B, lanes 3 and 4) precipitated a 67-kDa protein from ^{35}S-labelled proteins in young and old cells in relative amounts similar to those obtained with Western blots. Previous reports using these antibodies indicate that SRF is a low-abundance protein with a long (12-h) half-life which results in weak immunoprecipitation signals similar to those obtained here (29). These results, together with data from mobility shift assays, show that SRF is expressed in young and old cells; however, only the SRF expressed in young cells is capable of binding the SRE. The slight difference in expression levels of SRF in young and old cells cannot account for the almost total lack of SRF binding to the SRE in nuclear extracts from old cells, since shifted complexes are seen clearly when young-cell extracts diluted manyfold are used (data not shown). This suggests that a qualitative difference in the SRF proteins expressed in young and old cells might underlie the changes in binding avidity.

To examine posttranslational modifications to SRF in aging cells, we compared the phosphorylation state of the SRF protein in young cells with that in old cells. We incubated serum-deprived young and old cells with media containing 10% serum and $^{32}P_i$ and performed immunoprecipitations with α-SRF antibodies. As reported by others, a 67-kDa protein was seen to be phosphorylated under these conditions (29). This 67-kDa protein which was not detected by preimmune sera (Fig. 5B, lanes 7 and 8) reproducibly appeared to be much more heavily phosphorylated in old cells than in young cells (compare lanes 5 and 6 of Fig. 5B).

We also noted that the α-SRF immunoprecipitates obtained from old cells contained a band migrating with an M_r of 35,000 that was absent in young-cell lysates (Fig. 5B, lane 2). Our preliminary results suggest that the 35-kDa band is recognized directly by the α-SRF antibodies and is not phosphorylated. However, whether this protein shares an epitope with SRF or is a protein that specifically complexes with SRF in old cells remains to be determined.

Intracellular localization of SRF is similar in cells at different passage levels. To corroborate our findings that levels of SRF did not change markedly during cellular aging by an independent method and to examine the intracellular localization of SRF, indirect immunofluorescence studies were undertaken. Figure 6 shows that similar levels of immunostainable SRF were present in young and old cells and that staining was primarily nuclear in all cells examined. Thus, the inability of proteins extracted from the nuclei of senescing fibroblasts to bind the SRE in gel shift assays does not appear to be due to either a loss of SRF expression or to inappropriate intracellular localization of SRF protein, in agreement with results using immunoprecipitation and immunoblotting.

Endogenous phosphatase activity potentiates the DNA binding activity of SRF extracted from late-passage fibroblasts. To directly test whether phosphorylation affected the ability of SRF to bind to the SRE, we preincubated total nuclear extracts with potato acid phosphatase prior to gel shift assays. Such nonspecific phosphatase treatment did not reproducibly change the binding characteristics of proteins extracted from young or old cells to the SRE (data not shown). In a second approach, we isolated nuclear proteins in the absence of sodium fluoride (NaF, a phosphatase inhibitor), which should allow dephosphorylation of proteins by endogenous phosphatases. As shown in Fig. 7, proteins isolated from old cells in the absence of this phosphatase inhibitor are able to bind to the SRE with an avidity similar to that seen in proteins isolated from young cells (compare lanes 1 and 2 to 3 and 4). Furthermore, complexes formed on the SRE by proteins extracted from old cells in the absence of NaF contained SRF, since preincubation of the extracts with α-SRF antibodies (lanes 8 and 9) but not with preimmune serum (lanes 6 and 7) resulted in the disruption of specific shifted bands and the appearance of a supershift as shown previously for young-cell nuclear extracts (Fig. 4). Extraction in the absence of NaF also increased the amount of SRE-protein complexes observed when nuclear proteins isolated from young cells were used (compare lanes 2 and 4), suggesting that under these conditions, phosphorylation inhibits the DNA binding activity of SRF from both low- and high-passage cells. This possibility is presently being tested.

DISCUSSION

Previous studies have shown that the activity of the transcription factor Fos is required for the growth of fibroblast cells in culture (19, 22, 32, 37). Senescent cells that are unable to grow in response to mitogens express much lower levels of Fos in response to serum stimulation, and Fos proteins that are expressed appear to be much more heavily phosphorylated and fail to bind DNA (34, 35, 46). In the present study, we show that SRF, a transcription factor that is known to be required for transcriptional induction of the c-*fos* gene through its interaction with the SRE, fails to bind the SRE in senescent cells. This may explain the reduction of c-*fos* expression observed in old cells.

Several lines of evidence suggest that the loss of SRE binding activity is not due to a nonspecific artifact of protein isolation from senescent cells. First, the binding activity to a consensus TATA box oligonucleotide was maintained in senescent cell extracts. Although senescent cells contained variable amounts of an additional shifted complex compared with young, proliferation-competent cells, both complexes were specific to the TATA box. Second, binding of CREB, a well-characterized sequence-specific transcription factor, to DNA was also maintained in old cells. Third, a non-SRF-containing complex (S_1) forms specifically on the SRE in nuclear extracts from both young and old cells. Additionally, extracts prepared in parallel from young and old fibroblasts showed very similar ^{35}S-labelled protein profiles, showed no evidence of protein degradation, and showed similar abilities to bind heparin-agarose columns (data not shown).

What might be the basis of such differential binding of SRF to the SRE? Several possibilities, including a lack of SRF expression, rapid degradation of SRF, inappropriate intracellular localization of SRF, binding of inhibitors to SRF or to the SRE, or altered posttranslational modification of SRF, could underlie the lack of SRF binding that we have observed in senescent cells. By several criteria, we have noted that the absence of SRF binding is due neither to a lack of SRF expression (Fig. 5 and 6) nor to inappropriate localization (Fig. 6). Similarly, we have no evidence for altered stability of SRF in senescent cells, since similar amounts of the protein were recovered from young and old cells in both immunoblotting and immunoprecipitation experiments. Since immunoblotting experiments detect total levels of SRF, immunoprecipitation experiments detect newly synthesized SRF, and the half-life of SRF (12 h) is much longer than our labelling times, these two assays would give different results if the stability of SRF

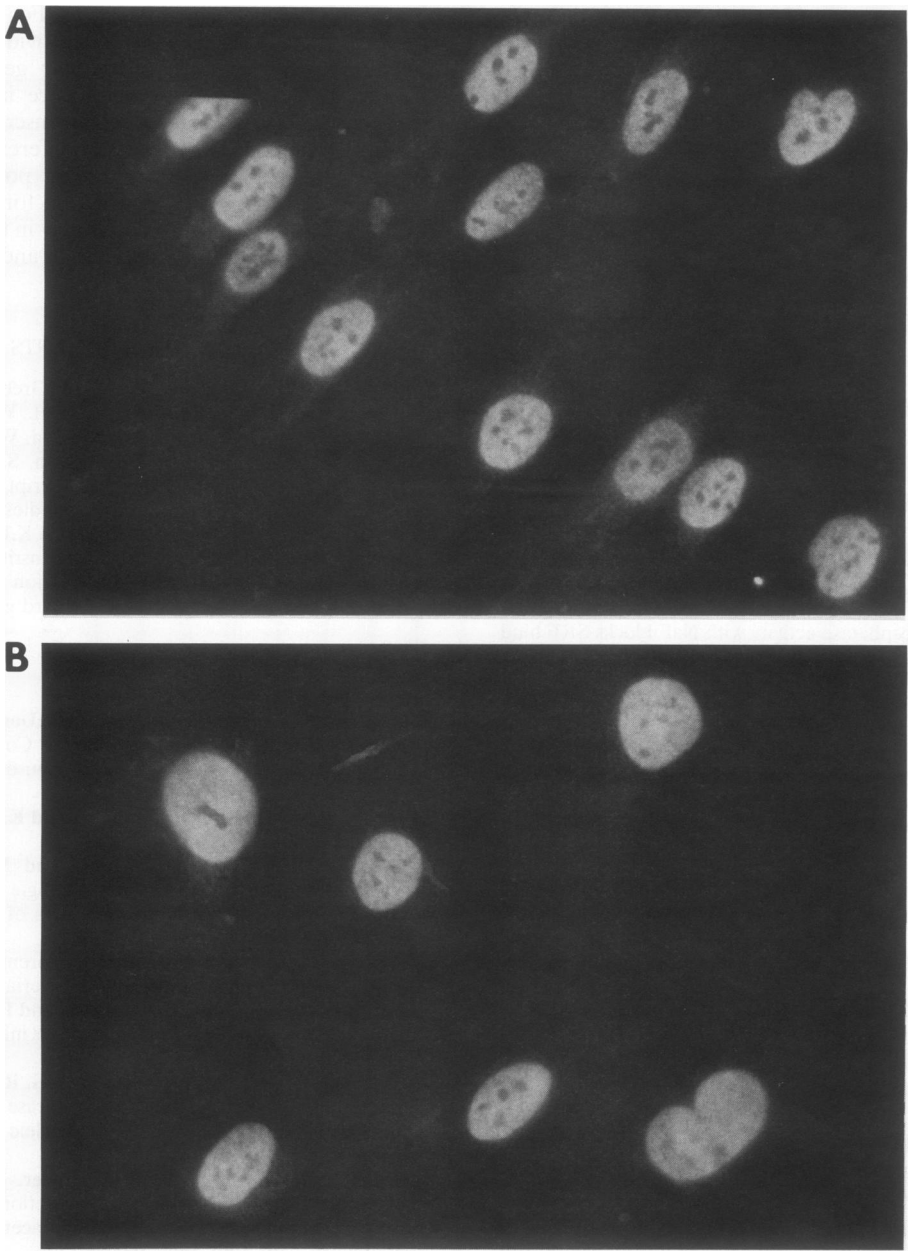

FIG. 6. Indirect immunofluorescence staining of young (A) and old (B) cells using α-SRF antibodies. Micrographs for young and old cells were done under the same conditions of fixation, staining, exposure, and printing to allow direct comparison.

protein were altered in old cells. The possibility that an inhibitor binds the SRE (preventing SRF binding) appears highly unlikely, since such binding should be observed in mobility shift assays, which was not the case. The remaining possibilities are that the SRF protein is bound by an inhibitor of DNA binding or that SRF is posttranslationally modified in a way that precludes DNA binding. Regarding the possibility that interaction of SRF with other proteins may inhibit binding to the SRE, we have noted the presence of a 35-kDa protein that is precipitated by α-SRF antibodies from old but not from young nondenatured cell extracts (Fig. 5B). This protein appears to be recognized directly by α-SRF antibodies, since it is also seen in denaturing immunoprecipitations (data not shown) and might therefore represent a degradation product of SRF or an additional protein that shares an antigenic determinant with SRF. We are presently attempting to differentiate among these possibilities. The remaining possibility, that posttranslational modification of SRF blocks its ability to bind DNA, is supported by our data. In addition to the observation that the SRF expressed by senescent cells migrates as at least two diffuse bands on acrylamide gels (Fig. 5A and B), immunoprecipitation of ^{32}P-labelled cell lysates shows that SRF is phosphorylated approximately three- to fivefold more heavily in senescent versus proliferation-competent young cells (Fig. 5C). Previous reports have indicated that phosphorylation of SRF increases the binding of this protein to the SRE and that the structure of SRF was highly sensitive to phosphorylation (24, 26). However, here we are proposing that hyperphosphorylation of SRF may alter its structure in a manner that blocks its ability to recognize the SRE, an idea that is

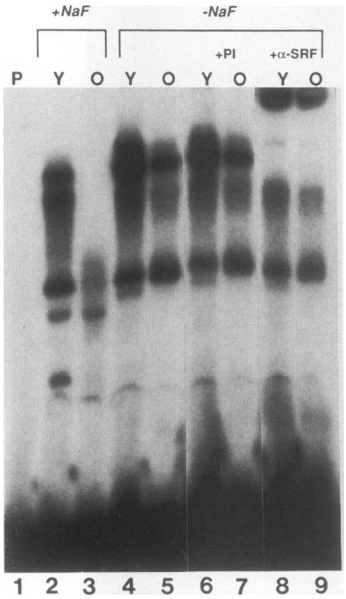

FIG. 7. Inhibiting phosphatase activity with NaF blocks SRF binding activity. Proteins were isolated from young and old cells in the absence or presence of 5 mM NaF. Lanes 2 and 3 contain complexes formed by nuclear extracts prepared from young (Y) and old (O) cells, respectively, in the presence of sodium fluoride. Lanes 4 and 5 show complexes formed by proteins isolated from young and old cells, respectively, in the absence of sodium fluoride. Reaction mixtures containing proteins isolated in the absence of sodium fluoride were incubated with α-SRF antibodies (lanes 8 and 9) or with preimmune serum (PI) (lanes 6 and 7). P is a negative control containing probe but no nuclear extract.

directly supported by the results of our experiments, in which the degree of phosphatase activity was varied in old-cell extracts. Although preliminary, these experiments strongly suggest that phosphorylation of SRF interferes with its ability to bind to DNA differentially in young and old cells, a possibility that we are presently testing.

We have also previously presented data showing that hyperphosphorylation of the Fos protein occurs in high-passage cells, and we have suggested that such phosphorylation might underlie the inability of the low levels of Fos produced in near-senescent cells to bind to AP-1 sites (34, 35). Indeed, phosphorylation of transcription factors including Myb, Jun, Max, myogenin, Oct1, Pit1, and Ets1 has been shown to inhibit binding activity (for a review, see reference 20). Taken together, these data therefore present the intriguing possibility that a common mechanism might underlie the modification of transcription factor activities in senescent cells. Such changes might then play a role in differential gene expression during cellular aging. Identification of the relevant age-specific kinases or phosphatases as well as their activators and inhibitors would prove useful in elucidating specific signalling pathways that operate during cellular senescence and in further understanding the process of cell aging in general.

In conclusion, we find that c-*fos* SRE binding activity decreases dramatically in senescent cells and that the complexes which fail to form are those that contain SRF. Similar levels of SRF are expressed in young and old cells; however, SRF is hyperphosphorylated in old cells. Allowing increased endogenous phosphatase activity increases the binding of SRF to the SRE, and this effect is much more pronounced in near-senescent cells. Differential phosphorylation of SRF and other transcription factors such as Fos in old cells may therefore alter transcription and provide a basis for the observation that a subset of cellular genes are expressed differentially during cellular senescence in vitro and during aging in whole animals. As the transcriptional regulatory regions of genes that are expressed differentially during aging become better defined, the intriguing possibility arises that there may be coordinate regulation for subsets of genes affected by cellular aging. If so, this may in turn provide targets that could allow modification of in vitro and in vivo lifespans of human cells.

ACKNOWLEDGMENTS

We thank L. Berkowitz, M. Gilman, M. Greenberg, R. Meyers, R. Misra, and P. Sharp for antibodies and H. Wong for providing a plasmid containing the c-*fos* upstream region. We are also grateful to A. Gibson, M. D. Hollenberg, R. Johnston, S. Lees-Miller, and D. Young for critical reading of the manuscript and to the SACRC hybridoma facility for preparation of antibodies.

This work was supported by grants to K.R. from the Medical Research Council and the National Cancer Institute of Canada. K.S. is a recipient of an Alberta Heritage Foundation for Medical Research stipend, and P.A. is an Alberta Cancer Board graduate student.

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