Insulin-like growth factor binding protein-3 (IGFBP-3) is a well documented growth inhibitor and pro-apoptotic factor. IGFBP-3 mRNA and its protein are overexpressed by senescent human diploid fibroblasts. However, the mechanism responsible for the up-regulation of its expression is still unclear. This report describes a novel transcriptional regulatory element, IGFBP-3 enhancer element (IEE), identified in the 5′ untranslated region of the IGFBP-3 gene. This element differentially activates IGFBP-3 expression in senescent versus young fibroblasts. Electrophoretic mobility shift assays revealed abundant complexes in senescent cell nuclear extracts compared with young cell nuclear extracts. Similar to young proliferative cells, young quiescent cells showed reduced binding activity; enhancement of this activity was specific to senescent cells and not an effect of cell cycle arrest. The DNase I footprint revealed the protein-binding core sequence within the IEE through which the protein binds the IEE. Site-directed mutagenesis within IEE abolished binding activity and selectively decreased IGFBP-3 promoter activity in senescent (but not young) cells. Furthermore, introduction of an IEE decoy suppressed the endogenous IGFBP-3 gene expression specifically in senescent cells. These results point to the IEE as being a positive transcriptional regulatory element that contributes to the up-regulation of IGFBP-3 during cellular senescence.

IGFBP-3 inhibits IGF action by competitively binding IGFs and thereby preventing their binding to the IGF receptor I (IGF-IR). This action has been demonstrated by numerous experiments in various cell types using destripeptide-(1,3)-IGF-I, an IGF-I analog that binds IGF-IR and stimulates DNA synthesis but cannot bind IGFBP-3 (5–7). By preventing IGFs from stimulating the IGF-IR, IGFBP-3 inhibits the IGF signaling pathway. However, the effects of IGFBP-3 on IGF-dependent cellular functions are complex, with both stimulatory and inhibitory actions reported, even within the one cell type (8).

Evidence from several studies has shown that, in addition to its ability to regulate access of IGF to its receptor, IGFBP-3 may have important IGF-independent antiproliferative effects. For example, transfection of human IGFBP-3 cDNA inhibits growth in various cell types, including human breast cancer cells (9) and murine 3T3 fibroblasts (10). In addition, Valentinis et al. (11) showed that transfection of human IGFBP-3 cDNA inhibits proliferation of fibroblasts with a targeted disruption of the IGF-IR. Further evidence has come from studies showing that IGFBP-3 gene expression is induced by potent growth-inhibitory proteins and agents, including tumor suppressor protein p53 (12), transforming growth factor (TGF)-β (13, 14), retinoic acid (15), tumor necrosis factor α (16, 17), and anti-estrogen (18), which suggests that these agents may mediate their inhibitory effects through IGFBP-3. More recently, it has been suggested that the growth-inhibitory effects of IGFBP-3 may be mediated via an induction of apoptosis. Indirect evidence has come from reports that an increase in IGFBP-3 expression is associated with the induction of apoptosis (19, 20). More directly, Nickerson et al. (21) demonstrated that the addition of recombinant IGFBP-3 induces apoptosis in breast carcinoma cells. This effect was abrogated in the presence of an IGF-1 analog that binds IGF-IR (but not IGFBP), which suggests that, in this system, IGFBP-3 induces apoptosis indirectly by reducing the bioavailability of ligands for IGF-IR. However, direct pro-apoptotic effects of IGFBP-3 have been reported in cells lacking IGF-IR (22) and under conditions where IGF-I could not elicit a survival effect (23), pointing to the existence of an IGF-independent mode of IGFBP-3 action.

Compared with young cells, senescent fibroblasts show substantially up-regulated IGFBP-3 mRNA and protein (24–26), and the protein also accumulates in the growth medium of young cells to levels directly correlated with the chronological age of the donor (25–27). Furthermore, Grigoriev et al. (28) found that IGFBP-3 accumulation in the medium of senescent human diploid fibroblasts can bind and sequester IGFs and thereby attenuate the response of senescent cells to IGF-I. Whether IGFBP-3 exerts its influence on senescent cells through an IGF-independent pathway is still unclear. In-
increased IGFBP-3 expression appears to suppress growth (but not induce) apoptosis in senescent fibroblasts, because senescent fibroblasts are more resistant to apoptotic stimuli than young cells (29).

Buckhinder et al. (12) first identified IGFBP-3 as one of the p53-inducible genes by employing EB1 colon carcinoma cells carrying an inducible wild-type p53 transgene and saos-2-D4H cells containing a temperature-sensitive p53 mutant. Furthermore, ultraviolet radiation and doxorubicin induced IGFBP-3 expression in fibroblasts containing the wild-type (but not mutant) p53. As well, on the basis of homology to the p53-binding consensus sequence (30), two p53-binding sites within the IGFBP-3 gene were identified and confirmed by electrophoretic mobility shift analyses (EMSAs) (12). Thus, IGFBP-3 expression is positively regulated by p53. However, IGFBP-3 expression could also be induced by a p53-independent pathway (31).

During cellular senescence, p53 activity increases greatly (32). Thus, we wondered whether the increased p53 activity contributes to overexpression of IGFBP-3 in senescent cells. However, treating senescent cells with the HPV-E6-expressing vector or another p53 inhibitor, PFT-α, did not reduce IGFBP-3 expression. This report describes our subsequent examination of potential 5′ regulatory regions and mechanisms that may be responsible for the up-regulation of IGFBP-3 in senescent human diploid fibroblasts. Analysis of the IGFBP-3 promoter via transient transfection of nested promoter deletions into young and old fibroblasts has identified an enhancer element. This element is located in the 5′-UTR of IGFBP-3 and binds a protein of 27 kDa more avidly in senescent cells than in young cells, which suggests that enhanced binding to this element in senescent cells contributes to the increased expression of IGFBP-3 during senescence.

MATERIALS AND METHODS

Cells and Cell Culture—Human embryonic lung diploid fibroblast 2BS cells (obtained from the National Institute of Biological Products, Beijing, China) were previously isolated from female fetal lung fibroblast tissue and have been fully characterized (33). The current expected life span is ~70 population doublings (PD). 2BS cells were considered to be young at PD30 or below and fully senescent at PD55 or above. The cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum in a 10-cm diameter dish by subculturing at a split ratio of 1:2. The cells were changed to 10% fetal calf serum when they reached confluence. At 15–20% confluence, the cells were harvested, and the labeled probe was subsequently purified using Sephadex G-50. Binding assays involved the use of a mixture containing a 5′-labeled oligonucleotide (0.3 ng), 5 μg of nuclear protein, 1 μg of poly(dI-dC) (Amersham Biosciences) adjusted to 20 μl with binding buffer (20 μl HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 7.5 μg/μl). Binding reactions were carried out for 30 min at room temperature. A 15-μl aliquot of each reaction was loaded onto a 5% nondenaturing polyacrylamide gel and run in 0.5× TBE buffer at 120 V. Following electrophoresis, the gels were dried and autoradiographed.

To measure the affinity of the potential binding site, the dissociation constant of the binding site was determined as described previously (34), DNase I footprinting experiments were performed with a fragment corresponding to –63 to +59 of the IGFBP-3 gene transcription starting site. The fragment was labeled with [α-32P]dATP and incubated with nuclear extracts at room temperature for 30 min in a reaction mixture containing 4% glycerol, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2.5 mM MgCl2, 0.5 mM dithiothreitol, and 2 μg of poly(dI-dC). The binding reaction was continued for exactly 1 min. Footprinting was done using a Core footprinting system kit from Promega, according to the manufacturer’s instructions. The samples were analyzed on a 6% acrylamide/7 M urea denaturing gel.

Site-directed Mutagenesis—Bases were mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). A mutagenic primer (45mer) was synthesized and annealed to the double-stranded –305 construct vector DNA. Plaque-forming unit DNA polymerase was used to synthesize the mutagenic promoter, followed by digestion of the parental plasmid by DpnI according to the manufacturer’s instructions. The mutated plasmid was transformed into XL1-Blue competent cells, and the resulting plasmid was isolated and sequenced. These clones were designated as –2031 (full-length) –1303, –778, –305, and –141 relative to the translational start site.

UV Cross-linking of DNA-Protein Complexes—To estimate the relative molecular mass of the DNA-binding proteins, binding reactions with oligonucleotide 5′-UTR-2 were performed. Twice the amount of the labeled probe (4 × 10^4 counts/min) and nuclear extracts (10 μg) were used. After 30 min of incubation at room temperature, the binding reaction was subjected to UV light, and unprotected DNA was digested with DNase I. The samples were irradiated by a 305-nm inverted UV
transilluminator at 7 milliwatts/cm² for 5 min. The cross-linked reactions were electrophoresed through 10% SDS-polyacrylamide gels, dried, and visualized by autoradiograph.

Dual Luciferase Reporter Assays—Constructs containing the wild-type and mutant IEE were made using pGL3 promoter plasmids containing the SV40 promoter (Promega). A total of 30 bp of oligonucleotides was cloned into the MluI/BglII in the multicloning site region upstream of the SV40 promoter and the HindIII/NcoI between the SV40 promoter and the luc reporter gene. HindIII/NcoI was chosen because of its proximity to the functional gene (i.e. the in vivo IEE is located in the 5'-UTR of the IGFBP-3 gene). MluI/BglII was chosen to determine whether a changed IEE location affects its transcriptional regulation. These constructs were then co-transfected into young and senescent 2BS cells with pRL-CMV as a transfection efficiency control. Luciferase activity was measured using an Optocomp luminometer and normalized on the basis of pRL-CMV expression.

RESULTS

Increased p53 Activity Does Not Contribute to IGFBP-3 Overexpression in Senescent 2BS Cells—Northern blot analysis with IGFBP-3 cDNA as a probe confirmed whether IGFBP-3 expression is increased in senescent 2BS cells. As shown in Fig. 1A, IGFBP-3 expression increased substantially in senescent cells as compared with young cells (24–26). Many studies have shown that the induction of the IGFBP-3 gene is associated with p53 activity. Coincidentally, p53 activity greatly increases in senescent cells. However, we wondered whether increased p53 activity caused IGFBP-3 overexpression. The expression vector expressing HPV-E6 protein, which is a well known p53 inhibitor, was used to examine whether or not p53 activity is
associated with IGFBP-3 expression in senescent 2BS cells. For the positive control, we used a pp53-TA-Luc vector (Clontech) containing a p53 Cis-acting enhancer element upstream of the luciferase reporter gene, because p53 target genes have not been definitely documented in senescent cells. We co-transfected senescent cells with both vectors; after 48 h, luciferase activity was assayed. The result from Fig. 1B showed that luciferase activity was decreased in HPV-E6 vector-treated senescent cells in a dose-dependent manner, suggesting HPV-E6 vector is functional in antagonizing p53 function in this system. Subsequently, senescent cells were treated with the same method as above; RNA were isolated and analyzed by Northern blotting with IGFBP-3 cDNA as a probe and GAPDH as an internal control. As shown in Fig. 1C, no change in IGFBP-3 expression was observed in HPV-E6 vector-transfected senescent cells, suggesting that elevated p53 activity does not contribute to IGFBP-3 gene overexpression in senescent cells. Such a view was further supported by the results with another p53 inhibitor, PFT-a (as shown in Fig. 1, D and E). Thus, we subsequently cloned the IGFBP-3 promoter and analyzed potential regulatory regions that may be responsible for the up-regulation of IGFBP-3 seen in the senescent 2BS cells.

Identification of the Functional Region in the 5′-UTR of IGFBP-3 That Contributes to Overexpression of IGFBP-3 in Senescent Fibroblasts by Deletion Analysis—To determine the basal activity of the IGFBP-3 promoter in young and senescent cells, a 2-kb IGFBP-3 promoter fused to the luciferase reporter gene (Fig. 2A) was used in transient transfection studies. Various 5′-deleted pGFPB-3 constructs were generated (Fig. 2B) and transiently co-transfected with a cytomegalovirus-driven Renilla luciferase vector (as an internal control) into young and senescent cells. After 36 h of incubation, the cells were harvested and analyzed for luciferase activity. As shown in Fig. 2B, luciferase activity caused by the full-length IGFBP-3 promoter was 3-fold greater in senescent than in young cells. Deletion of segments from the 5′ end, from −2031 to −778, slightly reduced the difference in expression between young and senescent cells. However, further deletion of the IGFBP-3 promoter, to 305 bp upstream of the ATG translation initiation site, did not reduce but rather increased the difference in expression by 4–5-fold, which indicates that the region from −305 to +59 strongly contributes to increased expression in senescent cells.

We then generated a 3′-nested deletion of the −305 to +59 region to further define the region involved in the differential regulation of IGFBP-3. Fig. 3A shows that deletion of the region from +59 to −58 substantially attenuated luciferase expression in senescent cells, but the luciferase activity in young cells remained relatively unchanged, thus reducing the difference in expression between senescent and young cells (Fig. 3B). Further deletion from +59 to −192 completely abolished luciferase expression in both cells, probably because the transcription initiation site had been removed (Fig. 3A). Thus, a potential binding site for a transcriptional repressor in young cells and/or a transcriptional activator in senescent cells exists in the 100-bp region between −58 and +59.

Identification of a 30-bp Enhancer Element with Overlapping Oligonucleotides—To define the region involved in protein binding, we used electrophoretic mobility gel shift assays (but not DNaseI) footprinting assays, because the former can semiquantitate protein-DNA binding. As shown in Fig. 4A, five doubled-stranded 30-bp oligonucleotides, designated 5′-UTR-1 to 5′-UTR-5, were synthesized and used in EMSA of young and senescent cell nuclear extracts. No detectable activity was observed with the 5′-UTR-1, -3, -4, and -5 oligonucleotides (Fig. 4B, lanes 6, 8, 14, 16, 18, 20, 22, 24). A specific complex was formed when 5′-UTR-2 was used as a probe with senescent cell extracts (Fig. 4B, lane 12), whereas the level was dramatically reduced with young cell extracts (Fig. 4B, lane 10). Specificity of the complex was confirmed by incubation with a 100-fold excess of unlabeled oligonucleotide, which competed with the labeled probe (Fig. 4B, lanes 11 and 13). Thus, the 30-bp region was involved in protein binding and termed IEE.
To rule out the possibility that the nuclear extracts from young 2BS fibroblasts were deficient in DNA binding ability, we performed gel shift studies using the same extracts with an oligonucleotide containing the Sp1-binding site. As shown in Fig. 4C, Sp1-binding activity actually was slightly higher in young cell extracts (lane 2) than in senescent extracts (lane 4). As expected, 100-fold competition with unlabeled oligonucleotide bearing the same Sp1 sequence competed with the complex formed on the labeled Sp1 (Fig. 4C, lanes 3 and 5). Given previous reports that Sp1-binding activity is about equal in young and senescent cells (35), the specific binding activity of the complex shown in Fig. 4B would actually be reduced 4-fold in extracts from young as compared with senescent cells (Fig. 4D).

Increased expression of IGFBP-3 is also seen upon serum withdrawal or contact inhibition-induced cell cycle arrest (25). To test whether the increased binding activity was specific to senescence, we incubated the 30-bp element with extracts from young proliferating cells, young quiescent cells, and subconfluent senescent cells. As shown in Fig. 4E, increased binding was not seen in young quiescent cells (lane 6), which indicates that elevated binding depends on senescence rather than growth.

To help determine the specific transcription factor producing the band shift shown in Fig. 4B, we first performed a detailed computer analysis using the MatInspector program but did not find highly homologous consensus with known transcription factors. To further confirm the bases within the IEE responsible for protein binding, oligonucleotides of 5'-UTR-2 containing various mutations (m1–m15) were generated as shown in Fig. 5A. In each case, purines and pyrimidines were exchanged for noncomplementary pyrimidines and purines, respectively. Surprisingly, EMSA of senescent cell nuclear extracts with these oligonucleotides (Fig. 5B) revealed that these mutations greatly reduced (lanes 3 and 14) or completely abolished (other lanes) the binding activity, suggesting that these bases within the IEE were necessary for transcription factor binding.

DNase I Footprint Identified the Protein-binding Core Sequence within the IEE—That seemingly every base pair within the oligonucleotide is important for the protein-DNA complex is a surprising phenomenon. To define the protein-binding sequence within the IEE, in vitro DNase I footprinting was performed with nuclear extracts from young and senescent 2BS cells and single end-labeled probe (-63 to +59) covering the IEE region. As seen in Fig. 6, two DNase I-protected regions, designated as sites A and B, were detected in this region. Coincidently, the two sites are located in the 30-bp IEE region, with site A covering the sequence CTGCCA and site B covering the sequence GCGTGCCCCG. Regions A and B were separated only by four nucleotides. Because a single protein-DNA complex was detected in EMSA, the protein (or protein complex) binds the probe likely through sites A and B, two contacting sites within the DNA probe. Besides the bases within the regions A and B, other base mutations in the 30-bp oligonucleotide also affected protein-DNA interaction, perhaps reflecting stabilization of the complexes by these bases.

Mutation of the IEE Decreases IGFBP-3 Promoter Activity in Senescent Cells—Three sets of 2-bp bases (randomly selected within the IEE) changed in the 305 construct, and corresponding to the mutation in 5'-UTR-m6, -m8, and -m10 (Fig. 7A), were introduced by site-directed mutagenesis. We trans-
affected the promoter −305 construct and the mutated constructs into young and senescent cells and measured the luciferase activity resulting from each construct after normalizing for pRL-CMV control. As shown in Fig. 7B, all three mutations within the IEE resulted in a nearly 3-fold decrease in luciferase activity in senescent cells as compared with the wild-type construct. In contrast, the luciferase activity from the mutant construct was slightly decreased as compared with that in the wild-type construct in young cells. Fig. 7C shows the results of the same experiment plotted as -fold difference in senescent

**Fig. 4. Identification of the IEE with overlapping oligonucleotides.** A, a portion of the IGFBP-3 promoter 5'-UTR showing a 117-bp region divided into five overlapping 30-bp oligonucleotides (5'-UTR-1 to 5'-UTR-5). B, electrophoretic mobility shift assay of the overlapping oligonucleotides shown in A with young (Y) and senescent (S) cell nuclear extracts. Lanes 1–5 are control reactions in the absence of nuclear extracts. The formation of a complex was evident in senescent cell nuclear extracts (lane 12) and weaker in young cell nuclear extracts with 5'-UTR-2 used as a probe (lane 10), but the signal was not detected with 5'-UTR-1, -3, -4, and -5 used as probes (lanes 6, 8, 14, 16, 18, 20, 22, and 24). Unlabeled 5'-UTR-1, -2, -3, -4, and -5 oligonucleotides were also used as probes for a competition experiment at a 100-fold excess as indicated in lanes 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25 to determine the specificity of binding. C, control gel shift with use of a consensus Sp1-binding site with the same young (Y) and senescent (S) nuclear extracts described in B. Lane 1 shows the probe alone in the absence of nuclear extracts. Lanes 2 and 4 show the formation of a complex with the addition of young senescent nuclear extracts. Lanes 3 and 5 show the reaction in the presence of 100-fold of a cold Sp1 oligonucleotide. D, the binding activity of senescent and young cells with 5'-UTR-2 in B was quantified as determined against the Sp1 control in C. E, electrophoretic mobility shift assay with independently isolated nuclear extracts from senescent subconfluent cells (S), young growing cells (Y), and young quiescent cells (Q), with 5'-UTR-2 used as a probe. Lane 1 shows 5'-UTR-2 in the absence of the nuclear extract, and lanes 2, 4, and 6 show the degree of binding activity of three nuclear extracts to 5'-UTR-2. Lanes 3, 5, and 7 show the reaction in the presence of 100-fold of a cold 5'-UTR-2 oligonucleotide.
versus young cells. Mutations of the IEE resulted in a nearly 60% decrease in the difference in luciferase activity between mutated and unmutated controls. These results suggest that the IEE within the 5′-UTR of IGFBP-3 constitutes a binding site for a potential transcriptional activator in senescent cells, the activity and/or levels of which are reduced in young cells.

Inhibition of the IGFBP-3 Gene Expression by Introducing IEE Decoy into Senescent 2BS Cells—To demonstrate the feasibility of suppressing the endogenous IGFBP-3 gene expression by the decoy strategy, we examined the effect of the IEE decoy on the IGFBP-3 mRNA expression in senescent cells. Either 0–14 μg of IEE decoy oligonucleotides or control mutant IEE oligonucleotides (shown in Fig. 10A) was introduced into senescent cells cultured in 10-cm diameter dishes by the lipofection method. When IEE decoy was introduced, the IGFBP-3 mRNA level was decreased in a dose-dependent manner compared with the level in cells transfected with a control (mutant) IEE oligonucleotides shown in Fig. 5A. Introduction of 2-bp changes reduced (lanes 3, 6, 14) or abolished (lanes 1, 2, 4, 5, 7–13, 15) binding activity compared with the wild-type 5′-UTR-2 oligonucleotide (lane 16).

Estimation of the Molecular Weight of the Protein Binding IEE—To estimate the molecular mass of any protein(s) bound specifically to IEE, nuclear extracts from young and senescent cells were incubated with the 30-bp oligonucleotide (5′-UTR-2) and subjected to UV cross-linking and then underwent SDS-PAGE. To demonstrate the sequence-specific nature of the protein, we added a 500-fold excess of cold competitors to the binding reaction mixture. As shown in Fig. 9, a complex was detected in both kinds of cells and was slightly more apparent in senescent than in young cells. Because the size of the complex was 45 kDa, the molecular mass of the protein is expected to be ~27 kDa.

The IEE Preferentially Activates Gene Expression in Senescent Fibroblasts—To test the activity of the 30-bp wild-type and mutant IEE, both forms were ligated into the HindIII/NcoI site between the luciferase reporter gene and the SV40 promoter and the MuLV/BglII multicloning site upstream of the SV40 promoter in the pGL3 promoter vector. These recombinant plasmids were transfected into young and senescent 2BS fibroblasts. Transfection efficiencies were normalized on the basis of Renilla luciferase (pRL-CMV) expression. As shown in Fig. 10B, the level of reporter was increased by >6.5-fold in senescent cells, with the wild-type IEE located in its natural site (HindIII/NcoI between the luciferase reporter gene and the SV40 promoter (but only slightly in young cells). No change was found with the ligation of the mutant IEE form in senescent or young cells. Therefore, although the IEE enhanced transcription in both young and senescent cells, the effect was markedly greater in senescent cells. The same phenomenon was observed in cells with the IEE cloned into its non-natural site (MluI/BglII) (Fig. 10B), although the transcription effect was weaker than with cloning into the natural site.

**DISCUSSION**

In this study, we cloned the IGFBP-3 promoter. Deletion analysis showed that the 117-bp region from –58 to +59 strongly contributes to increased IGFBP-3 gene expression in senescent cells. Use of overlapping oligonucleotides corresponding to this region narrowed the protein-binding region further to a 30-bp sequence in the 5′-UTR of IGFBP-3, which we have termed the IEE (from –37 to –8). Further EMSA with young quiescent and senescent nuclear extracts showed that the elevation of the protein-binding activity depends on senescence. Although mutation experiments showed that every base within the IEE appears to be important for forming protein-DNA complexes, the DNaseI footprint revealed the protein-binding core sequence within the IEE, through which the pro-
tein bind the IEE. Site-directed mutagenesis within IEE may markedly reduce IGFBP-3 promoter activity in senescent (but not young) cells; furthermore, introduction of the IEE decoy suppressed the endogenous IGFBP-3 gene expression specifically in senescent cells, indicating that the IEE is a positive transcription regulatory element. Luciferase reporter con-

**FIG. 7.** Luciferase activity in senescent versus young cells with use of an IGFBP-3 promoter containing a mutant IEE. A, three sets of 2-bp changes randomly selected in the IEE region were introduced into the −305 construct (−305 to +59 of IGFBP-3 promoter fused to the pGL3-basic vector) by site-directed mutagenesis. The resulting mutants were transfected into young and senescent cells. B, relative luciferase (LUC) activity from senescent (black bars) and young (white bars) cells after normalizing for transfection efficacy with co-transfected pRL-CMV. An average of three independent transfections were performed, with the S.D. indicated by error bars. C, results from B plotted as fold difference in luciferase (LUC) activity in senescent versus young cells.

**FIG. 8.** Down-regulation of the endogenous IGFBP-3 mRNA by introducing the IEE decoy in senescent cells. A, the indicated amount of IEE or mutIEE (control mutant IEE oligos shown in Fig. 10A) was introduced into senescent cells. At 24 h after transfection, RNA was isolated and analyzed by Northern blotting. GAPDH mRNA was used as the control. B, the bar graph shows the level of expression of IGFBP-3 mRNA in Fig. 8A after normalizing for GAPDH mRNA expression.
It has been well documented that the IGFBP-3 gene is activated by p53. However, we showed that increased p53 activity during cellular senescence was not required for induction of IGFBP-3, which indicates a p53-independent regulation of IGFBP-3 expression. A similar phenomenon was observed for the resistance of senescent cells to apoptosis. Although p53 is an important apoptotic factor in other cells, increased p53 activity does not sensitize senescent fibroblasts to apoptotic stimuli. In fact, senescent fibroblast cells are refractory to apoptosis (29). This difference in function probably results from the differential post-translational modification of p53 in senescent cells. It is well known that p53 is activated mainly through chemical modification, such as phosphorylation, acetylation, and ADP-ribosylation. A detailed analysis of p53 phosphorylation in senescent fibroblasts showed that senescence is associated with a major change at a putative regulatory site in the N and C termini of p53, with increased phosphorylation at Ser-15, Thr-18, and Ser-376 and decreased phosphorylation at Ser-392.

Ionizing and UV radiation generated an overlapping but distinct profile of response, with increased serine-15 phosphorylation being the only common change (36). This observation suggests that the difference in modification likely makes p53 function slightly differently in senescent than in other cells.

Fig. 1A shows that IGFBP-3 expression in senescent cells was >20-fold higher than that in young cells, but subsequent luciferase assay (Fig. 2B) showed only an ~4–5-fold difference in the promoter activity of the wild-type and mutant IEE sequences. The results in senescent (black bars) and young (white bars) cells were normalized to pRL-CMV activity and represent the mean ± S.D. for three independent experiments (right graph).
in expression. This observation indicates that other elements exist and contribute to the differential expression of IGFBP-3 in young and senescent cells.

Although the expression of a number of genes changes during senescence (37, 38), the mechanism by which senescence depends on regulates this expression has not been extensively studied. However, a few reports have suggested that regulation of some gene expression in senescent cells could differ from that in other cells. For example, Wang et al. (39) showed that a novel negative regulatory element at −495 to −485 bp of the p16INK4a promoter contributes to overexpression of p16 in senescent fibroblasts. As well, Berardi et al. (40) reported that a novel transcriptional inhibitory element in the 5′-UTR of cyclin D1 was related to the up-regulation of cyclin D1 in senescent fibroblasts. Thus, inactivation or activation of some unidentified element in the 5′-UTR of the IGFBP-3 gene in young fibroblasts (40). In this paper, another novel transcription enhancer D1 was related to the up-regulation of IGFBP-3 in senescent fibroblasts. As well, Berardi et al. (40) reported that a novel transcriptional inhibitory element in the 5′-UTR of IGFBP-3 was identified and shown to contribute to the increased IGFBP-3 expression in senescent cells. Thus, inactivation or activation of some unidentified transcription factor databases revealed no homologies to known human transcription factor binding sites, which suggests that the protein(s) binding the IEE are novel. Experiments to identify the putative enhancer protein by a one-hybrid assay are under way and should reveal important insights regarding its role(s) in cellular aging.

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