Human Fibroblasts. Irradiation, and Its Regulation Is Impaired in Senescent Human Fibroblasts

Cells undergoing replicative senescence display an altered pattern of gene expression. Senescent fibroblasts show significant changes in the expression of mRNAs encoding extracellular matrix-remodeling proteins; among these mRNAs, the mRNA encoding fibromodulin is highly decreased in these cells. To understand the molecular basis of this phenomenon, we explored the regulatory mechanisms of the human fibromodulin gene. We found that fibromodulin gene promoter contains a cis-element, crucial for its basal expression, that forms a DNA-protein complex when exposed to nuclear extracts from exponentially growing human fibroblasts and not to extracts from cells undergoing senescence by repeated in vitro passages or by mild oxidative stress.

The purification of this complex showed that it contains the damage-specific DNA-binding protein DDB-1. The latter is known to be induced by UV irradiation; therefore we checked whether fibromodulin gene promoter is regulated upon the exposure of the cells to UV rays. The results showed that, in exponentially growing fibroblasts, the promoter efficiency is increased by UV irradiation and the DDB-1-containing complex is robustly enriched in cells exposed to UV light. Accordingly, in these experimental conditions the endogenous fibromodulin mRNA accumulates to very high levels. On the contrary, senescent cells did not show any activation of the fibromodulin gene promoter, any induction of the DDB-1-containing complex, or any accumulation of fibromodulin mRNA. These phenomena are accompanied in senescent cells by a decrease of the UV-damaged DNA binding activity.

Normal human cells are not able to grow indefinitely in culture, and after a number of cell divisions, usually 50–100 in vitro, they stop growing and undergo a condition known as replicative senescence. The presence and the role of senescent cells in vivo remain to be definitively addressed; however, there is a significant interest in the study of this cellular phenotype for at least two reasons: i) accumulation of senescent cells in the tissues of living organisms could have deleterious effects, actually favoring the development of age-related dysfunctions and diseases, and ii) cellular senescence could represent a strategy adopted by the cells to prevent the development of malignancies. Senescent cells have a well defined microscopic and molecular phenotype characterized by enlarged and flattened shape, expression of β-galactosidase activity, shortened telomeres, and a specific gene expression profile.

The mechanisms underlying the changes of gene expression profile accompanying replicative senescence are not completely understood. Senescent human fibroblasts show significant differences, compared with early passage cells, in the expression of genes encoding matrix-remodeling proteins and growth factors and cytokines involved in wound healing. Among these genes, stromelysin 1 and 2, plasminogen activators 1 and 2 and urokinase plasminogen activator, and metalloelastase 4, 5 are overexpressed, whereas elastin, stromelysin 3, and prostaglandin-1 synthase are suppressed. Similarly, fibromodulin (FM) mRNA was found to be significantly underexpressed in human fibroblasts undergoing senescence by repeated in vitro passages or mild oxidative stress and in postmitotic fibroblasts. Although experimental evidence suggests that the observed up-regulation of genes expression is due to the stabilization of APA-1 transcription factor in senescent cells, the mechanisms underlying the observed gene down-regulation are not understood.

FM belongs to the family of leucine-rich proteoglycans, which regulates the assembly of extracellular matrix proteins. FM binds to collagen and is involved in the generation of collagen fibrils.

Received for publication, December 30, 2004, and in revised form, July 1, 2005 Published, JBC Papers in Press, July 6, 2005, DOI 10.1074/jbc.M414677200

Maria Assunta Bevilacqua, Barbara Iovine, Andrea Scaloni, Tommaso Russo, and Filiberto Cimino

From the Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, CEINGE Biotecnologie avanzate, 80131 Napoli, Italy and Proteomics and Mass Spectrometry Laboratory, ISPAAM, Consiglio Nazionale delle Ricerche, 80147 Naples, Italy

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To whom correspondence may be addressed: CEINGE Biotecnologie avanzate, via Comunale Margherita 482, 80145 Napoli, Italy. Tel.: 390813722806; Fax: 390813722808; E-mail: russo@dbbm.unina.it.

To whom correspondence may be addressed: Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, via S. Pansini 5, 80131 Napoli, Italy. E-mail: cimino@dbbm.unina.it.

1 The abbreviations used are: FM, fibromodulin; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; CSA, Cockayne syndrome protein A; DDB, damage-specific DNA-binding protein; DEM, diethylmaleate; EMSA, electrophoretic mobility shift assay; HF-28, IMR-90 fibroblasts at 28 PDL; HF-60, IMR-90 fibroblasts at 60 PDL; PDL, population doubling level; dsRNA, double-stranded RNA; siRNA, small interfering RNA; GFP, green fluorescent protein.
senescence-associated days of culture in the presence of DEM, the cells stopped growing and treated solution of DEM in 10 ml of freshly prepared medium. After 3 positive for senescence-associated (HF-28 cells) or at 60 PDL (HF-60 cells). More than 50% of HF-60 were number of cells at each passage (21). The cells used were at 28 PDL

is induced by UV irradiation. Experimental evidence indicates that the machinery responsible for the basal transcription of the SPT3-TAF(II)31-GCNSL acetylase complex (17), and to be part transcription regulation. In fact, the DDB dimer was demonstrated to act as a co-factor of the E2F1 transcription factor (16), as

DDB-1 and DDB-2 have been proposed to play a role in trans-

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ponent of the damage-specific DNA-binding heterodimeric complex DDB (12–14). It is involved in nucleotide excision repair of the global genome repair, and the DDB-

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(80 to +20), FP-CAT5 (122 to +20), and FP-CAT6 (122 to –42) were obtained by PCR using the FP-CAT1 as template. In FP-CAT5 and FP-CAT6, a region of 50 bp located upstream from the transcription start site of the herpesvirus thymidine kinase gene precedes the CAT gene. FP-CAT6mut vector was generated by PCR using the same for- ward oligonucleotide as for FP-CAT6 and a reverse primer containing four mismatches corresponding to the mutations present in the oligonucleotides m4 and m8 (see below).

cDNA encoding human DDB-1 was obtained by retrotrans- scribing total RNA from IMR-90 cells. It was then cloned into the pRC-CMV vector. Cloned cDNA was completely sequenced. The RNA

interference experiments were performed by using two dsRNA: iDDB-1, 5'-CCCCUUUGAGAGACCUCUA-3', and iGFP, 5'-GAAAGCUGCCCUUGAGGUCAU-3'. Conditions of the siRNA experiments were as described previously (23).

Cell transfections were carried out with Lipofectamine-plus (Invitro- gen) in 60-mm dishes, according to the manufacturer's instructions. Each transfection was performed in quadruplicate with 2 μg of plasmid DNA plus 0.2 μg of CMV-luciferase vector, to correct for variations in DNA uptake and transfection efficiency. CAT gene expression was assessed by measuring the CAT protein levels by enzyme-linked immu-
nosorbent assay (Roche Applied Science).

Nuclear Extracts, EMSAs, and Purification Procedure—Nuclear extracts were prepared from IMR-90 and HeLa cells as described previously (24). EMSAs were carried out as described previously (25). The nucleotide sequences of the probes and of the competitor DNA were as follows: B1, 5'-GGGTTCTCTCAGCCCTTTTTCAC-3'; B2, 5'-ACAATAT-TTGAATGATTTGGGC-3'; B3, 5'-GAATTTGGCCGCGAGGCAC-3'; B4, 5'-CAGGAAGACCTCAGTGACTCT-3'; B5, 5'-CACCCGCACCTCAGTGACTCT-3'; and CTF, 5'-TTT-ATTTTGGATGAACCACATATGATAA-3'.

The EMSA binding to the B4 oligonucleotide were purified from HeLa cell extracts in 5 ml of buffer A (50 mM Hepes, pH 7.9, 12.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 17% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) as described previously (26). Brieﬂy, 50 mg of HeLa nuclear extracts in buffer A were loaded on a DEAE column (2 ml bed volume) (Amersham Biosciences). Retained proteins were eluted by step-increasing the concentration of KCl from 100 to 500 mM. The 200 μM KCl active fraction (7.6 mg) was diazylated against buffer 2 (25 mM Hepes, pH 7.6, 100 mM KCl, 12.5 mM MgCl2, 1 mM dithiothreitol, 20% (v/v) glycerol, 0.1% (v/v) Nonidet P-40), challenged with 400 μg of competitor (poly(dI-dC), Sigma), and loaded on a DNA affinity column constructed with the multimeric B4 oligonucleotide covalently linked to CNBr-activated Sepharose. The retained sample was eluted with increasing KCl concentration (from 250 mM to 1.5 M).

The active fraction was concentrated on Centricron filters (3-kDa cut-off), resolved by SDS/PAGE (12% (w/v) gel), and silver-stained with a commercially available kit (Sigma). The bands were excised from the gel and digested with trypsin, and the peptide mixture was analyzed by mass-assisted laser desorption ionization time-of-flight mass spectrometry as reported previously (22).

For Western blot analyses, total extracts from IMR-90 cells were challenged with antibodies against DDB-1, DDB-2, CSA, and tubulin (Santa Cruz Biotechnology), and the signals were detected using the ECL kit (Amersham Biosciences).

UV Treatment and Real-Time PCR—1.0 × 106 fibroblasts were seeded onto 100-mm culture plates in 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After incubation for 4 days at 37 °C in 5% CO2, the medium was replaced with 1 ml of cold phosphate-buffered saline, and the monolayer of subconfluent cells was irradiated with 15 J/m2 of UV light (254 nm). The phosphate-buffered saline was then replaced with 10 ml of fresh culture medium, and the cells were allowed to recover for 12–72 h. Total RNA was prepared from IMR-90 cells by using the RNeasy mini kit (Qiagen) and subjected to cDNA synthesis with random hexanucleotide primers and MultiScribe reverse transcriptase (Invitrogen) at 48 °C for 1 h. The

MATERIALS AND METHODS

Cell Cultures—IMR-90 human primary fibroblasts and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen). Cultures were maintained at 37 °C in a 5%

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RESULTS

Transcription from Fibromodulin Gene Promoter Is Decreased in Senescent Fibroblasts—We have observed previously that human senescent fibroblasts express low levels of FM mRNA (6). To ascertain whether this phenomenon is due to a decreased transcription of the FM gene, we generated a reporter construct (FP-CAT1) in which the CAT gene is under the control of about 2,000 bp flanking the transcription start site of the FM gene cloned upstream from the CAT gene. In FP-CAT5 and FP-CAT6 the CAT gene is preceded by the transcription start site of the human thymidine kinase gene (mesh pattern). The FP-CAT6mut vector is identical to FP-CAT5 but for four nucleotide changes contained in the oligonucleotides m4 and m8 reported in Fig. 2. B, the vectors reported in A (from FP-CAT1 to FP-CAT6) were assayed for their transcription in HF-28, HF-60, and HF-DEM cells, as indicated. White bars refer to the transcription efficiencies of the various reporter vectors reported as percentages of the mean values obtained in HF-28 cells transfected with the FP-CAT1 vector. Black bars refer to the control experiments in which the efficiency of the CMV-CAT vector, transfected in the three different cell types, is reported as percentages of the mean value of transcription of this vector in HF-28 cells. All of the results are mean values ± S.D. of triplicate experiments. C, β-galactosidase staining of HF-28, HF-60, and HF-DEM performed as reported under “Materials and Methods.”
the human FM gene (see Fig. 1A). As shown in Fig. 1B, the reporter gene in this construct is efficiently transcribed in exponentially growing IMR-90 fibroblasts at 28 PDL (HF-28 cells). The same construct was transfected in IMR-90 fibroblasts grown in culture for several passages thus reaching 60 PDL (HF-60 cells) and showing the phenotype of senescent cells, including the expression of β-galactosidase in more than 50% of the cells. As shown in Fig. 1B, the transcription of the FP-CAT1 vector transfected in HF-60 cells was dramatically suppressed, compared with the transcription efficiency observed in HF-28 cells, whereas that of CMV-CAT was very similar to that observed in HF-28 cells.

Another method to induce the senescence in cultured cells is based on the exposure of cells to a mild oxidative stress (29). Therefore, HF-28 cells were treated for 3 days with 100 μM DEM (HF-DEM cells), and this treatment resulted in the arrest of the growth and the appearance of β-galactosidase staining. Also in this case, although the efficiency of the CMV promoter was the same in HF-28 and HF-DEM cells, the results reported in Fig. 2B clearly show that the transcription from the promoter in the FP-CAT1 vector is suppressed in both the HF-28 and the HF-DEM cells.

To identify the minimal promoter region still able to drive an efficient transcription of the reporter gene in HF-28 cells and not in HF-60 and HF-DEM cells, we generated a collection of 5′-deletion mutants of the promoter region (see Fig. 1A). As shown in Fig. 1B, the deletion of a great part of the 2,000 bp flanking the transcription start site at the 5′ terminus, down to position −122 (FP-CAT2 and FP-CAT3), did not modify the promoter efficiency, whereas a further deletion down to nucleotide −54 (FP-CAT4) resulted in a promoter that drives a weak transcription to the same extent in HF-28, HF-60, and HF-DEM cells. Similarly, we have deleted the region encompassing the transcription start site, and the obtained fragments were cloned upstream from the transcription start site of herpesvirus thymidine kinase gene (FP-CAT5 and FP-CAT6). Fig. 1A shows that the fragment from −122 to −42 contains a promoter element that supports the transcription in HF-28 and not in HF-60 or HF-DEM cells.

**DDB-1 Protein Interacts with the Fibromodulin Gene Promoter**—To identify the cis-element(s) that is contained in the −122 to −42 region of the FM gene promoter and that is possibly responsible for the observed decline of transcription efficiency in senescent cells, five overlapping double-stranded oligonucleotides covering this region were tested by EMSA for their ability to bind proteins present in the nuclear extracts from HF-28 and HF-60 cells. As shown in Fig. 2B, three of these oligonucleotides (B1, B3, and B5) form specific shifted complexes with either HF-28 or HF-60 nuclear extracts. The oligonucleotide B4, which partly overlaps B3 and B5 sequences, forms a DNA-protein complex when challenged with HF-28 nuclear extracts. On the contrary, this complex was barely visible when the nuclear extracts from HF-60 cells were used.

To better characterize the cis-element involved in the formation of the complex, we generated a collection of mutant B4 oligonucleotides. Fig. 2C shows that two of these mutant oligonucleotides (m4 and m8) were unable to compete for the formation of the complex, thus suggesting a sequence-specific binding. On this basis, the FP-CAT6 construct (see Fig. 1A) was mutagenized through the insertion of the nucleotide changes present in the m4 and m8 oligonucleotides. This vector was transfected in HF-28, HF-60, and HF-DEM cells, and, as shown in Fig. 2D, the mutant vector drives low levels of transcription both in HF-28 and in HF-60 and HF-DEM cells. These results support the possibility that the region of the FM gene promoter covered by the B4 oligonucleotide contains a specific cis-ele-

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**FIG. 2.** The FM gene promoter contains a cis-element that forms a specific complex when challenged with HF-28 nuclear extracts. A, schematic representation of the oligonucleotides covering the −122 to −42 region of the FM gene. For the B4 oligonucleotide the complete nucleotide sequence is reported along with the nucleotide changes present in the oligonucleotides from m1 to m8 used in the competition assays. B, wild type. B, the oligonucleotides from B1 to B5 were challenged in EMSA experiments with the nuclear extracts of HF-28 and HF-60 cells. Specific shifted bands are reported along with specific competitions (s.c.) with a 50-fold molar excess of the wild type B4 oligonucleotide. The B4 oligonucleotide was also challenged with nuclear extracts from HeLa cells. The CTF/NF1 oligonucleotide (see under “Materials and Methods”) was used as a control of the quality and protein concentration of the nuclear extracts used in the experiments mentioned in this legend. C, a 50-fold molar excess of the oligonucleotides from m1 to m8 (see A) was used as competitor in the gel shift experiment with labeled B4 oligonucleotide. In the second lane (B4) the competition with the unlabeled wild type B4 oligonucleotide is shown, and the third lane (m5) reports the competition with an unrelated oligonucleotide. D, the FP-CAT6 and FP-CAT6mut vectors were transfected in HF-28, HF-60, and HF-DEM cells. The bars indicate the transcription efficiencies as percentages of the mean value obtained by transfecting FP-CAT6 in HF-28 cells. All of the results are mean values ± S.D. of triplicate experiments.
experiments with the B4 oligonucleotide, as described under “Materials and Methods.” The fraction eluted with 0.5 M KCl and containing an activity that shifts the B4 oligonucleotide was examined for the specificity of the shift with 

The results show that antibodies directed against DDB-1 or DDB-2 and CSA. The fraction was analyzed by SDS-PAGE, the silver staining of which is reported in Fig. 3B. The bands were excised from the gel, digested with trypsin, and analyzed by mass spectrometry. Peptide mass fingerprint analysis with 12 of 15 measured tryptic fragments allowed us to identify one band of about 120 kDa as the DDB-1. We were unable to identify the other bands present in the 0.5 M KCl fraction; mass spectrometry analysis suggests that some of them could have been DDB-1 degradative products.

To confirm that DDB-1 is present in the protein-DNA complex responsible for the electrophoretic shift of the oligonucleotide B4, we did the supershift experiments reported in Fig. 3D. The results show that antibodies directed against DDB-1 or against the two known partners of this protein, i.e., DDB-2 and CSA, did not induce any evident supershift. On the contrary, when we challenged the nuclear extract with a combination of anti-DDB-1 and anti-DDB-2 antibodies, a clear supershift was observed, whereas no supershift appeared with the combination of anti-DDB-1 and anti-CSA antibodies. No specific band shift was observed in control experiments in which the oligonucleotide B4 was challenged with the above mentioned antibodies without nuclear extracts (see supplemental Fig. 1).

DDB-1 Is Necessary for the Basal Transcription from the FM Gene Promoter—To ascertain whether DDB-1 is involved in the regulation of the FM gene promoter, we overexpressed this protein in IMR-90 cells. As shown in Fig. 4A, this overexpression significantly activated the transcription of the FM gene promoter in the FP-CAT3 vector and not of the FP-CAT4 vector.

We then tested the effect of the suppression of the endogenous DDB-1 by using an siRNA targeting the DDB-1 mRNA. We observed that a 50% decrease of the DDB-1 protein concentration in transfected cells was accompanied by a significant reduction of the transcriptional efficiency of the FM gene promoter in the FP-CAT3 vector and not of the CMV-CAT vector. A control siRNA, which suppresses the expression of enhanced GFP had no effect on the FP-CAT3 transcription (see Fig. 4B).

Nuclear extracts from cells overexpressing DDB-1 or transfected with siRNA targeting the DDB-1 mRNA were analyzed by EMSA. As shown in Fig. 4C, DDB-1 overexpression resulted in an increased intensity of the shifted band, whereas DDB-1

Fig. 3. Purification of the proteins interacting with the cis-element present in the FM gene promoter. A, nuclear extracts from HeLa cells were fractionated on a DEAE column. Load, flow-through (f.t.), and three fractions eluted with 0.1, 0.2, and 0.5 M KCl were used in gel shift experiments with the B4 oligonucleotide, as described under “Materials and Methods.” B, the fraction eluted from the DEAE column with 0.2 M KCl and that had the ability to shift the B4 oligonucleotide was affinity-chromatographed on a Sepharose column bearing the multimerized B4 oligonucleotide. The fractions eluted with various concentrations of KCl (from 0.25 to 1.5 M) were analyzed by EMSA with the B4 oligonucleotide. The fraction eluted with 0.5 M KCl and containing an activity that shifts the B4 oligonucleotide was examined for the specificity of the shift with a 50-fold molar excess of the unlabelled B4 oligonucleotide (s.c.) or of an Sp1 site-containing oligonucleotide (n.s.c.). C, four fractions eluted from the affinity chromatography described in B were concentrated and run on SDS-PAGE as described under “Materials and Methods.” The arrow indicates the 120-kDa band present in the 0.5 M lane identified by mass spectrometry as DDB-1. D, nuclear extracts from HeLa cells were analyzed by EMSA with the B4 oligonucleotide. The extracts were preincubated as indicated with unlabelled B4 (s.c.) or Sp1 site-containing (n.s.c.) oligonucleotides, with mouse IgG (m IgG), with antibodies against DDB-1, CSA, or DDB-2, or with antibodies against a combination of DDB-1 and DDB-2 or of DDB-1 and CSA. The arrowhead indicates the specific shifted complex; the arrowhead plus the asterisk indicates the super-shifted band.

ment that interacts with the transcription factor(s) responsible for the different efficiency of the promoter in exponentially growing cells compared with senescent cells.

Considering that a robust shifted complex was observed when the B4 oligonucleotide was challenged with HeLa cell nuclear extracts (see Fig. 2A), we decided to use the latter as a source to purify the protein(s) present in the shifted complex. Nuclear extracts from HeLa cells were chromatographed on a DEAE column, as described under “Materials and Methods.” The fraction eluted with 0.2 M KCl was able to shift the B4 oligonucleotide (see Fig. 3A). A further purification was obtained by affinity chromatography on a Sepharose column bearing the multimerized B4 oligonucleotide. As shown in Fig. 3B, the factor was eluted from the column with 0.5 M KCl. This fraction was analyzed by SDS-PAGE, the silver staining of which is reported in Fig. 3C. The bands were excised from the gel, digested with trypsin, and analyzed by mass spectrometry. Peptide mass fingerprint analysis with 12 of 15 measured tryptic fragments allowed us to identify one band of about 120 kDa as the DDB-1. We were unable to identify the other bands present in the 0.5 M KCl fraction; mass spectrometry analysis suggests that some of them could have been DDB-1 degradative products.

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filters were reprobed with B; the same transfected with dsRNA interfering with DDB-1 or GFP (and DDB-1-transfected cells) and in mock-transfected cells or in cells

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tubulin tubulin tubulin

mock s.e. s.e. s.e.

DDB-1 DDB-1 DDB-1

IDDB-1 iGFp IDDB-1 iGFp

FP-CAT6 FP-CAT3 FP-CAT4 CMV-CAT

FIG. 4. DDB-1 levels modulate the basal transcription from the FM gene promoter. A, HF-28 cells were co-transfected with the vector driving the expression of human DDB-1 and with CMV-CAT (black bars) or FP-CAT3 or FP-CAT4 (white bars, see Fig. 1A). The values are reported as percentages of the mean expression of CMV-CAT without DDB-1 (black bars) and as percentages of the mean expression of FP-CAT3 without DDB-1 (white bars). B, HF-28 cells were co-transfected with the dsRNAs targeting DDB-1 or GFP and with CMV-CAT (black bars) or FP-CAT3 (white bars). The values are reported as percentages of the mean expression of CMV-CAT without dsRNA (black bars) and as percentages of the mean expression of FP-CAT3 without dsRNA (white bars). All of the data are mean values ± S.D. of triplicate experiments. The Western blots report the amount of DDB-1 in mock- and DDB-1-transfected cells (A) and in mock-transfected cells or in cells transfected with dsRNA interfering with DDB-1 or GFP (B); the same filters were reprobed with α-tubulin antibody as a control of sample loading. C, nuclear extracts from cells transfected with DDB-1 expression vector (DDB-1) or with dsRNAs interfering with the expression of DDB-1 (iDDB-1) or GFP (iGFp) were analyzed by EMSA with the B4 oligonucleotide. The extracts from mock-transfected cells were preincubated as indicated with unlabeled B4 (s.e.) or Sp1 site-containing (ns.c.) oligonucleotides.

suppression caused a decrease of the shifted band. These results demonstrated that the basal levels of transcription driven by the FM gene promoter are, at least in part, dependent on DDB-1 concentration.

Fibromodulin Gene Expression Is Activated by UV Irradiation—It has been demonstrated that DDB-1 is translocated into the nucleus upon UV irradiation (30); therefore we asked whether UV irradiation is associated with a change in the transcription from the FM gene promoter. To this aim, HF-28 cells were transfected with the FP-CAT6 vector (see Fig. 1A) and exposed to UV irradiation (15 J/m²). CAT assay allowed us to observe that UV irradiation activates FM gene promoter efficiency, leading to a 3-fold increase 72 h after the irradiation (Fig. 5A).

To ascertain whether endogenous fibromodulin gene is similarly regulated, we extracted RNA from HF-28 cells before and at various times after the exposure to UV irradiation and analyzed the RNA by real time PCR. As shown in Fig. 5B, a dramatic accumulation of FM mRNA was observed, reaching the maximal expression 72 h after irradiation. These results support the hypothesis that the binding of DDB-1 to the FM gene promoter not only affects the basal levels of transcription but also plays a role in the regulation of this gene upon UV irradiation.

Fibromodulin Gene Does Not Respond to UV Irradiation in Senescent Human Fibroblasts—Considering that the FM gene transcription is repressed in senescent cells, we explored its response to UV irradiation in these cells. To this aim, FM mRNA was measured in HF-60 cells at various times after the exposure to UV, and, in contrast to that observed with HF-28 cells, no significant increase of FM mRNA was observed (Fig. 5B). Another two mRNAs, encoding GADD45 and p21waf1, showed a robust accumulation upon UV irradiation in both HF-28 and HF-60 cells (Fig. 5C).

Therefore, we first addressed whether the interaction between the B4 element of the FM gene promoter and the DDB-1 protein is modified upon UV treatment. To do this, we used EMSA with the B4 oligonucleotide to analyze nuclear extracts from HF-28 cells exposed to UV irradiation. Fig. 6 shows that the intensity of the shifted band of the B4 oligonucleotide is significantly increased in the extracts from UV-treated cells. Then, we addressed whether the enhancement of the B4 oligonucleotide shift upon UV irradiation is abolished in senescent cells. As shown in Fig. 5, no effects on the intensity of this band were seen by using extracts from HF-60 cells exposed to UV rays, thus suggesting an impairment of DDB-1 function in these cells.

DDB Activity Is Impaired in Senescent Fibroblasts—One possible explanation of the results presented above is that DDB-1 expression is suppressed in senescent cells. To address this point, we measured by Western blot the levels of DDB-1, DDB-2, and CSA in exponentially growing and in senescent IMR-90 fibroblasts. Fig. 7A shows that these three proteins are similarly expressed in HF-28 and HF-60 cells.

Given that we demonstrated that the DDB-1-dependent transcription of the FM gene, both in basal conditions and after UV irradiation, is impaired in senescent cells, it could be hypothesized that the well known ability of DDB-1 to bind UV-damaged DNA, regardless of its sequence, is also impaired in senescent cells. To test this possibility we used an enzyme-linked immunosorbent assay-based method, which measures the binding of nuclear proteins to DNA fragments exposed to UV rays (17). An oligonucleotide with a sequence that is not related to that of the B4 oligonucleotide was UV-irradiated (15,000 J/m²) and challenged with nuclear extracts from HF-28 and HF-60 cells. Nuclear extracts from HF-28 cells have the capacity to bind this irradiated oligonucleotide, which is decreased in cells transfected with the siRNA for DDB-1 (see supplemental Fig. 2). As shown in Fig. 7B, although the irradiated cold oligonucleotide efficiently competed for this binding, a 50-fold excess of the unirradiated cold probe was unable to compete. On the contrary, HF-60 extracts, when challenged with the irradiated probe, led to a very faint band, thus suggesting that the binding activity of the damaged DNA in these cells was greatly reduced.

DISCUSSION

In this study, we have explored the regulation of FM gene expression in normal human fibroblasts, either exponentially
growing or arrested by repeated in vitro passages. These experiments support several conclusions on the regulation of this gene and the phenotype of fibroblasts undergoing replicative senescence.

First, we have found that the FM gene is regulated by UV irradiation and that this leads to a dramatic (50-fold) accumulation of FM mRNA. Our knowledge of the functions of this protein is mostly based on the study of the phenotype of the FM gene knock-out in the mouse (9, 10). These studies show several abnormalities mostly concerning defects in collagen fibrillogenesis. A significant amount of experimental data indicate that UV irradiation provokes skin damage localized in the dermis, where collagen fibrils are deposited (31). Chronic exposure to UV rays provokes the so-called photoaging of the skin, which is characterized by disorganization of collagen fibrils, reduced levels of procollagens I and III due to increased degradation by collagenase and metalloproteinases, and increased levels of elastin. Therefore, it is absolutely not surprising to find that UV treatment of cultured fibroblasts induces the expression of the FM gene, the product of which plays a crucial role in collagen fibrillogenesis. Thus, it could be speculated that FM overexpression is aimed at correcting, or at counteracting, the connective tissue trouble induced by exposure to UV light by assisting in the assembly of new collagen fibrils. On the other hand, chronic exposure to UV light could induce sustained overexpression of FM that could contribute to the development of the photoaging phenotype.

The second point that deserves comment concerns the find-

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**FIG. 5.** FM gene expression is induced by UV irradiation in exponentially growing human fibroblasts and not in senescent cells. A, FP-CAT6 vector (white bars) and CMV-CAT (black bars) were transfected in HF-28 cells exposed or not to UV rays (15 J/m²). CAT expression was measured 48 h after the UV irradiation or mock treatment. The efficiencies of the promoters are indicated as percentages of the mean values observed with the two vectors in the mock-treated cells. B, FM mRNA was measured by real time PCR in total RNA preparations from HF-28 or HF-60 cells before and at various times after the exposure to UV irradiation. White bars indicate the relative abundance of the mRNA, considering as +1 the abundance of the FM mRNA in HF-28 cells not exposed to UV light. The values of black bars have been calculated by considering +1 the FM mRNA concentration in HF-60 cells not exposed to UV light. All of the values represent the mean ± S.D. of triplicate experiments on two distinct total RNA preparations. C, the same total RNAs analyzed in the experiments of B were used to calculate the changes in the concentrations of GADD45 and p21waf1 mRNAs. The bars indicate the relative abundance of the two mRNAs, considering as +1 their abundance in HF-28 cells not exposed to UV light.

**FIG. 6.** The interaction of DDB-1 with the FM gene promoter is induced upon UV irradiation only in HF-28 cells. Nuclear extracts from UV-irradiated or unirradiated HF-28 or HF-60 cells were used for EMSAs to test the formation of the complex with the oligonucleotide B4. The last two lanes indicate the competition experiments performed on the unirradiated HF-28 nuclear extracts, by using a 50-fold molar excess of unlabeled B4 oligonucleotide (s.c.) or of Sp1 site-containing oligonucleotide (ns.c.).
cleotide 1 was UV-irradiated (15,000 J/m²) and incubated with HF-28 or HF-60 cells. Tubulin was used as a control of loading. A Western blot analysis of DDB-1, DDB-2, and CSA in HF-28 and blasts is decreased compared with exponentially growing cells. The mechanism into the nucleus after UV irradiation (30). The mechanism of nucleotide excision repair is altered in senescent cells. Repair of UV-induced DNA damage on nontranscribed strands (global genomic repair) is reduced in terminally differentiated cells (34), and the number of UV photoproducts found in normal skin increases with age (35). This is in agreement with the dramatic decrease we observed in the UV-irradiated DNA binding activity in nuclear extracts from HF-60 cells. It is conceivable that replicative senescence could be associated with a decreased efficiency of the DDB-1/DDB-2-dependent global genomic repair. This is not expected to provoke significant alterations because senescent cells do not replicate their DNA; thus the accumulation of mutations in nontranscribed strands is not harmful. On the contrary, if DDB-1 is involved in gene regulation, as suggested by our results, the effects on the functions of senescent cells and on the tissues in which they are present should be relevant. The understanding of the complete array of genes regulated by DDB-1 is expected to make a significant contribution to address this point.

Third, FM gene expression is impaired in senescent human fibroblasts, and this appears to be dependent on the suppression of the DDB-1 functions. In fact, although DDB-1 is expressed in senescent fibroblasts at levels comparable with those observed in exponentially growing cells, the B4 cis-element of the FM gene promoter fails to form any detectable complex when challenged with HF-60 nuclear extracts. On the other hand, UV-damaged DNA binding activity of senescent cells is severalfold lower than that present in the extracts from growing HF-28 cells. This phenomena are apparently due to the failure of the mechanisms governing the intracellular trafficking of DDB-1. It has been demonstrated that DDB-1 is mostly cytosolic in unirradiated cells, whereas it translocates into the nucleus after UV irradiation (30). The mechanism regulating this trafficking and its relationship with UV irradiation are not known. However, our results, indicating that this mechanism is suppressed in senescent cells, suggest a possible explanation; the molecular machinery driving DDB-1 into the nucleus could be related to the cell cycle progression. This is obviously supported by the main peculiarity of senescent cells compared with exponentially growing cells, that is, the close association of replicative senescence with the arrest of the cell cycle. Experimental evidence further supports this hypothesis. DDB-1 nuclear translocation does not take place at the same time in all of the cells exposed to UV; 24 h after UV irradiation DDB-1 is localized in the nucleus of about 40% of the cells, and this percentage increases to 70% after 48 h and to 100% only after 72 h (30). This supports that the DDB-1-nuclear translocation mechanism, to sense the DNA damage-induced signaling, should pass through a specific cell cycle step. In agreement with this hypothesis, there are several results indicating that DDB-1-containing complexes functionally or even physically interact with cell cycle regulatory proteins (16).

The altered regulation of the FM gene expression in senescent cells could have significant effects. Because the number of senescent fibroblasts is high in the elderly, decreased basal levels of FM expression could alter the extracellular matrix assembly in these individuals, leading to a condition similar to that observed in FM knock-out mice that is characterized by skin fragility, reduced corneal transparency, and age-dependent osteoarthritis. In addition, the lack of response of the FM gene to UV irradiation could have deleterious effects on the sensitivity of aged tissue exposed to sun rays.

Solid experimental evidence indicates that the efficiency of repair of DNA double strand breaks is decreased in senescent cells (probably because of a decline of end-joining activity (32)), and an accumulation of unreparable double strand breaks is suggested to have a causative role in aging (33). On the contrary, it is not completely clear whether nucleotide excision repair is altered in senescent cells. Repair of UV-induced DNA damage on nontranscribed strands (global genomic repair) is reduced in terminally differentiated cells (34), and the number of UV photoproducts found in normal skin increases with age (35). This is in agreement with the dramatic decrease we observed in the UV-irradiated DNA binding activity in nuclear extracts from HF-60 cells. It is conceivable that replicative senescence could be associated with a decreased efficiency of the DDB-1/DDB-2-dependent global genomic repair. This is not expected to provoke significant alterations because senescent cells do not replicate their DNA; thus the accumulation of mutations in nontranscribed strands is not harmful. On the contrary, if DDB-1 is involved in gene regulation, as suggested by our results, the effects on the functions of senescent cells and on the tissues in which they are present should be relevant. The understanding of the complete array of genes regulated by DDB-1 is expected to make a significant contribution to address this point.

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J. Biol. Chem. 2005, 280:31809-31817.
doi: 10.1074/jbc.M414677200 originally published online July 6, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M414677200

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