Senescent human diploid fibroblasts are unable to initiate DNA synthesis following mitogenic stimulation and adopt a unique gene expression profile distinct from young or quiescent cells. In this study, a novel transcriptional regulatory element was identified in the 5′-untranslated region of the cyclin D1 gene. We show that this element differentially suppresses cyclin D1 expression in young versus senescent fibroblasts. Electrophoretic mobility shift assays revealed abundant complexes forming with young cell nuclear extracts compared with senescent cell nuclear extracts. Binding was maintained in young quiescent cells, showing that loss of this activity was specific to senescent cells and not an effect of cell cycle arrest. Site-directed mutagenesis within this cyclin D1 inhibitory element (DIE) abolished binding activity and selectively increased cyclin D1 promoter activity in young but not in senescent cells. Sequences with homology to the DIE were found in the 5′-untranslated regions of other genes known to be up-regulated during cellular aging, suggesting that protein(s) that bind the DIE might be responsible for the coordinate increase in transcription of many genes during cellular aging. This study provides evidence that loss of transcriptional repressor activity contributes to the up-regulation of cyclin D1, and possibly additional age-regulated genes, during cellular senescence.

Normal human diploid fibroblasts (HDFs) are widely used as a model system to study the process of replicative or cellular senescence (1, 2). These cells have a finite proliferative life span, at the end of which they are unable to enter the S phase in response to mitogenic stimuli, but they remain metabolically active for long periods of time (3). They have prominent and functional Golgi apparatus, a large, flat morphology, invaginated and lobed nuclei, large lysosomal bodies, and an increase in cytoplasmic microfibrils compared with young cells (reviewed in Ref. 4). They also show a senescence-associated β-galactosidase (β-gal) activity, staining blue under acidic conditions (pH 6.0), whereas young cells do not (5). Some of the biochemical hallmarks that accompany cellular senescence include up-regulation of cyclin D1 (6–8), p21Waf1/Cip1/Sdi1 (9), p16INK4a (10, 11), and insulin-like growth factor binding protein-3 (12). Furthermore, senescent cells arrest with a DNA content characteristic of the G0 and G1 phases of the cell cycle; yet the expression and activity of many cell cycle regulatory proteins during G0, G1, and senescence are distinct, implying that senescent cells exist in a unique, nonproliferative state that we have termed G0 (13). Cyclin D1 is a member of the D-type family of cyclins that associates with cyclin-dependent kinases 4 and 6 (14, 15). Cyclin D1- cyclin-dependent kinase 4 promotes the G1 to S phase transition of the cell cycle by cooperating with cyclin E-cyclin-dependent kinase 2 to sequentially phosphorylate the retinoblastoma tumor suppressor protein (16). However, cyclin D1 knockouts are rescued by cyclin E expression, whereas the reverse does not hold, suggesting that cyclin D1 does not play a central role in regulating the cell cycle, which is further suggested by the phenotype of cyclin D1 knockout mice (17–19). Normal human diploid fibroblasts that have reached the end of their in vitro life span (senescent cells) express 3-fold higher levels of cyclin D1 protein than low passage cells. Individual cells in mass culture that fail to initiate DNA synthesis in response to serum addition have severalfold higher levels of this cyclin than proliferation competent cells (7). It has been shown that cyclin D1 overexpression may inhibit this entry into S phase through binding to proliferating cell nuclear antigen and cyclin-dependent kinase 2 (7, 20). Cyclin D1 dysregulation and gene amplification have been implicated in a variety of cancers (21), suggesting that deregulated expression of cyclin D1 contributes to abnormal cell proliferation.

Ectopic overexpression of cyclin D1 in normal HDFs, the mammary epithelial cell line MCF-7, Dami megakaryotic cells, and rat embryo fibroblasts inhibits DNA synthesis and cell growth (7, 20, 22–25). Cyclin D1 has also been shown to be up-regulated in numerous nonproliferative differentiated cell types (26–29) and during apoptosis (30), lending further mass to the idea that it may also provide growth suppressive functions. In senescent fibroblasts, the cyclin D1 mRNA and protein levels are constitutively up-regulated by ~3–5-fold compared with serum-stimulated young cells (6–8, 31).

The 5′-regulatory region of the cyclin D1 gene has been well characterized, and the regions responsible for serum-inducible transcription have been identified (32, 33). However, the regulation of cyclin D1 gene expression is not well understood under conditions of senescence-associated growth arrest where
induction is at least as great as seen in response to serum. The increased expression of cyclin D1 is specific for senescence-associated growth inhibition in HDFs and is not apparent in contact-inhibited or serum-deprived cells arrested at a similar place in the cell cycle (24). This is in contrast to the closely related cyclin D2 where expression is increased in senescent, quiescent and contact-inhibited cells (24). Thus, unique transcriptional mechanism(s) may strongly contribute to cyclin D1 expression and senescence. In this report, we examine potential 5′ regulatory regions and mechanisms that may be responsible for the up-regulation of cyclin D1 seen in aging HDFs. Analysis of the cyclin D1 promoter via transient transfections of nested promoter deletions into young and old fibroblasts has identified a 15-bp cyclin D1 inhibitory element (DIE). This element is located in the 5′-UTR of cyclin D1 and has been shown to bind a low molecular mass protein more avidly in young cells compared with senescent cells, suggesting that loss of binding to this element in senescent cells contributes to the increased expression of cyclin D1 seen during cellular senescence. Consistent with a role in cell aging, it was noted that several other genes that are up-regulated during cell aging were found to have DIE-like elements in their 5′-UTRs, strongly supporting the idea that this element may bind a common regulatory protein that contributes to senescence-specific gene expression.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—HDFs (Hs68; ATCC CRL 1635 from newborn foreskin, reaches 85 mean population doublings (MPDs) in culture; WI-38: CCL 75, from embryonic lung, reaches 56 MPDs under our culture conditions) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. All experiments were performed in a humidified atmosphere with 5% CO2. Cells were incubated at 37°C in 5% CO2. Cells were between 28–39% confluent on day 7 of culture conditions) 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 harvested using passive lysis buffer (Invitrogen) and assayed for luciferase expression using an EG & G Berthold luminometer.

β-Galactosidase and CAT Assays—Electroporated cells were washed twice with ice-cold PBS, harvested, and centrifuged. The cell pellet was washed with PBS, resuspended in 100 µl of lysis buffer (0.25% Triton X-100, 50 mM HCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM PMSF, 100 µg/ml aprotinin, 100 µg/ml leupeptin, 10% glycerol, 5% β-mercaptoethanol) and 150 mM potassium ferricyanide, and 1 mM MgCl2.

The cells were trypsinized, suspended in 400 µl of lysis buffer (0.25% Triton X-100, 50 mM HCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM PMSF, 100 µg/ml aprotinin, 100 µg/ml leupeptin, 10% glycerol, 5% β-mercaptoethanol) and 150 mM potassium ferricyanide, and 1 mM MgCl2.

Nuclear extracts from young HDFs were prepared as described previously (36, 37). A double-stranded 64-bp oligonucleotide containing the cyclic AMP response element (CRE) and binding conditions for this element have been described previously (36). Briefly, 20,000 cpm (0.1–0.5 ng) of gel-purified [α-32P]dCTP-labeled probe was incubated with 5 µg of nuclear extract from young or old HDFs. The reactions were incubated for 30 min at room temperature, electrophoresed through 5% nondenaturing polyacrylamide gels at 150 V at room temperature, dried, and visualized by autoradiography.

Oligonucleotides corresponding to the cyclic AMP response element (CRE) and binding conditions for this element have been described previously (36). Briefly, 20,000 cpm (0.1–0.5 ng) of gel-purified [α-32P]dCTP-labeled probe was incubated with 5 µg of nuclear protein extract in a buffer that contained 20 mM Tris (pH 7.6), 4% Ficoll, 50 mM KCl, 1 mM EDTA, 0.2 mM dithiothreitol, 10 µM ZnCl2, 1 µg of partially denatured salmon sperm DNA, and 30 µg of bovine serum albumin in a final volume of 20 µl for 30 min at room temperature. Unlabeled wild type and mutant competitor DNA was added to a 100-fold excess and incubated for 10 min at room temperature before the addition of hot probe.

Site-directed Mutagenesis—A total of 8 bp between +117 and +131 of the 5′ flanking region were mutated to alanine. The QuickChange Site-Directed Mutagenesis kit (Stratagene). A mutagenic primer (46-mer) was synthesized and annealed to the double-stranded pBLCAT3 construct containing the full-length cyclin D1 promoter. Pfu DNA polymerase was used to synthesize the mutagenic promoters followed by digestion of the parental plasmid by DpnI as per the manufacturer’s instructions. The mutated

Electrotransfection—5 × 105 log phase young and senescent (arrested) Hs68 fibroblasts were seeded in 150-mm plates and harvested when ~80% confluent. The cells were trypsinized, suspended in 400 µl of serum-free modified Eagle’s medium (Invitrogen) and transferred to 10-cm plates. The samples were transferred to 10-cm plates. The cells were harvested 48 h post-transfection and assayed for CAT activity.

Lipopectamine 2000—5 × 105 log phase (unsynchronized) young and senescent (arrested) Hs68 fibroblasts were seeded in 6-well plates and treated with ~95% confluent. The cells were washed in PBS (pH 7.2), and the medium was replaced with OPTI-MEM (Invitrogen). Lipofectamine 2000/OPTI-MEM (Invitrogen) solution (250 µl) was incubated for 20 min with DNA/OPTI-MEM solution (250 µl). Immediately after incubation 500 µl was added to each well of the 6-well plate. Approximately 6 h later, the medium was replaced with Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. The cells were harvested using passive lysis buffer (Invitrogen) and assayed for luciferase expression using an EG & G Berthold luminometer.
plasmid was transformed into XL1-Blue competent cells, and the resulting plasmid was isolated and sequenced to confirm the mutations.

UV Cross-linking of DNA-Protein Complexes—To estimate the relative molecular mass of the DNA-binding proteins, binding reactions using various oligonucleotides as described for the band shift assays were performed. Twice the amount of labeled probe (40,000 cpm) and nuclear extract (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. The samples were irradiated by a 305-nm inverted UV transilluminator at 7 mW/cm² for 5 min. The cross-linked reactions were electrophoresed through 15% SDS-polyacrylamide gels, dried, and visualized by autoradiography.

Sequence Analysis—All of the sequence analyses were performed using the Wisconsin Package™ (version 9.1) from the Genetics Computer Group available through the Canadian Bioinformatics Resources website. Promoter sequences in the GenBank™ DNA sequence data base were obtained from the NCBI. The accession numbers identifying particular sequences are listed under “Results.” The sequences were searched for the 15-bp DIE using the FINDPATTERNS program, allowing for 33.3% (5 of 15) random mismatch. Further DIE-like element comparison was done using the PRETTY program to find those elements with highest sequence similarity to the DIE.

Dual Luciferase Reporter Assays—Constructs containing wild type and mutant DIE elements were made using pGL3 Control reporter plasmids (Promega). The 15-bp high pressure liquid chromatography-purified oligonucleotides were cloned into the HindIII site found between the SV40 promoter and the luc+ reporter gene. This cloning site is found outside of the pGL3 control multi-cloning site and was chosen because of its proximity to the functional gene (i.e., the in vivo DIE is located in the 5′-UTR of the cyclin D1 gene). Therefore, the DIE was cloned upstream of the luc+ start codon but downstream of the SV40 promoter (see Fig. 8A). These constructs were then co-transfected into young and old HDFs with a Renilla luciferase construct (pRL-TK) as a transfection efficiency control. Firefly luciferase expression was measured using an EG & G Berthold luminometer and normalized based on pRL-TK expression.

RESULTS

Removal of a 64-bp Region in the 5′-UTR of Cyclin D1 Abolishes Increased Promoter Activity in Old Cells—To determine the basal activity of the cyclin D1 promoter in young and old cells, a 1.3-kb cyclin D1 promoter fused to a CAT reporter gene (Fig. 1A) was used in transient transfection studies. Various 5′ deleted cyclin D1 promoter-CAT reporter constructs were also generated (Fig. 1B) and transiently co-transfected with a cyto-

![Candidate Transcriptional Repressor Element Identification](image)

**Fig. 1.** 5′-nested deletion analysis of the cyclin D1 promoter. A, schematic representation of several potential transcription factor binding sites in the cyclin D1 promoter. TRE, TPA response element; Egr1, immediate-early growth response gene; WT1, Wilms' tumor suppressor gene product; E2F, transcription factor E2F-binding site; Sp1, promoter-specific transcription factor; Oct1, octamer-like transcription factor binding site; E-box, insulin-responsive region. B, a series of 5′-nested deletions of the cyclin D1 promoter in pBluescript vector was generated by exonuclease III digestion, and the resulting deletions were subcloned into the pBLCAT3 vector for transient transfections into young and old fibroblasts (left panel). The bar graphs in the right panel represent the fold difference in CAT activity in old versus young cells after normalizing for transfection efficiencies by measuring activity from co-transfected β-galactosidase constructs. The results represent CAT activity from three independent transfections with the standard error indicated.

**Fig. 2.** 5′ and 3′ nested deletion analysis of the −23 to +138 region of the cyclin D1 promoter. A, 5′ and 3′ deletions of the cyclin D1 promoter (left panel) and the resulting CAT activity in young (black bars) and old (white bars) cells plotted after normalizing for β-galactosidase activity (right panel). B, fold difference in CAT activity in old versus young cells from A.
megalovirus-driven β-galactosidase construct (as internal control) into young and old cells. CAT activity caused by the full-length cyclin D1 promoter was 4–5-fold greater in old than in young cells (Fig. 1B), consistent with endogenous levels of cyclin D1 expression in those cells (7). Deletion of segments from the 5′ end from −1154 to −85 progressively reduced the expression difference between young and old cells to levels observed with the promoterless pBlcAt3 vector (Fig. 1B). However, the smallest deletion construct containing −23 to +138 of the cyclin D1 promoter retained 3–4-fold higher expression levels in old compared with young cells, indicating that this region is sufficient to confer increased expression in old cells.

We then generated further 5′ and 3′ nested deletions of the −23 to +138 region to further define the region involved in the differential regulation of cyclin D1. As shown in Fig. 2A, removal of the transcription initiation site resulted in approximately equivalent levels of inhibition of CAT activity in young and old cells. However, when 64 bp (+75 to +138) of the 5′-UTR were deleted, it resulted in a further 44% decrease in old cells, but the CAT activity in young cells remained relatively unchanged, thus reducing the fold difference in CAT activity to levels observed with the control vector (Fig. 2B, compare −23 to +138 with −23 to +74). Further removal of the 5′-UTR did not have any differential effect on CAT activity because it resulted in an approximately 77% decrease in young and an 82% decrease in old cells. These results indicate that together, other regions of the promoter are likely to contribute as strongly to the differential expression of cyclin D1 in young and old cells. The 64-bp sequence between +75 and +138 appeared to be a potential binding site for a transcriptional repressor in young cells and/or a transcriptional activator in old cells.

**Decreased DNA Binding Activity in Extracts of Old Cells to the +75 to +138 Region of the Cyclin D1 Promoter**—To determine whether any proteins could be detected binding to this 64-bp region, electrophoretic mobility shift assays with young and old cell nuclear extracts were performed. Specific complexes were formed with young cell extracts, whereas the levels were dramatically reduced with old cell extracts (Fig. 3A, lanes 2 and 3). Specificity of the complexes was confirmed by incubation with a 100-fold excess of unlabeled oligonucleotide, which effectively competed with the labeled probe (Fig. 3A, lanes 4 and 5), whereas a 100-fold excess of unlabeled, unrelated CRE oligonucleotide did not compete efficiently (Fig. 3A, lanes 6 and 7).

To rule out the possibility that the nuclear extracts from old Hs68 fibroblasts were deficient in their ability to bind DNA through some nonspecific mechanism, we performed gel shift studies using the same extracts with an oligonucleotide containing the CRE present in the c-fos promoter (36). As shown in Fig. 3B, CRE binding activity was actually about 50% higher in old cell extracts than in young (lanes 2 and 3). As expected, 100-fold competition with unlabeled oligonucleotide bearing the same CRE sequence competed with the complexes formed on the labeled CRE (Fig. 3B, lanes 4 and 5), whereas mutant sequences did not (Fig. 3B, lanes 6 and 7). Given previous reports that CRE binding activity is about equal in young and old cells, specific binding activity of the complex in Fig. 3A may actually be even more dramatically reduced in extracts from old compared with young cells (36).

Although extracts from young cells showed more binding activity than extracts from old cells, it was possible that reduced binding in old cells was due to cells exiting the cell cycle when senescent rather than to a senescence-specific event per se. For example, a clear passage-related up-regulation of cyclin D2 is seen in all strains of primary HDFs examined, but increased expression is also seen upon serum withdrawal or con-
Fig. 4. Identification of the DIE using overlapping oligonucleotides. A, a portion of the cyclin D1 5′-UTR showing a 64-bp region divided into five overlapping 22-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 old (O) cell nuclear extracts. Lanes 1–7 are control reactions in the absence of nuclear extract. Detection of complex formation was evident in young cell nuclear extracts and weaker in old cell extracts when using 5′-UTR-4 and 5′-UTR-5 as a probe (lanes 14–17), but signal was not detected when 5′-UTR-1 or 5′-UTR-3 was used as a probe (lanes 8, 9, 12, and 13). A very weak complex was detected preferentially in young cell extracts when 5′-UTR-2 was used as a probe (lanes 10 and 11). Complex formation on the 64-bp and control CRE oligonucleotide is also shown (lanes 18–21). C, the 5′-UTR-4 and 5′-UTR-5 oligonucleotides were used as probes for competition experiments either alone or with the other unlabeled probes at a 100-fold excess as indicated, to determine the specificity of binding.

Identification of a 15-bp DIE in the 5′-UTR with Overlapping Oligonucleotides—DNase I footprinting assays were attempted but did not prove useful in identifying sequences within the 64-bp region that were differentially protected using young and old cell nuclear extracts (data not shown). An alternative approach using overlapping oligonucleotides was undertaken in an attempt to better define the region involved in protein binding. As shown in Fig. 4A, five double-stranded 22-bp oligonucleotides designated 5′-UTR-1 to 5′-UTR-5 were synthesized and used in mobility shift assays using young and old cell nuclear extracts. No detectable binding activity was observed when the 5′-UTR-1 and 5′-UTR-3 oligonucleotides were used (Fig. 4B, lanes 8, 9, 12, and 13), whereas a very weak complex was detected in young cell extracts when the 5′-UTR-2 probe was used (lanes 10 and 11). Complexes were readily detected when using 5′-UTR-4 and 5′-UTR-5 as probes with young cell but not with old cell extracts (lanes 14–17). The migration of the complexes with these oligonucleotides was slightly slower than the migration of the complexes with the 64-bp oligonucleotide (lanes 18 and 19), probably reflecting the smaller charge-to-mass ratio with the shorter oligonucleotides. Complexes with the shorter oligonucleotides were also less readily detectable than those with the 64-bp oligonucleotide, perhaps reflecting stabilization of the complexes by peripheral sequences present in the 64-bp oligonucleotide. Because both 5′-UTR-4 and 5′-UTR-5 share a 15-bp overlap as shown in Fig. 4A, the region involved in protein binding was mapped to this site in the 5′-UTR that we have termed the cyclin D1 inhibitory element (DIE).

To determine whether this interaction was specific, the labeled 5′-UTR-4 and 5′-UTR-5 probes were incubated with a 100-fold excess of unlabeled 5′-UTR-4, 5′-UTR-5, the 64-bp oligonucleotide, or 5′-UTR-3 as a negative control. As shown in Fig. 4C, incubation with unlabeled oligonucleotides containing the DIE (lanes 3–8 and 13–18), but not the 5′-UTR-3 oligonucleotide (lanes 9, 10, 19, and 20) competed with the labeled probe for binding proteins, and the degree of competition varied when using different unlabeled probes.

Mutations within the DIE Abolish Binding Activity—To identify the bases within the DIE that were responsible for protein binding, we attempted to better define the region involved in protein binding by using overlapping oligonucleotides designated 5′-UTR-1 to 5′-UTR-5.
binding, oligonucleotides of 5'-UTR-4 containing various mutations (5'-UTR-4a to 5'-UTR-4d) were generated as shown in Fig. 5A. In each case, purines and pyrimidines were exchanged for noncomplementary pyrimidines and purines, respectively. Electrophoretic mobility shift assays of young and old cell nuclear extracts using the mutant 5'-UTR-4 oligonucleotides shown in A. Lanes 1-5 are control lanes in the absence of nuclear extracts. Introduction of 2 or 4-bp changes inhibited binding activity (lanes 8-13), whereas an 8-bp change within the DIE nearly abolished binding activity (lanes 14 and 15) compared with the wild type 5'-UTR-4 oligonucleotide (lanes 6 and 7).

FIG. 5. Mutations of oligonucleotides in the 5'-UTR-4 region. A, to determine the effect of various base pair changes on protein binding activity, four sets of oligonucleotides (5'-UTR-4a to 5'-UTR-4d) were synthesized and used in mobility shift assays. B, electrophoretic mobility shift assays with young and old cell nuclear extracts using the mutant 5'-UTR-4 oligonucleotides shown in A. Lanes 1-5 are control lanes in the absence of nuclear extracts. Introduction of 2 or 4-bp changes inhibited binding activity (lanes 8-13), whereas an 8-bp change within the DIE nearly abolished binding activity (lanes 14 and 15) compared with the wild type 5'-UTR-4 oligonucleotide (lanes 6 and 7).

Mutation of the DIE Increases Cyclin D1 Promoter Activity in Young Cells—An 8-bp change in the full-length cyclin D1 promoter-CAT reporter construct from +117 to +131, corresponding to the mutations in 5'-UTR-4d, was introduced by site-directed mutagenesis. The 1.3-kb full-length cyclin D1 promoter-CAT reporter construct and the mutated construct were transfected into young and old cells, and the CAT activity resulting from each construct was measured after normalizing for β-galactosidase activity. As shown in Fig. 6A, mutation of the DIE in the 5'-UTR resulted in nearly a doubling of CAT activity in young cells relative to old cells, compared with the activity of the wild type construct. In contrast, the CAT activity

FIG. 6. CAT activity in young versus old cells using a cyclin D1 promoter containing a mutant DIE. The 8-bp change corresponding to 5'-UTR-4d was introduced into the full-length cyclin D1 promoter by site-directed mutagenesis, and the construct was transfected into young and old cells. A, the graph represents CAT activity from young (black bars) and old (overlapping white bars) cells after normalizing for transfection efficacy using co-transfected β-galactosidase construct. B, results from A plotted as fold difference in CAT activity in old versus young cells. An average of three independent transfections were performed with the standard deviations indicated by error bars.
from the mutant construct was similar to that from the wild type construct in old cells. Fig. 6B shows the results of the same experiment plotted as fold difference in CAT activity in young versus young cells. Mutation of the DIE resulted in a nearly 50% decrease in the fold difference in CAT activity compared with the unmutated control. These results suggest that the DIE within the 5'-UTR of cyclin D1 constitutes a binding site for a potential transcriptional repressor in young cells, the activity and/or levels of which are dramatically reduced in old cells.

Low Molecular Mass Proteins Bind the DIE—To estimate the molecular mass of any protein(s) that bound specifically to the DIE, nuclear extracts from young and old cells were incubated with 5'-UTR-4, 5'-UTR-5, or the 64-bp oligonucleotide and were subjected to UV cross-linking. Following resolution by SDS-PAGE, complexes were apparent in young cell extracts that were decreased in old cell extracts (Fig. 7). Three different complexes were formed with the 5'-UTR-4, suggesting that this sequence might contain a more complete binding site(s) than 5'-UTR-5, with which only two complexes were seen (compare lane 4 with lane 9). Three complexes were also apparent with the 64-bp oligonucleotide (lanes 14 and 15). After subtraction of the molecular mass of the labeled probe (i.e., 649 Da/bp), an estimated molecular mass range of 20–45 kDa was calculated for proteins bound to both the 22- and 64-bp oligonucleotides, depending upon the amount of DNA protected by UV cross-linking.

The DIE Preferentially Inhibits Gene Expression in Young Fibroblasts—To test the activity of the 15-bp wild type and mutant, DIE elements in luciferase reporter containing these elements (Fig. 8A) were transfected into young and senescent Hs68 fibroblasts. Transfection efficiencies of 55 and 40% in young and senescent fibroblasts, respectively, were obtained using LipofectAMINE 2000. Transfection efficiencies were normalized based on Renilla luciferase (pRL-TK) expression.

As shown in Fig. 8B, young HDFs showed more than 80% reporter inhibition by the wild type DIE compared with the mutant element, whereas senescent HDFs were inhibited ~30%. Therefore, although the DIE element had an inhibitory effect upon transcription in both young and senescent cells, the inhibitory effect was markedly greater in senescent cells, even though the assay is done with a single DIE element (compared with several seen in most genes) outside the context of the native cyclin D1 promoter.

Identification of DIE-like Elements in Other Genes Up-regulated during Cellular Aging—We next examined whether sequences similar to the DIE were present in promoters of other genes up-regulated during cellular senescence. Well characterized genes whose expression is increased in senescent cells include 1) plasminogen activator inhibitor type-1 (38, 39), 2) insulin-like growth factor binding protein-3 (12), 3) p14ARF (40), 4) ING1 (41), 5) NF-κB (42, 43), and 6) osteonectin (44). As controls, the “housekeeping” genes glyceraldehyde-phosphate dehydrogenase expressed similarly in young and senescent fibroblasts, (10) and phenylalanine tRNA synthetase, whose expression decreases during cellular senescence (45), were used. The 15-bp DIE was searched for in the promoters of these genes using the FINDPATTERNS program (see “Experimental Procedures”). As shown in Fig. 9A, sequences with similarity to the DIE were found to be clustered within 400-bp of the transcription initiation site (often within the 5'-UTR) of the genes that are up-regulated in aging cells, whereas the frequency of DIE-like sequences in the control genes was much lower. Alignment of the DIE with the sequences detected in the other promoters showed the presence of six perfectly conserved bases in the group of genes up-regulated during cellular senescence, whereas only two bases were conserved in the controls (Fig. 9B). Furthermore, p14ARF had a second DIE that was very well conserved. Taken together, these results suggest that the regulatory function of the DIE may not be restricted to cyclin D1 gene expression but also to a selected number of genes that are overexpressed in senescent cells.

**DISCUSSION**

In this study, a region has been identified in the cyclin D1 5'-UTR that strongly contributes to selectively repressing cyclin D1 gene expression in young cells. Although deletion of most cyclin D1 promoter sequences affected reporter gene expression to a similar extent in young and old cells, deletion of a 64-bp sequence from +75 to +138 of the 5'-UTR selectively reduced expression in old cells. By using overlapping oligonucleotides corresponding to fragments of the 64-bp region, the protein-binding region was further narrowed to a 15-bp sequence that we have termed the DIE. Mutation of particular bases within the DIE reduced or abolished complex formation and selectively up-regulated cyclin D1 promoter activity in young cells. Luciferase reporter constructs effectively demonstrate the ability of this element to differentially regulate transcription in young versus senescent HDFs and gel shift assays suggest that protein(s) ranging from 20 to 45 kDa specifically bind the DIE. Finally, sequence similarity comparisons revealed that sequences with homology to the DIE were also found clustered in the promoters of a subset of genes whose expression is also up-regulated in aging cells at a frequency higher than seen in control genes whose expression is unaffected or reduced by replicative senescence.

The age-related increase in cyclin D1 transcripts observed in old cells could be the result of specific transcriptional and/or post-transcriptional regulation. Our observations suggested that δ-type cyclin transcripts are similarly stable over extended time courses (4- to 16-h) in both young and old cells (data not shown), suggesting that age-related up-regulation of these transcripts occurs at the level of transcription.

Mutation of the DIE between +117 to +131 increased cyclin D1 promoter activity in young cells but not to the levels seen in old cells. Thus, other regions of the cyclin D1 promoter are also likely to contribute to age-related expression. In fact, differential DNA binding activity in young and old cells has been reported for various regions of the cyclin D1 promoter (8),
although their contribution to gene expression is unknown. Interestingly, the sequence similarity comparison revealed two additional DIE-like sequences within the cyclin D1 5'-UTR (Fig. 9). This included a sequence with limited homology within the 5'-UTR-2 oligonucleotide, with which weak complex formation was observed in mobility shift assays (Fig. 4). Based on our results, it appears that specific interactions of the repressor are stabilized by multiple copies of the DIE such that mutation of one can only partly reverse cyclin D1 gene repression in young cells. This would be consistent with the stronger binding seen using the 64-bp versus the shorter 22-bp oligonucleotides.

Although regulation of gene expression via the 5'-UTR has not been widely reported, such mechanisms might act to repress transcription more commonly than previously thought. For example, p53 suppresses the expression of bcl-2 at least partly through a p53 response element located in the 5'-UTR of the bcl-2 gene (46). Similarly, a suppressor element has been identified in the 5'-UTR of the androgen receptor gene (47). Although the exact mechanisms operating to repress transcription are unknown, the results presented here raise the possibility that other genes that are up-regulated during cellular senescence could be regulated by a mechanism similar to if not identical with the DIE identified here. Indeed, comparison of the DIE with a selected number of genes up-regulated during cellular aging revealed the presence of DIE-like sequences in their 5'-UTRs and the absence of these elements in control

Fig. 8. Dual luciferase reporter assays using wild type and mutant DIE sequences. 26-bp sequences containing wild type or mutant forms of the 15-bp DIE were cloned into pGL3-control firefly luciferase reporter plasmids in the sense orientation. The assays show differential inhibition by the wild type versus mutant DIE on luc+ expression. Young HDFs show more than 80% reporter inhibition, whereas senescent HDFs show less than 30% inhibition. Mutant constructs show little to no inhibition of reporter expression.
Candidate Transcriptional Repressor Element Identification

Fig. 9. DIE elements in the promoter regions of other genes transcriptionally up-regulated during replicative senescence. A, schematic representation of the promoter regions of genes well established to be up-regulated with in vitro age. The white boxes indicate all DIE-like elements with at least 66.6% (10 of 15) homology to the DIE. The black boxes indicate the DIE-like elements most similar to the cyclin D1 DIE that are expanded in B. B, DIE-like elements from the 5′-UTR of the indicated genes from A showing the closest sequence homology to the cyclin D1 DIE.

genes. This raises the possibility that the expression of a group of age-related genes is coordinately regulated by one principal mechanism and that it may be possible to influence the expression of many genes by targeting a single repressor element or protein.

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REFERENCES
1. Haflick, L. (1965) Exp. Cell Res. 37, 614–636
2. Haflick, L., and Moorhead, P. S. (1961) Exp. Cell Res. 25, 585–621
3. Goldstein, S. (1990) Science 249, 1129–1133
4. Cristofalo, V. J., Volker, C., Fires, M. K., and Tresini, M. (1998) Crit. Rev. Eukaryotic Gene Expression 8, 43–80
5. Dumri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linkevich, M., Rubelj, I., Pereira-Smith, O., Peacecke, M., and Campisi, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9369–9377
6. Dulic, V., Drulliger, L. F., Lies, E., Reed, S. I., and Stein, F. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11034–11038
7. Atadja, P., Wong, H., Veillette, C., and Riabowol, K. (1995) Exp. Cell Res. 217, 205–216
8. Fukami-Kobayashi, J., and Mitani, Y. (1998) Exp. Cell Res. 241, 435–444
9. Noda, A., Ning, Y., Venable, S. P., Pereira-Smith, O. M., and Smith, J. R. (1994) Exp. Cell Res. 211, 90–98
10. Wang, H., and Riabowol, K. (1996) Exp. Gerontol. 31, 311–325
11. Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. (1996) Mol. Cell. Biol. 16, 859–867
12. Moerman, E. J., Thwaitt, R., Moerman, A. M., Jones, R. A., and Goldstein, S. (1993) Exp. Cell Res. 28, 361–376
13. Parr, E., Tousa, T., Hull, C., Meyyappan, M., Bestliny, L., Wheaton, K., and Riabowol, K. (1998) Curr. Sci. 74, 878–883
14. Matsushima, H., Ewen, M. E., Strom, D. K., Kato, J. Y., Hanks, S. K., Rousell, M. F., and Sherr, C. J. (1992) Cell 71, 225–234
15. Meyerson, M., and Harlow, E. (1994) Mol. Cell. Biol. 14, 2077–2086
16. Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999) Cell 98, 59–69
17. Geng, Y., Whorsley, W., Park, M. Y., Bronson, R. T., Medema, R. H., Li, T., Weinberg, R. A., and Sicinski, P. (1999) Cell 97, 767–777
18. Geng, Y., Yu, Q., Sicinski, E., Das, M., Bronson, R. T., and Sicinski, P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 194–199
19. Sicinski, P., Donaher, J. L., Parker, S. B., Li, T., Fazeli, A., Gardner, H., Haslam, S. Z., Bronson, R. T., Elledge, S. J., and Weinberg, R. A. (1995) Cell 82, 621–630
20. Pagano, M., Theodoras, A. M., Tam, S. W., and Draetta, G. R. (1994) Genes Dev. 8, 1627–1639
21. Lammie, G. A., Fantl, V., Smith, R., Schuuring, E., Brookes, S., Michalides, R., Dickson, C., Arnold, A., and Peters, G. (1991) Oncogene 6, 439–444
22. Han, E. K., Begemann, M., Spagnato, A., Sch, J. W., Doki, Y., Xing, W. Q., Liu, W., and Weinberg, R. A. (1998) Cell Growth Differ. 9, 689–710
23. Han, E. K., Spagnato, A., Jiang, W., Zhang, Y. J., Santella, R. M., Doki, Y., Cacace, A. M., Schieren, I., and Weinstein, I. B. (1995) Oncogene 10, 953–961
Candidate Transcriptional Repressor Element Identification

24. Meyyappan, M., Wong, H., Hull, C., and Riabowol, K. T. (1998) Mol. Cell. Biol. 18, 3163–3172
25. Wilhide, C. C., Van Dang, C., Dipersio, J., Kenedy, A. A., and Bray, P. F. (1995) Blood 86, 294–304
26. Burger, C., Wick, M., and Muller, R. (1994) J. Cell Sci. 107, 2047–2054
27. Horiguchi-Yamada, J., Yamada, H., Nakada, S., Ochi, K., and Nemoto, T. (1994) Mol. Cell Biochem. 132, 31–37
28. van Grunsven, L. A., Thomas, A., Urdiales, J. L., Machenaud, S., Choler, P., Durand, I., and Rudkin, B. B. (1996) Oncogene 12, 855–862
29. Yan, G. Z., and Ziff, E. B. (1995) J. Neurosci. 15, 6209–6212
30. Freeman, R. S., Estus, S., and Johnson, E. M., Jr. (1994) Neuron 12, 343–355
31. Fukami, J., Anno, K., Ueda, K., Takahashi, T., and Ide, T. (1995) Mech. Ageing Dev. 81, 139–157
32. Herber, B., Truss, M., Beato, M., and Muller R. (1994) Oncogene 9, 2105–2107
33. Motokura, T., and Arnold, A. (1993) Genes Chromosomes Cancer 7, 89–95
34. Severino, J., Allen, R. G., Balin, S., Balin, A., and Cristofalo, V. J. (2000) Exp. Cell Res. 257, 162–171
35. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
36. Atadja, P. W., Stringer, K. F., and Riabowol, K. T. (1994) Mol. Cell. Biol. 14, 4991–4999
37. Riabowol, K., Schiff, J., and Gilman, M. Z. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 157–161
38. Goldstein, S., Moerman, E. J., Fuji, S., and Sobel, B. E. (1994) J. Cell. Physiol. 161, 571–579
39. West, M. D., Shay, J. W., Wright, W. E., and Linskens, M. H. (1996) Exp. Gerontol. 31, 175–193
40. Wei, W., Hemmer, R. M., and Sedivy, J. M. (2001) Mol. Cell. Biol. 21, 6748–6757
41. Garkavtsev, I., and Riabowol, K. (1997) Mol. Cell. Biol. 17, 2014–2019
42. Supalcor, P. C., Jang, M. H., Song, C. S., Chatterjee, B., and Roy, A. K. (1995) J. Biol. Chem. 270, 837–842
43. Smith, J. R., and Pereira-Smith, O. M. (1989) Genes Chromosomes Cancer 31, 386–389
44. Murano, S., Thweatt, R., Shmookler Reis, R. J., Jones, R. A., Moerman, E. J., and Goldstein, S. (1991) Mol. Cell. Biol. 11, 3905–3914
45. Lee, C. K., Klopp, R. G., Weinrich, R., and Prolla, T. A. (1999) Science 285, 1390–1393
46. Miyashita, T., Harigai, M., Hanada, M., and Reed, J. C. (1994) Cancer Res. 54, 3131–3135
47. Grassmann, M. E., Lindzey, J., Kumar, J., and Tindall, D. J. (1994) Mol. Endocrinol. 8, 448–455