Regulation of Dihydrofolate Reductase Gene Expression and E2F Components in Human Diploid Fibroblasts During Growth and Senescence

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The induction of dihydrofolate reductase (DHFR), a key enzyme in DNA biosynthesis that is induced just before the onset of S phase, is markedly attenuated in senescent human fibroblasts (Pang and Chen, 1994, J. Cell. Physiol., 160:531–538). Footprinting analysis of the 365 bp promoter region of the human DHFR gene (−381 to −17) indicated that nuclear proteins bind to a cluster of cis-elements, including two overlapping E2F binding sequences, two Sp1 sites, and one Yi sequence. Gel mobility shift assays were performed to assess the role of each cis-element in the regulation of DHFR gene expression. We found that 1) Sp1 binding activity was constitutively expressed throughout the cell cycle in early passage and senescent cells; 2) Yi binding activity was undetectable in both early passage and senescent cells; and 3) E2F binding activity was serum-inducible, senescence-dependent, and prominent in presenescent cells but strikingly diminished in senescent cells. Northern blot analysis of the expression of E2F and DP family members showed that the E2F-1, E2F-4, and E2F-5 mRNA was growth- and senescence-dependent, whereas E2F-3, DP-1, and DP-2 expression was constitutive and senescence-independent. In contrast, E2F-2 mRNA was not detectable in IMR-90 or WI-38 human fibroblasts. Western blot analysis showed that among the E2F-associated proteins, the expression of E2F-1, cyclin A, and cyclin B but not p107 was cell cycle- and senescence-dependent. A nuclear extract mixing experiment suggested that an inhibitory factor may further reduce E2F binding activity in senescent cells. © 1996 Wiley-Liss, Inc.

Normal diploid fibroblasts have only limited doubling potential in culture (Hayflick and Moorhead, 1961) and most probably in vivo (reviewed by Campisi et al., 1995). This phenomenon has been termed the finite replicative life span of cells, and the process that limits cell division potential is known as cellular or replicative senescence. The hallmark of cellular senescence is an essentially irreversible failure to initiate DNA synthesis, even after growth factor stimulation (Cristofalo and Sharf, 1973). Senescent cells certainly retain the ability to receive growth factor signals, because many mid-G1 genes are induced by serum in senescent cells as well as in presenescent cells (Rittling et al., 1986; Chang and Chen, 1988; Seshadri and Campisi, 1990; Pang and Chen, 1994). However, many genes that are normally expressed at the G1/S boundary are not expressed by senescent cells, even after serum stimulation (Chang and Chen, 1988; Seshardri and Campisi, 1990; Stein et al., 1991; Pang and Chen, 1994). Since a suppression of G1/S genes may be an immediate cause for the loss of dividing potential in senescent cells, understanding the mechanisms that regulate these genes in senescent cells should shed light on the molecular basis for the stringent growth arrest associated with cellular senescence.

We are particularly interested in these questions: 1) Do G1/S genes that are repressed in senescent cells share a common regulatory mechanism? 2) Is there a master switch that represses these genes? 3) What are the upstream regulatory elements responsible for the attenuation of these genes in senescent cells? It may well be necessary to examine the mechanism that regulates each of these G1/S genes in order to answer the above questions.

DHFR is one of the key enzymes needed for DNA synthesis, because it is required for the synthesis of deoxyribonucleotides. The human DHFR gene has been cloned (Chen et al., 1984; Yang et al., 1984) and the promoter region shows high homology among several mammalian species. A retinoblastoma (pRB) binding protein, E2F, has been shown to be a positive regulator

of murine DHFR gene transcription (Slansky et al., 1993).

E2F was initially identified as a DNA binding protein for the sequence TTTCGCGC within the adenovirus E2 promoter (Kovesdi et al., 1986). Several cell cycle-dependent genes, including DHFR, thymidylate synthase (TS), and proliferating cell nuclear antigen (PCNA), contain sequences similar to this E2F binding site (Nevins, 1992; Farnham et al., 1993). By virtue of E2F's association with pRB (Helin et al., 1992; Kaelin et al., 1992), a family of related proteins (E2F-1, E2F-2, and E2F-3) has been cloned (Ivey-Hoyle et al., 1993; Lees et al., 1993). Recently, E2F-4 and E2F-5 were identified and cloned (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995; Vairo et al., 1995). Both E2F-4 and E2F-5 are expressed earlier in the cell cycle than E2F-1, and both associate with one or more pRB-related protein, as opposed to pRB itself. E2F is a heterodimer, consisting of one E2F family protein and one DP family protein, of which DP-1 and DP-2 have thus far been cloned (Girling et al., 1993; Helin et al., 1993; Zhang and Chellapan, 1995). Why cells require more than one E2F complex is not clear. Recent evidence suggests that different E2F complexes may act at different points in the cell cycle (Sardet et al., 1995).

We have previously demonstrated a senescence-dependent attenuation of DHFR gene expression and enzyme activity in human diploid fibroblasts (Pang and Chen, 1994). Moreover, E2F binding activity has been shown to be diminished in senescent human fibroblasts (Dimri et al., 1994). In this report, we show that, among the cis-elements identified in the human DHFR promoter, only E2F binding activity exhibited a senescence-dependent attenuation. We also show that, among the E2F family and DP family members, attenuation of E2F-1 gene expression at both the mRNA and protein level correlates closely with the expression of DHFR in senescent cells. In addition, senescent cells are also deficient in E2F-4 and E2F-5 expression, relative to proliferating presenescent cells. Moreover, mixing experiments suggest the presence of an inhibitory activity for E2F binding in senescent cells. Thus, the senescence-dependent repression of DHFR expression in human fibroblasts may result from the failure to express several E2F components and the presence of an inhibitory factor targeted to E2F binding.

MATERIALS AND METHODS
Cell culture

IMR-90 and WI-38 human embryonic lung fibroblasts at low passage numbers (population doubling level (PDL) at 25 or below) were obtained from the

Coriell Institute for Medical Research (Camden, NJ). Cultures were grown and made senescent (PDL greater than 46) as previously described (Chang and Chen, 1988; Seshadri and Camipisi, 1990). HeLa cells and SV-40 transformed IMR-90 cells were from the American Type Culture Collection (Rockville, MD).

Transient expression driven by the DHFR promoter

The 365 bp AvaII/AvaII fragment of the human DHFR gene was used to create pB365 in vector pBLUESK+. The HindIII/XbaI fragment of pB365 was excised and inserted into pCATBasic (Promega, Madison, WI) to give pB365CAT. The transfection was carried out using 20 μg plasmid DNA per 100 mm dish of cells by the calcium phosphate mediated precipitation procedure as described by Gorman et al. (1982). After a 3 min glycerol shock, cells were incubated in fresh Dulbecco's medium containing 10% fetal bovine serum for a 24 h period of recovery. The transfected cells were then serum-deprived for another 24 h before serum stimulation. At various time points after stimulation, cells were washed with Eagle's balanced salt solution and homogenized in a Tris buffer (250 mM, pH 8.0). The homogenate was centrifuged at 14,000 rpm for 30 min, and aliquots of the supernatant fraction were used for the CAT enzyme assay (Gorman et al., 1982). A standard reaction mixture containing 150 μg protein, 0.1 μCi [^{14}C]chloramphenicol, and 440 nmol acetyl CoA in a 180 μl of Tris buffer (250 mM, pH 8.0) was incubated at 37°C for 60 min, and the reaction product was extracted by ethyl acetate and analyzed by thin layer chromatography and autoradiography. The assay was in the linear range since only two forms of the acetylated chloramphenicol were detected. Control experiments using a plasmid carrying the β-galactosidase gene (pRSVβgal) showed similar transfection efficiency in presenescent and senescent cells, as reported previously (Liu et al., 1989).

DNase I footprinting

DNase I footprinting was carried out by the standard technique of Blake and Azizkhan (1989). The gels were exposed to X-ray film overnight at −70°C with an intensifying screen.

Northern blot analysis

Northern blot analysis was performed as previously described (Pang and Chen, 1994). The SalI/XhoI fragment of pBSKglob E2F-1, the BamHI fragment of pBSKHAE2F-2, and the BamHI fragment of pBSKHAE2F-3 were used, respectively, for probing E2F-1, E2F-2, and E2F-3 mRNA. Probes for DP-2, E2F-4, and E2F-5 were generated by RT-PCR. The primers were DP-2, 5'-dGCACACCACGAGACTAACCAC-3' (Zhang and Chellapan, 1995); E2F-4, 5'-dAGCTGT-ACGCAGAAGCGGCGGA-3' (Ginsberg et al., 1994); and E2F-5, 5'-dAGGAGAATTTATGATATCACCA-3' (Sardet et al., 1995). The primers amplified fragments of 268, 325, and 320 bp, respectively, and were verified by sequencing. GAPDH (G3PDH) was amplified using a commercially available primer set (Clontech, Palo Alto, CA). The PCR fragments (50 ng) were labeled by random priming and hybridized to RNA as described (Dimri et al., 1994).

Gel mobility shift assays

The preparation of nuclear extracts and gel mobility shift assays were carried out as previously described (Pang and Chen, 1993). Oligonucleotides were synthesized on a Pharmacia LKB Gene Assembler in our laboratory and purified by passing through a NAP-10 column (Pharmacia, Piscataway, NJ). Complementary sequences were annealed and end-labeled with [γ-^{32}P]-ATP using T4 kinase for gel mobility shift assays.

Western blot analysis

Whole cell lysates were prepared and fractionated by SDS-PAGE. Proteins separated within the gel were transferred to a nitrocellulose membrane for immunoblotting using various antibodies. All antisera were used undiluted for gel mobility shift assays and at 1,000-fold dilution for Western blot analysis. The ECL immunodetection kit (Amersham, Arlington Heights, IL) was used for detection.

Materials

All tissue culture media and sera were obtained from GIBCO-BRL (Gaithersburg, MD) [γ-^{32}P]ATP (3,000 Ci/mmol) was from Amersham or ICN Chemical Radioisotope Division (Irvine, CA). Restriction enzymes and other molecular biological supplies were from Promega or Pharmacia. All other chemicals were of reagent grade. Anti-p107 (polyclonal) was given by Dr. Mark E. Ewien (Dana Farber Cancer Institute, Boston, MA), the anti-E2F1 (KH95) (monoclonal) by Drs. Kristian Helin and Ed Harlow (MGH Cancer Center, Charlestown, MA), the anti-cyclin A (polyclonal) by Dr. Tony Hunter (The Salk Institute, San Diego, CA), the plasmid pDHFR1.8 by Dr. Arthur Nehuis (National Institutes of Health, Bethesda, MD), and the E2F cDNAs by Dr. K. Helin (Danish Cancer Society, Copenhagen, Denmark).

RESULTS

Comparison of human DHFR promoter activity in early passage and senescent cells

We first compared DHFR promoter activity in early passage and senescent cells by measuring CAT activity driven by a 365 bp human DHFR promoter fragment (−382/−17 bp). Figure 1 shows that CAT activity in transfected presenescent cells was at least fourfold higher than that in senescent cells 24 h after serum stimulation. This result suggests that the attenuation of DHFR gene expression in senescent human fibroblasts (Pang and Chen, 1994) is regulated, at least partially, at the level of transcription.

Multiple DNA-protein interactions within the human DHFR 5′-flanking region

To correlate protein-DNA interactions within the human DHFR 5′-flanking region with the high promoter activity in presenescent cells, the 365 bp fragment of the human DHFR promoter was mapped for sites of protein-DNA interaction by DNase I footprinting. Figure 2A shows that two regions of the DNA were protected from DNase digestion. Region I contained an Sp1 and E2F binding site; Region II contained only an Sp1. As shown in Figure 2B, the Sp1 site in Region I also overlaps with a perfect Yi binding sequence. The E2F
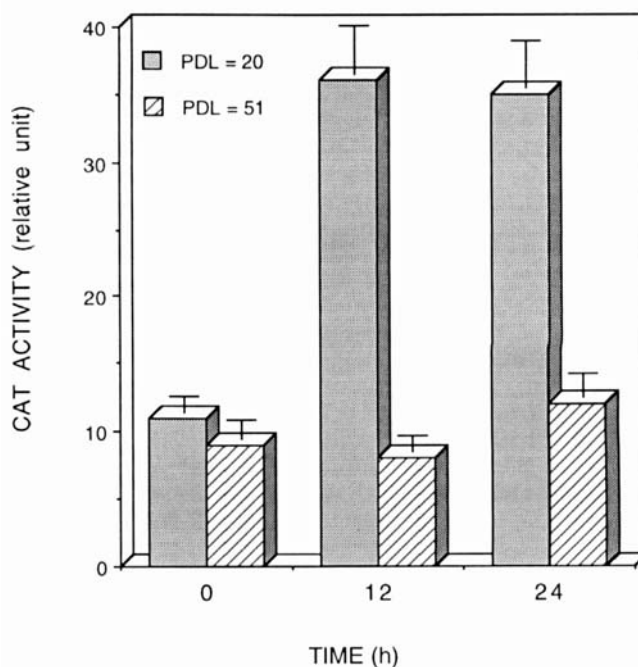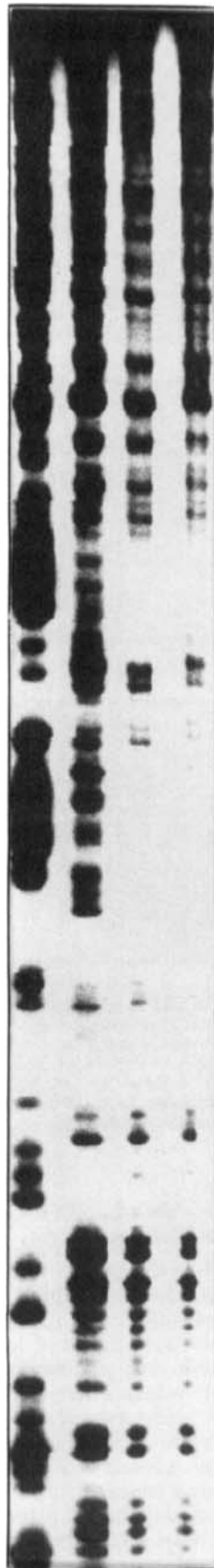

Fig. 1. Induction of CAT activity driven by a DHFR promoter fragment in serum-deprived and serum-stimulated early (PDL = 20) and late passage (PDL = 51) cells. IMR-90 cells were transfected, serum-deprived, and serum-stimulated for the indicated intervals, lysed, and assayed for CAT activity. Densitomeric scanning of the relative intensity of the major product of CAT activity was presented. Both CAT assay and X-ray film exposure were carried out in the linear range to ensure quantitative comparison.

site comprises two E2F consensus binding sites, arranged in opposing directions.

E2F, SP-1, and Yi binding activity in presenescent and senescent IMR-90 cells

Gel mobility shift assays were performed to evaluate each cis-element, E2F, Sp1, and Yi, for their ability to bind proteins expressed by early passage and late passage cells. Figure 3 shows that protein binding to only the E2F site was both cell cycle- and senescence-dependent, in agreement with previous results using WI38 human fibroblasts (Dimri et al., 1994). E2F binding was low to undetectable in senescent cells, whether or not they were serum-stimulated. In quiescent presenescent cells, it was low, and binding increased and shifted to slower mobility complexes upon serum stimulation. In contrast, Sp1 binding activity was constitutively present in both presenescent and senescent cell extracts (Fig. 3B). Since the probe used in the assay contains a tandom copy of the Sp1 consensus sequence, the lower band is likely caused by the protein bound to only one or two Sp1 sites. Yi binding was completely undetectable in normal human diploid fibroblasts extracts but could be detected in AG03204 cells, an SV-40 transformed IMR-90 cell strain (Good et al., 1995). The significance of the Yi binding activity in transformed human cells remains to be investigated. Thus, among the three cis-elements present in the DHFR promoter, only the E2F binding site is a candidate for contributing to the cell cycle- and senescence-dependent regulation of DHFR.

(B)

DHFR 50-bp SEQUENCE PROMOTER(-94/-45)


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            Sp1                    E2F
           ———▶                  ———▶
5' AGGGACGAGGGGGCGGGGCGGCCACAATTTCGCGCCAAACTTGACCGCGC 3'
   TCCGTGCTCCCCCGCCCCGCCGGTGTTAAAGCGCGGTTTGAACTGGCGCG
           ◀———                  ◀———
            Yi                     E2F
```
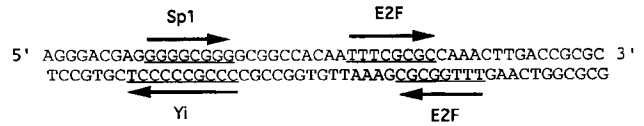
Fig. 2. Footprinting analysis of a 365 bp fragment of the human DHFR promoter region using extracts from serum-stimulated early passage IMR-90 cells. A: Identification of protein binding sites on the human DHFR promoter. ^{32}P-end-labeled DNA fragment spanning nucleotides −17 to −382 (numbers corresponding to translational initiation site) was used for DNase I footprinting as described in Materials and Methods. Lane 1: G-ladder. Lane 2: No DNase I. Lane 3: Nuclear extract (60 µg), 1 unit DNase I. Lane 4: Nuclear extract (80 µg), 1.5 units of DNase I. B: 50 bp promoter sequence (−94/−45) containing the protein binding site as identified in A (Region I). The consensus sequence of each cis-element is underlined. The arrows indicate the direction of each cis-element.

Expression of E2F and DP family members

Figure 4 shows a Northern blot analysis of the mRNA corresponding to three E2F family members in presenescent and senescent IMR-90 cells after serum stimulation. The E2F-1 transcript (~2.5 kb) showed a cell cycle-dependent increase beginning 6–12 h after serum stimulation, increasing through 18–24 h. The magnitude of E2F-1 mRNA in presenescent cells was five- to eightfold higher than in senescent cells at all time points. The low level of E2F-1 mRNA in senescent cells can be accounted for by the small fraction of presenescent cells present in the population (see Seshadri and Campisi, 1990; Dimri et al., 1994). In contrast, E2F-3 mRNA was constitutively expressed at high levels in both presenescent and senescent cells, with or without serum stimulation. E2F-2 mRNA was essentially undetectable in both presenescent and senescent cells. Very similar results were obtained in WI-38 human fibroblasts (data not shown).

Figure 5A shows a Northern blot of E2F-4 mRNA in early and late passage WI-38 human fibroblasts. E2F-4 was expressed, and to a similar level, in serum-deprived early (quiescent) and late passage cells. Presenescent cells proliferating in serum expressed slightly greater levels of E2F-4 mRNA (two- to threefold), but serum had no effect on senescent cells. By contrast, E2F-5 mRNA was undetectable in both quiescent and senescent cells. Serum induced E2F-5 expression in quiescent but not senescent cells (Fig. 5B). In quiescent cells, E2F-5 was induced within 4 h of serum stimulation, in agreement with previous results in keratinocytes (Sardet et al., 1995). Finally, the DP-1 component of E2F was shown to be constitutively expressed in human fibroblasts, independent of serum stimulation or passage number (Dimri et al., 1994). Recently, a second DP family member, DP-2, was cloned. DP-2 gives rise to multiple transcripts (approximately 1.4–1.5, 1.8–2.0, 2.4–2.5, 2.6–3.0, and 9.5–10 kb), many of which are cell type- or tissue-specific (Zhang and Chellappan, 1995; Wu et al., 1995). Figure 5C shows that the major DP-2 transcripts expressed by human
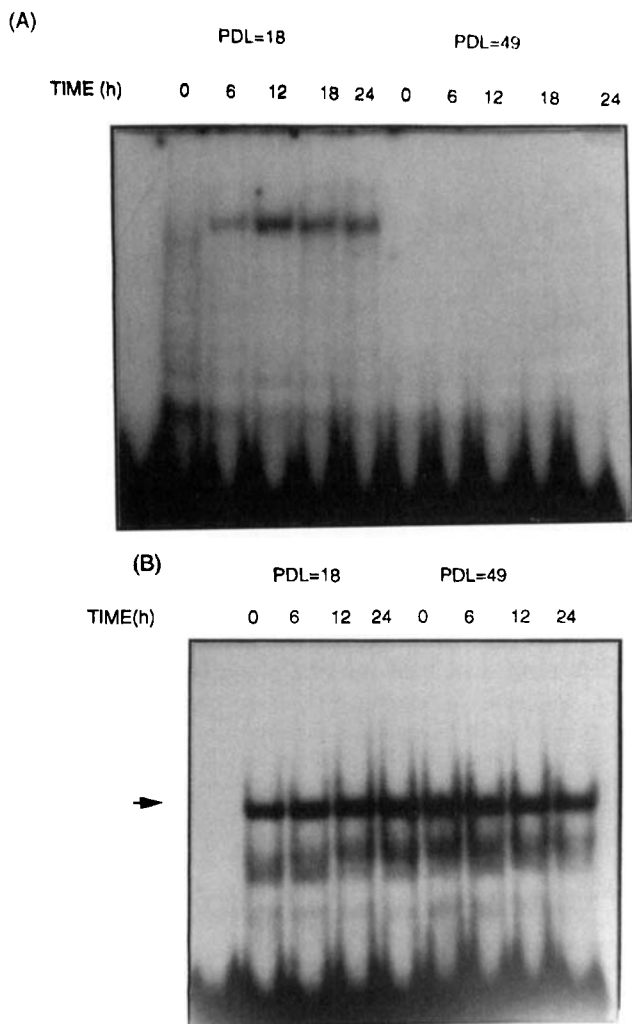
Region II

Region I

(A)

(B)

Fig. 3. DHFR promoter cis-element binding activity in presenescent and senescent IMR-90 cells. A: E2F binding activity. B: Sp1 binding activity. Cells were serum-deprived for 48 h (0 h) and then stimulated to enter cell cycle by serum addition. Cells were harvested at the indicated times following stimulation. Nuclear extracts were prepared as described in Materials and Methods, and gel mobility shift assays were carried out using a ^{32}P-labeled probe. The arrow indicates the position for the Sp1 binding complex.

fibroblasts (1.4 and 2.4 kb) are also constitutively expressed. A minor 9.5 kb transcript was less abundant in senescent cells, relative to presenescent cells. However, its low abundance and the inefficient transfer of large transcripts in Northern analyses make the significance of this finding unclear until suitable antibodies are available. The identity of the diffuse band of 5–5.5 kb is not known. It could be a fibroblast-specific transcript, as normal human fibroblasts have not been previously examined for DP-2 expression, or an as yet unknown additional DP family member that cross-hybridizes with DP-2. The Northern blot results shown in Figures 4 and 5 are summarized in Table 1. Among the seven transcription factors, E2F-1 is likely to play the key role in regulating cell cycle- and senescence-dependent regulation of DHFR, although a role for E2F-3, E2F-4, and E2F-5 cannot be ruled out at this time.
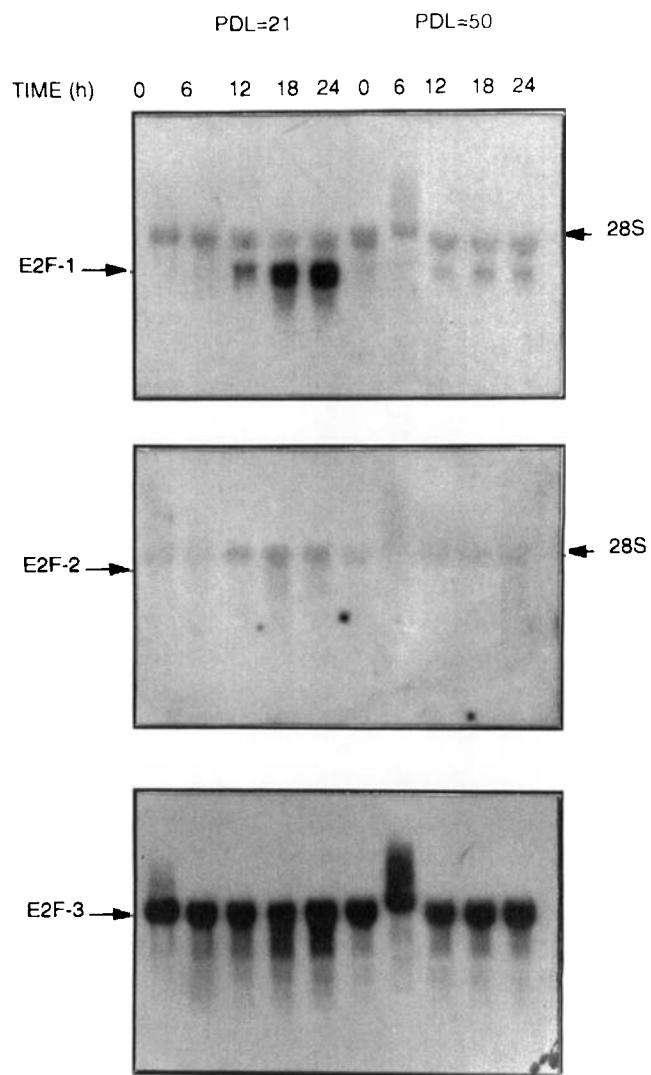

Fig. 4. Northern blot analysis of E2F-1, E2F-2, and E2F-3 mRNA levels in serum-deprived and serum-stimulated presenescent and senescent IMR-90 cells. Total RNA was isolated from cells at various times after serum stimulation. RNA was analyzed for E2F-1, E2F-2, and E2F-3 mRNAs as described in Materials and Methods. E2F-3 mRNA, which does not change in response to serum stimulation or passage, serves as a control for mRNA quantitation and integrity.

Western blot analysis of E2F-1 and other E2F binding proteins

Figure 6A shows that E2F-1 protein, evident as a doublet of approximately 60 kDa, was almost undetectable in serum-deprived presenescent and senescent cells. Serum stimulation induced high levels of E2F-1 protein in presenescent cells but not senescent cells (Fig. 6A). Thus, E2F-1 protein levels reflected the E2F-1 mRNA levels reported for presenescent and senescent human fibroblasts (Dimri et al., 1994). The more than tenfold difference in serum-induced E2F-1 protein between early passage cells and senescent cells supports the idea that the senescence-dependent attenuation of E2F binding activity is at least partially due to a lack of E2F-1 protein in senescent cells.
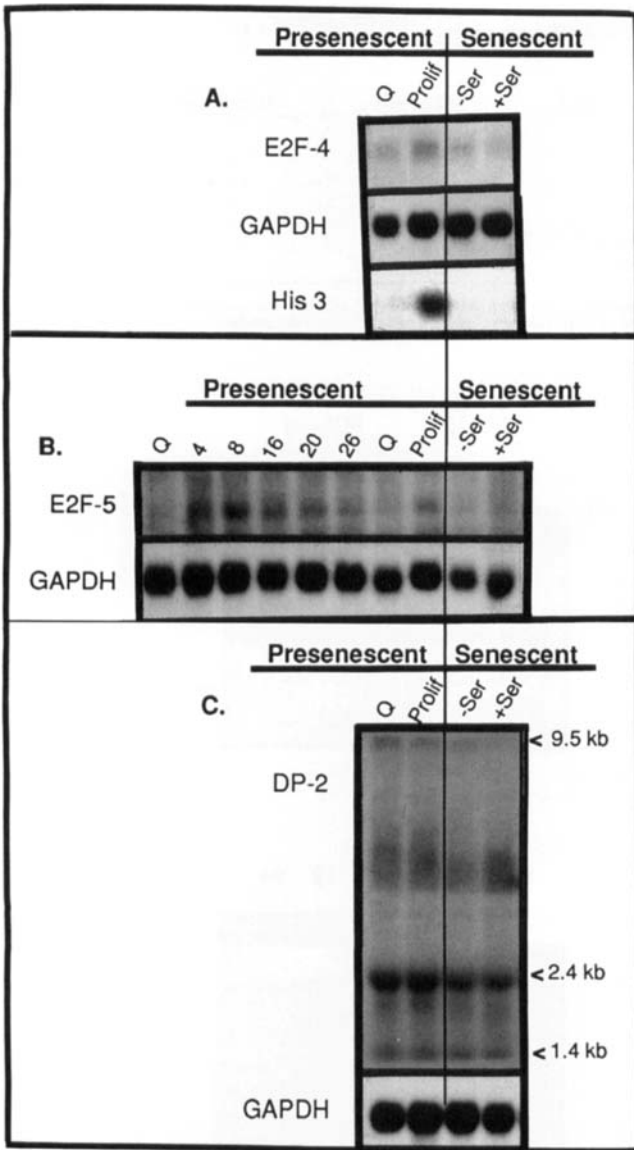
Fig. 5. Expression of E2F-4, E2F-5, and DP-2 mRNA in early and late passage human fibroblasts. Total RNA was isolated from presenescent (**left panels**) and senescent (**right panels**) WI-38 fibroblasts that were serum deprived (Q or –Ser), maintained in 10% serum (Prolif or +Ser), or serum-deprived and then stimulated for the indicated intervals (h). The RNA was analyzed for the indicated mRNAs as described in Materials and Methods. **A:** E2F-4, histone 3 (his 3) (to confirm proliferative state), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (to confirm RNA quantitation, integrity, and transfer) mRNA. **B:** E2F-5 and GAPDH mRNA. **C:** DP-2 and GAPDH mRNA.

E2F complexes contain several proteins important for cell cycle progression, including the pRB-related proteins p107 and p130, cyclin-dependent kinases, and cyclins (Nevins, 1992). Stein et al. (1990, 1991) have shown that senescent cells fail to phosphorylate pRB and fail to express cyclin A and B in response to serum. We found that the level of cyclin A protein, evident as a 52 kDa band, was cell cycle- and senescence-dependent (Fig. 6C), similar to E2F-1. Cyclin B protein, by con-

trast, was evident at all times in presenescent cells but undetectable in senescent cells (Fig. 6B). In contrast, p107 (Fig. 6D) and pRB protein (Stein et al., 1990) was constitutively expressed in both presenescent and senescent cells, regardless of serum stimulation.

Additional causes for the attenuated E2F binding activity in senescent cells

Although E2F-1 may be largely responsible for the cell cycle- and senescent-dependent E2F binding in human fibroblasts, a monoclonal antibody against E2F-1 abolished only about 70% of the total E2F binding activity in IMR-90 cells (data not shown). This finding is consistent with the idea that other E2F family members may contribute to this E2F binding activity as well (e.g., Sardet et al., 1995; Vairo et al., 1995). The undetectable E2F binding activity in senescent cells could be due to the absence or low abundance of E2F-1, E2F-4, or E2F-5. In addition, a senescence-associated inhibitory factor may prevent E2F-3 or as yet unidentified E2F proteins from binding to the E2F site. To test this latter possibility, we performed a mixing experiment in which we asked whether nuclear extracts from senescent cells could titrate the E2F binding activity in presenescent cell extracts. Figure 7 shows that a fixed amount of nuclear extract from senescent cells reduced the E2F binding activity in varying amounts of presenescent cell nuclear extracts. This result suggests the presence of an inhibitory factor in senescent cells that may suppress E2F binding activity. As a control, we showed that the binding activity of CBP/tk, another senescence-dependent trans-acting factor (Pang and Chen, 1993), remained constant in an identical mixing experiment.

DISCUSSION

A comparison of CAT activity driven by the human DHFR promoter in presenescent and senescent cells (Fig. 1) supports the notion that transcriptional regulation plays a significant role in the attenuation of DHFR gene expression in senescent cells. DNase I footprinting analysis of the 365 bp human DHFR promoter region confirmed that DNA-protein interactions occurred over the Sp1 (GGGGCGGGGC) and E2F (TTTCGCGC) consensus binding sites in the DHFR transcription control region (Fig. 2A). It can be noted that the 50 bp sequence (−94/−45) containing the proximal Sp1/Yi and E2F sequences (Fig. 2B) is conserved in several mammalian species (Chen et al., 1984; Yang et al., 1984). The proximal Sp1 site (−87/−76) overlaps with a binding sequence for Yi, a transacting factor that regulates the murine thymidine kinase gene (Dou et al., 1991). However, the Yi binding was not detectable in normal human fibroblasts (Good et al., 1995).

Within this 50 bp fragment, occupancy of only the E2F site was found to be serum-responsive and senescence-dependent (Fig. 3). Thus, despite protein binding at the sites of Sp1 and Sp1/Yi sites, E2F binding is the prime candidate for playing a the crucial role in the senescence-dependent attenuation of DHFR gene expression. Sp1 binding may be essential for DHFR transcription (Swick et al., 1989) but is unlikely to contribute to the cell cycle- and senescence-dependent regulation of DHFR expression.

Currently, five E2F family members and two DP fam-

TABLE 1. Expression of E2F family and DP family members in presenescent (early passage) and
senescent human diploid fibroblasts before (quiescent) and after serum stimulation (stimulated)[1]

	Presenescent cells		Senescent cells	
Transcription factor	Quiescent	Stimulated	Quiescent	Stimulated
E2F-1	−	+++	−	−
E2F-3	−	−	−	−
E2F-3	+++	+++	+++	+++
E2F-4	+	++	+	+
E2F-5	−	++	−	−
DP-1	++	++	++	++
DP-2	++	++	++	++

[1]The relative mRNA levels as estimated from densitometric tracing of Northern blot data shown in Figs. 4 and 5 were
qualitatively expressed as follows: −, mRNA signal very low or undetectable; +, mRNA signal weakly detectable; ++,
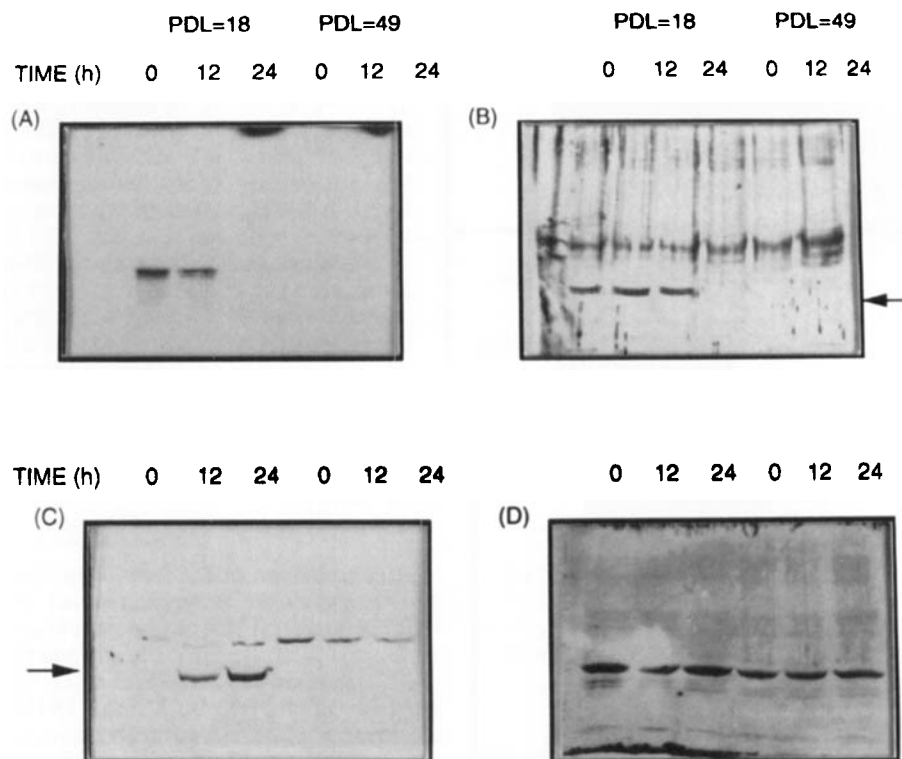moderate mRNA signal; +++, strong mRNA signal.

Fig. 6. Western blot analysis of E2F-1 and other E2F-associated
proteins in presenescent and senscent IMR-90 cells. The antibody
used for each immunoblot is (A) KH95, E2F-1 monoclonal antibody;
(B) cyclin B polyclonal antibody; (C) cyclin A polyclonal antibody; and
(D) p107 polyclonal antibody. The positions for cyclin A and cyclin
B are indicated with arrows. Additional bands were likely due to
nonspecific cross-hybridization. Cell extracts were prepared, cell pro-
teins were separated, and specific proteins were detected as described
in Materials and Methods.

ily members have been identified and cloned. As sum-
marized in Table 1, among the E2F family members,
E2F-1 and E2F-5 and, to a lesser degree, E2F-4 showed
cell cycle- and senescence-dependent expression. In the
case of E2F-1, for which antibodies are available, this
was true both at the mRNA and protein level (Figs. 4,
6). Our results are consistent with previous reports that
E2F-1 expression is cell cycle-dependent in various cell
lines (Krek et al., 1993; Helin et al., 1992; Kaelin et
al., 1992; Girling et al., 1993; Lees et al., 1993; Slansky
et al., 1993) and that E2F-1 mRNA is diminished in
senescent WI-38 cells (Dimri et al., 1994). E2F-5 be-
haved similarly to E2F-1. It was not expressed by quies-

cent early passage or serum-deprived senescent cells
and was strongly serum-inducible, albeit with earlier
kinetics than E2F-1, in presenescent but not senescent
cells. By contrast, E2F-4 was expressed by both serum-
deprived presenescent and senescent cells, but the
modest induction by serum in presenescent cells did
not occur in senescent cells. E2F-3 and DP-2 were con-
stitutively expressed at a high level in both early pas-
sage and senescent cells (Figs. 4, 5). Since an antibody
against E2F-1 could not completely abolish E2F bind-
ing activity in serum-stimulated cells (data not shown),
it is likely that E2F-3 and E2F-4 are responsible for
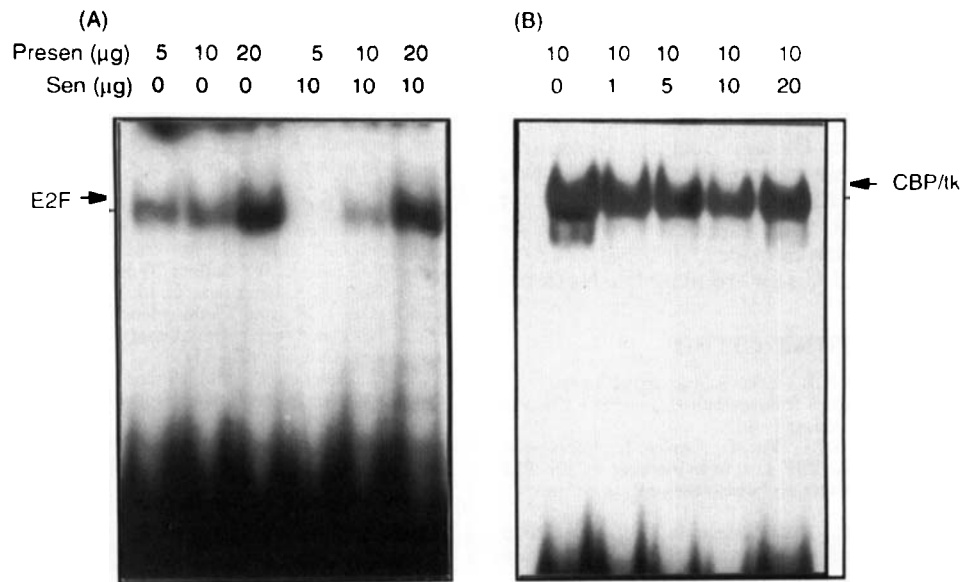some of the E2F binding activity.

Fig. 7. Presence of an inhibitory factor for E2F complex formation in senescent IMR-90 cells. Nuclear extracts from presenescent and senescent cells were mixed at the ratios indicated. E2F (A) and CBP/tk (B) DNA binding activity was assayed as described in Materials and Methods.

We considered the possibility that there may exist in senescent cells an inhibitory factor(s) that suppresses E2F binding even in the presence of E2F-3, E2F-4, DP-1, and DP-2, all of which are expressed in senescent cells. Indeed, an inhibitory activity for E2F binding has been reported in differentiated NEC14 cells (Hara et al., 1993). Mixing experiments using nuclear extracts from presenescent and senescent cells (Fig. 7) did suggest the presence of an inhibitory activity for E2F binding in senescent cells. Our preliminary evidence suggests that the inhibitory activity can be abolished by preincubation of extracts with the cAMP-dependent protein kinase (L. Good and K.Y. Chen, unpublished observation). We have not yet been able to footprint the relevant regions of the DHFR promoter using nuclear extracts from senescent cells and are currently working to understand the reasons for this.

The activity of E2F is thought to be regulated by association with other proteins, including pRB and the pRB-related proteins, p107 and p130 (Farnham et al., 1993). Association of pRB, p107, or p130 with various E2F complexes prevents transcriptional activity (Bandara and La Thangue, 1991). Both pRB and p107 did not show a significant change in relative abundance during serum stimulation of either presenescent or senescent cells (Fig. 6) (Stein et al., 1990). Stein et al. (1990) have reported that senescent cells contain the underphosphorylated form of pRB exclusively. Whether p107 also exists in an underphosphorylated form in senescent cells remains to be investigated. Among other E2F associated proteins, we found that levels of cyclin A and cyclin B were significantly less in senescent cells relative to early passage cells (Fig. 7). Stein et al. (1991) have shown that cyclin A, cyclin B, and cdc2 mRNA are attenuated in senescent cells. Microinjection of either antisense oligonucleotides or antibodies against cyclin A causes a block or delay in DNA synthesis (Girard et al., 1991; Pagano et al.,

1992). Taken together, these results indicate that several components of E2F complexes are altered during senescence. Although it is not yet possible to construct the hierarchy of gene regulation that eventually leads to the senescence-dependent loss of dividing potential, the lack of cycA, cycB, cdc2, E2F-1, and E2F-5 and overexpression of the cyclin-dependent kinase inhibitor p21 (Noda et al., 1994) suggest that there may be coordinated control of these genes in human cells during replicative senescence. More detailed studies of the regulation of each of these genes will be needed to understand whether and how they are hierarchically related. For example, Dulic et al. (1993) have shown that cyclin E-associated kinase activity was very low in senescent cells, despite the presence of abundant cyclin E-Cdk2 complexes. This deficiency in kinase activity is very likely due, at least in part, to p21 overexpression. In addition, recent data suggest that the inhibition of cyclin-dependent kinases by p21 is an upstream event that downregulates the expression of E2F-1 and E2F-dependent transcription (Dimri et al., 1996).

In summary, our data indicate that the absence of E2F-1 protein and the presence of an inhibitory activity in senescent cells may be largely responsible for the loss of E2F binding activity. This absence of E2F binding is primarily responsible for the senescence-dependent attenuation of DHFR gene expression. We speculate that the loss of E2F binding in senescent cells may very well be the cause for the senescence-dependent attenuation of other G1/S genes, such as thymidylate synthetase, proliferating cell nuclear antigen, and histone 2B.

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