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Interleukin-6 (IL-6) and/or Soluble IL-6 Receptor Down-regulation of Human Type II Collagen Gene Expression in Articular Chondrocytes Requires a Decrease of Sp1·Sp3 Ratio and of the Binding Activity of Both Factors to the *COL2A1* Promoter*

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Type II collagen is composed of $\alpha 1(\text{II})$ chains encoded by the *COL2A1* gene. Alteration of this cartilage marker is a common feature of osteoarthritis. Interleukin-6 (IL-6) is a pro-inflammatory cytokine that needs a soluble form of receptor called sIL-6R to exert its effects in some cellular models. In that case, sIL-6R exerts agonistic action. This mechanism can make up for the partial or total absence of membrane-anchored IL-6 receptors in some cell types, such as chondrocytes. Our study shows that IL-6, sIL-6R, or both inhibit type II collagen production by rabbit articular chondrocytes through a transcriptional control. The cytokine and/or sIL-6R repress *COL2A1* transcription by a -63/-35 sequence that binds Sp1·Sp3. Indeed, IL-6 and/or sIL-6R inhibit Sp1 and Sp3 expression and their binding activity to the 63-bp promoter. In chromatin immunoprecipitation experiments, IL-6/sIL-6R induced an increase in Sp3 recruitment to the detriment of Sp1. Knockdown of Sp1·Sp3 by small interference RNA and decoy strategies were found to prevent the IL-6- and/or sIL-6R-induced inhibition of *COL2A1* transcription, indicating that each of these Sp proteins is required for down-regulation of the target gene and that a heterotypic Sp1·Sp3 complex is involved. Additionally, Sp1 was shown to interact with Sp3 and HDAC1. Indeed, overexpression of a full-length Sp3 cDNA blocked the Sp1 up-regulation of the 63-bp *COL2A1* promoter activity, and by itself, inhibits *COL2A1* tran-

scription. We can conclude that IL-6, sIL-6R, or both in combination decrease both the Sp1·Sp3 ratio and DNA-binding activities, thus inhibiting *COL2A1* transcription.

Extracellular matrix (ECM)⁶ of articular cartilage contains tissue-specific macromolecules including types II, IX, and XI collagens and the large aggregating proteoglycan (PG) aggrecan (1). Type II collagen is the major collagen synthesized by chondrocytes in mature articular cartilage. Each $\alpha 1(\text{II})$ procollagen chain of the triple helix is encoded by the *COL2A1* gene, whose transcription is regulated by DNA elements within both the promoter and the first intron regions (2). Thus, several binding sites of the intronic enhancer were shown to interact with transcription factors such as Sox9, L-Sox5, and Sox6 (3, 4), required for cartilage-specific expression of type II collagen during chondrogenesis *in vivo* (5), as well as with zinc finger transcription factors Sp1, Sp3, and C-Krox (6, 7). The three latter proteins are also able to bind to several binding sites identified in a 266-bp promoter of the human *COL2A1* gene (6–8). Sp1 was shown to be a strong activator of *COL2A1* gene expression via the promoter binding sites, whereas Sp3 was found to prevent the Sp1 up-regulation of the *COL2A1* promoter activity by binding to the same *cis*-acting elements (7).

In healthy cartilage, chondrocytes maintain steady-state expression of collagens and PGs and are sensitive to a number of growth factors and cytokines that either enhance or reduce type II collagen synthesis. In osteoarthritis and rheumatoid arthritis, structural and functional breakdown of the ECM is accompanied by phenotypic changes of the resident chondro-

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⁶ The abbreviations used are: ECM, extracellular matrix; PG, proteoglycan; IL-1, interleukin-1; MMP, metalloproteinase; LIF, leukemia inhibitory factor; STAT, signal transducers and activators of transcription; IL-6R, IL-6 receptor; RAC, rabbit articular chondrocyte; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcription; RLU, relative luciferase unit(s); siRNA, small interference RNA; EMSA, electromobility shift assay; ChIP, chromatin immunoprecipitation; TGF, transforming growth factor; HDAC, histone deacetylase 1.

cytes. Pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor- α , and interferon- γ play key roles in these pathologies, inducing overexpression of metalloproteinases (MMPs) and decreased synthesis of tissue-specific macromolecules, including type II collagen and aggrecan (9). IL-6 expression, which is induced by IL-1, is likely to take part in the degradation process.

IL-6 cytokine family consists of IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor (10), cardiotrophin-1 (11), and cardiotrophin-like cytokine (12). These factors activate target genes involved in hematopoiesis as well as in acute-phase and immune responses of the organism (12). Cytokines of the IL-6 family bind to plasma membrane receptor complexes containing the common signal transducing receptor chain glycoprotein 130 (gp130). Signal transduction generally involves the activation of Janus kinase tyrosine kinase family members, leading to the activation of transcription factors of the signal transducers and activators of transcription (STATs). Another major signaling pathway for the cytokines of the IL-6 family is the mitogen-activated protein kinase cascade (13, 14).

IL-6 binds to a specific IL-6 receptor (IL-6R), and this complex associates with two molecules of the ubiquitously expressed gp130 leading to initiation of signaling (15, 16). Although cells that do not express IL-6R are nonresponsive to IL-6 alone, these cells can be stimulated by a complex of IL-6 and its soluble IL-6R (15, 17), generated by proteolytic cleavage (shedding) or differential IL-6R mRNA splicing (15, 18, 19). Thus, the cells that release the soluble IL-6R render other cells that only express gp130 responsive toward IL-6, through a pathway named *transsignaling* (20).

Accumulating evidence suggests that IL-6 and its soluble receptor are implicated in both inflammatory and degenerative joint diseases. First, increased levels of IL-6 and sIL-6R have been found in synovial fluids and sera from osteoarthritis and rheumatoid arthritis patients (21), and these levels correlate with the increased leukocyte infiltration in synovial tissue (22). Furthermore, in IL-6-deficient mice immunized with type II collagen, a decrease of inflammatory cells in knee joints and a reduced antibody response to type II collagen have been observed, suggesting that IL-6 plays a crucial role in the development of autoimmune collagen-induced arthritis (23). In rabbit articular chondrocytes (RACs), IL-6 treatment was found to repress PG synthesis and enhanced PG degradation induced by IL-1 β (24). However, the role of IL-6 on the depletion of the cartilage ECM remains controversial. Indeed, some authors could not demonstrate any effect of IL-6 on the expression of PG (25). Other studies have demonstrated that injection of recombinant IL-6 in the joint cavity corrected the IL-6 deficiency and significantly reduced cartilage destruction in the IL-6 gene knockout mouse model (26). Furthermore, IL-6 acts synergistically with IL-1 β and oncostatin M to up-regulate matrix metalloproteinase-1 (MMP-1) and MMP-13 in bovine and human cartilage explant cultures (27, 28). By contrast, it was also reported that the IL-6·sIL-6R complex induces the up-regulation of tissue inhibitor of metalloproteinases-1 and blocks IL-1-induced collagenolytic activity (29). Part of the controversy related to these data is probably due to the fact that

most of *in vitro* studies were performed in the absence of sIL-6R. Indeed, levels of membrane-bound IL-6R on chondrocytes are lower compared with those of other cell types such as hepatocytes, and the addition of sIL-6R to IL-6 is required to observe the full effect of the cytokine (30). In this way, the sIL-6R·IL-6 complex may partially account for the loss of PG commonly associated with arthritic lesions, because it has been shown to increase aggrecanase-mediated catabolism of PG in articular cartilage (31). In cultured articular chondrocytes, we have reported that IL-6·sIL-6R decreased the amount of aggrecan core and link protein mRNA steady-state levels (32), whereas they increase those of MMP-1, MMP-3, and MMP-13 (33).

Very little is known on the effect of IL-6·sIL-6R on type II collagen expression. In bovine articular chondrocytes, IL-6·sIL-6R was shown to reduce the steady-state levels of *COL2A1* mRNA (32), but the nature of transcription factors mediating IL-6·sIL-6R inhibition of *COL2A1* gene is still largely unknown. Extensive studies have established the central role of the STAT family of transcription factors in IL-6 cytokine family signaling. In response to IL-6 stimulation, STAT1 and STAT3 are tyrosine-phosphorylated and translocate to the nucleus where STAT homo- and/or heterodimers bind to specific DNA elements in the promoters of IL-6 target genes (34, 35). Previous data from our laboratory have shown that IL-6·sIL-6R induced activation of STAT1/STAT3 proteins in bovine articular chondrocytes and that this activation was accompanied by an increase of the DNA binding of the STAT proteins on a STAT consensus sequence.

We have already reported that two other cytokines, TGF- β 1 and IL-1 β , exert a repressive effect on type II collagen expression in primary RAC by a transcriptional regulatory mechanism, which involves a -41/-33-bp promoter sequence binding Sp1 and Sp3 (8, 36). The inhibitory effect of these cytokines results from a decrease in Sp1·Sp3 ratio, which prevents Sp1-induced transactivating effects. In the present study, we show for the first time that IL-6, sIL-6R, or both repress *COL2A1* gene transcription through the same promoter region and that this effect implicates both Sp1 and Sp3 transcription factors. It seems therefore that cytokines that have a repressive effect on type II collagen expression involve similar transcriptional regulatory mechanisms that converge on the same complex in which the Sp1 and Sp3 binding activities and the Sp1·Sp3 ratio are decreased.

EXPERIMENTAL PROCEDURES

General Materials—Hyaluronidase and trypsin were purchased from Serva (Heidelberg, Germany). Collagenase, guanidium chloride, benzamidine, *N*-caproic acid, EDTA, pepstatin, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, Nonidet P-40, EGTA, lauryl sulfate (SDS), and trichostatin A were from Sigma-Aldrich. Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), RNase inhibitor, di-deoxynucleosidtriphosphate (dNTPs), recombinant Moloney murine leukemia virus reverse transcriptase, and phosphate-buffered saline were purchased from Invitrogen. [3 H]Proline was from PerkinElmer Life Sciences. Triton X-100, trichloroacetic acid, Tris, and glycine were purchased from Acros Organics (Noisy-le-Grand, France). Protein Assay Brad-

IL-6/sIL-6R Repress COL2A1 Gene Transcription

ford solution was from Bio-Rad. Random hexamers and 2× SYBR Green Master Mix were purchased from Applied Biosystems (Courtabœuf, France).

Cell Culture—RACs were released from articular cartilage slices of shoulders and knees of 3-week-old rabbits, by enzymatic treatment, as described previously (6). The cells were seeded generally at 2×10^5 cells per 9.6-cm² dishes or 1×10^6 cells per 55-cm² dishes in DMEM supplemented with 10% heat-inactivated FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml). They were grown at 37 °C in a 5% CO₂ humidified atmosphere, with medium change every 2–3 days.

Collagen Labeling—To assay newly synthesized collagen, RAC cultures at 80% confluency were preincubated in 10% FCS-containing DMEM in 9.6-cm² dishes for 15 h in the presence of 50 µg/ml sodium ascorbate. Then, the medium was changed to fresh medium supplemented with 50 µg/ml sodium ascorbate, 100 µg/ml β-aminopropionitrile, and 2 µCi/ml [³H]proline containing or not increasing concentrations (5–100 ng/ml) of human recombinant IL-6 (Upstate Biotechnology, Inc.), human recombinant sIL-6R (R&D Systems), or both. 24 h later, the culture medium and the cell layer were collected, and the amount of labeled collagen was assayed as previously described (36).

Quantitative Real-time RT-PCR—Total RNA was extracted by the guanidium hydrochloride method (37), and the integrity of samples was checked by 1% agarose-formaldehyde gel electrophoresis. Then, 2 µg of total RNA were reverse transcribed into cDNA in the presence of 50 pmol of random hexamers, 40 units/µl of RNase inhibitor, 10 mM of each dNTPs, first-strand buffer 5×, and 60 units/µl of Moloney murine leukemia virus in a final reaction volume of 25 µl. The mixture was incubated at 37 °C for 1 h, and the cDNA synthesis was stopped by heating the reaction mixture at 90 °C for 15 min. To test the efficiency of reverse transcription, glyceraldehyde-3-phosphate dehydrogenase cDNA was submitted to 40 cycles of amplification (1 cycle: 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min) in an Omni-E-Hybrid thermocycler and using a PCR kit (Invitrogen). Then, the samples were separated on a 2% agarose gel and visualized by ethidium bromide staining. Real-time RT-PCR reactions were performed (38), using sequence-specific primers (Table 1) (Eurogentec, Angers, France) defined with “Primer Express” software (Applied Biosystems, Courtabœuf, France). Analysis of relative gene expression was done by using the 2^{-ΔCT} method (39).

Transfection Experiments—80% confluent chondrocytes were transiently transfected by the calcium phosphate precipitation method using chimeric COL2A1-luciferase reporter vectors and pSV40β-gal plasmid, as described previously (6–8). The reporter constructs pGL2-3.774 kb, pGL2-3.316 kb, pGL2-3.059 kb, pGL2-0.387 kb, pGL2-0.110 kb, and pGL2-0.082 kb cover, respectively, the -932/+2842 bp, -932/+2384 bp, -932/+2127 bp, -266/+121 bp, -63/+47 bp, and -35/+47 bp sequences of COL2A1 gene, as previously described (6, 8).

In some experiments, reporter plasmids were also cotransfected with Sp1 and/or the full-length Sp3 isoform expression vectors kindly provided by Dr. G. Suske (University of Marburg, Germany) (pEVR2/Sp1 and pN3/Sp3, respec-

tively) as described previously (8, 40). The corresponding insertless expression plasmids were used as controls (pEVR2 and/or pN3). In these experiments, the pSV40β-gal construct has not been cotransfected, because Sp1 is able to increase SV40 promoter activity, due to the presence of several Sp1 DNA-binding sites in this promoter (8).

10–15 h after transfection, the medium was changed to 2% FCS/DMEM containing or not IL-6, sIL-6R, or both at concentrations ranging from 1 to 100 ng/ml. 24 h later, the samples were harvested and protein content, luciferase, and β-galactosidase activities were determined as previously described (6). Protein amount was determined by the Bradford procedure (Bio-Rad). Luciferase activities were normalized, and means ± S.D. of three independent samples were expressed as relative luciferase units (RLU).

To inhibit Sp1 and Sp3 expression, we used the technique of small interfering RNA (siRNA). The Sp1 and Sp3 siRNAs were obtained from Santa Cruz Biotechnology and are directed against the human Sp1 and Sp3 mRNAs. 80% confluent RACs were transiently transfected by the calcium phosphate precipitation method using the pGL2-0.110 kb chimeric COL2A1-luciferase reporter vector, pSV40β-gal plasmid, and with 2 µg of Sp1 and/or Sp3 siRNA as described above. After the overnight transfection period, the cells were incubated for 24 h with IL-6, sIL-6R, or both. At the end of incubation, the cells were harvested and relative transcriptional activities were determined.

In parallel, similar transfection experiments were performed to follow the efficiency of Sp1 and/or Sp3 silencing, but in that case, the reporter vector was not transfected. The down-regulation of Sp1/Sp3 expressions was measured through their steady-state mRNA levels or DNA-binding activity, by real-time RT-PCR with the primers presented in Table 1, and electrophoretic mobility shift assay (EMSA) respectively.

Decoy Experiments—RACs were transfected with double-stranded oligonucleotides to interfere with Sp1 and Sp3 binding to their cognate *cis*-acting elements within the 63-bp short promoter of the COL2A1 gene. Two copies of the -50/+1 wild-type (wt) sequence found in the COL2A1 promoter as well as the corresponding mutated sequence were used as decoy oligonucleotides (Table 1). Medium was changed to fresh medium containing the decoy oligonucleotides 10–15 h after transfection, and the cultures were incubated for a further 24-h period with or without 25 ng/ml of IL-6, sIL-6R, or both. Relative luciferase and β-galactosidase activities as well as protein amounts were determined as described previously.

Preparation of Cytoplasmic and Nuclear Extracts, and Gel Retardation Assays—To prepare cytoplasmic extracts, RACs were seeded in 55-cm² Petri dishes, and incubated for 24 h at 80% confluency with or without IL-6, sIL-6R, or both at 10 ng/ml. Following treatment, chondrocytes were rinsed once with ice-cold phosphate-buffered saline and lysed for 30 min at 4 °C in RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 0.25% sodium deoxycholate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, and aprotinin at 10 µg/ml). The cell layer lysate was then centrifuged for 30 min at 14,000 × g and 4 °C. The supernatant containing protein extracts was collected for further analysis by SDS-PAGE and Western blot.

Nuclear proteins from RACs treated or not with 25 ng/ml of IL-6, sIL-6R, or both were extracted as minipreparations (41). EMSAs were performed with the oligonucleotides presented in

TABLE 1
Primers used in this study

Mutation substitutions are underlined.

Name	Sequence (5' → 3')
COL2A1 primer (forward)	5'-GGC AAT AGC AGG TTC ACG TAC A-3'
COL2A1 primer (reverse)	5'-CGA TAA CAG TCT TGC CCC ACT T-3'
Sp1 primer (forward)	5'-AGA ATT GAG TCA CCC AAT GAG AAC A-3'
Sp1 primer (reverse)	5'-GTT GTG TGG CTG TGA GGT CAA G-3'
Sp3 primer (forward)	5'-TCT GTT TCA GGG ATA GGA ACT GTT AA-3'
Sp3 primer (reverse)	5'-TTT ACT CCA TTG TCT CAT TTC CAG AA-3'
18S primer (forward)	5'-CGG CTA CCA CAT CCA AGG AA-3'
18S primer (reverse)	5'-GCT GGA ATT ACC GCG GCT-3'
GAPDH primer (forward)	5'-CTG ACT TCA ACA GCG ACA CC-3'
GAPDH primer (reverse)	5'-CCC TGT TGC TGT AGC CAA AT-3'
-50/-26 wt	5'-GCC TCG AAA GGG GCC GGG CGG ATA T-3'
ChIP COL2A1 primer (forward)	5'-GGG CGG GCG GTT CAG GTT AC-3'
ChIP COL2A1 primer (reverse)	5'-CCT TGG AGC AGG AGG AGG AA-3'
-50/+1 mc wt	5'-GCC TCG AAA GGG GCC GGG CGG ATA TAA CGG GCG CCG CGG GGA GAA GAC-3'
-50/+1 mc mut	5'-GCC TCG AAA GGT <u>TCC TTG</u> CGG ATA TAA CGG GCG CCG CGG GGA GAA GAC-3'

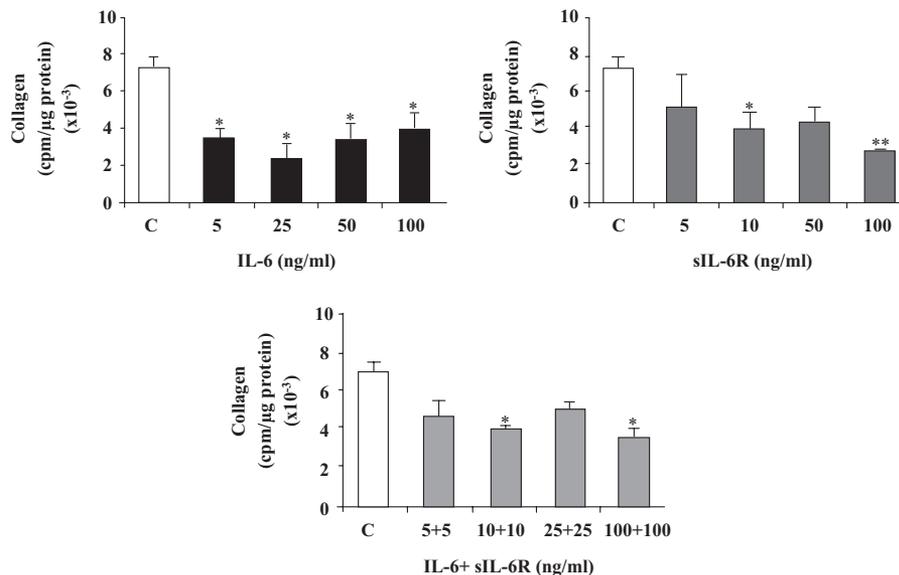


FIGURE 1. Effect of IL-6, sIL-6R, or both on collagen neosynthesis in primary chondrocytes. Primary RACs (80% confluency) were cultured as described under "Experimental Procedures" in the presence or absence of IL-6, sIL-6R, or both at concentrations ranging from 5 to 100 ng/ml. At the end of the experiment, the amount of radiolabeled collagen was assayed in both medium and cell layer as collagenase-digestible radioactivity. The values, normalized to the amount of total protein assayed by the Bradford colorimetric method, were expressed as cpm/μg of protein and represent the mean ± S.D. of triplicate dishes. C, control; *, $p < 0.05$; **, $p < 0.01$.

Table 1, as previously described (6–8). In antibody interference assays, 1 μl of anti-Sp1, anti-Sp3, or both antibodies, was added to each reaction for 15–20 min at room temperature and then for 15 min at 4 °C. The probe was finally added to the binding reaction, a further 15-min incubation at room temperature was performed, and the samples were finally submitted to electrophoresis on a polyacrylamide gel that was processed by autoradiography (6).

Western Blot Analysis—Western blot analyses of type II collagen, Sp1, Sp3, and HDAC1 were performed on cellular and/or nuclear extracts from RACs treated or not with 10 or 25 ng/ml IL-6, sIL-6R, or both as previously described (7).

Anti-type II collagen antibody was from Novotec (Lyon, France). Sp1, Sp3, and HDAC1 antibodies were from Santa Cruz Biotechnology. After incubation with the primary antibodies, the membranes were rinsed eight times for 5 min in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween 20), and incubated for 2 h with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody, 1/7000 dilution). The blots were then washed eight times for 5 min in TBST. Finally, type II collagen, Sp1, Sp3, and HDAC1 proteins were revealed, using a Western blot detection kit. Signals were captured with the Fluor-S Multimager video system (Bio-Rad, Marnes-la-Coquette, France) and then quantified with the ImageQuANT software (Amersham Biosciences). Type II collagen and Sp1 and Sp3 expressions were normalized to β-actin protein signal. Mouse monoclonal anti-actin and horseradish peroxidase-conjugated goat anti-rabbit were purchased from Tebu-Bio (Le Perray en Yvelines, France).

Immunoprecipitation Assays—100 μg of nuclear extracts from control RACs was pre-cleared with appropriate amounts of protein A-Sepharose at 4 °C for 2 h. Pre-cleared nuclear extracts were then centrifuged for 2 min at 4,000 × g and 4 °C to pellet protein A-Sepharose. The supernatant was collected and immunoprecipitated overnight at 4 °C using either anti-Sp1, anti-Sp3, or anti-HDAC1 antibodies. Then, protein A-Sepharose was added to each tube for 2 h at 4 °C and after immunoprecipitation, complexes were collected by centrifugation for 2 min at 4,000 × g. The unbound proteins were then removed by washing the solid phase four times with complete lysis buffer. Protein A was then resuspended in 50 μl of 2× reducing sample buffer and boiled for 10 min. Samples were centrifuged for 5 min at 14,000 × g, and supernatants were submitted to Western blot analysis.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation (ChIP) assays were performed via a commercially purchased chromatin immunoprecipitation kit (Active Motif). Briefly, RACs, treated or not with 25 ng/ml of IL-6 and sIL-6R, were cross-linked, scraped, and

IL-6/sIL-6R Repress COL2A1 Gene Transcription

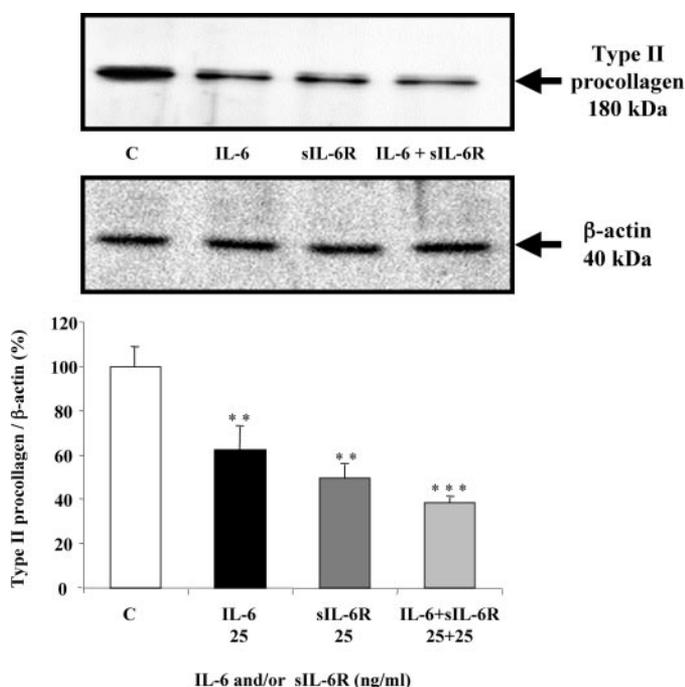


FIGURE 2. Effect of IL-6, sIL-6R, or both on type II collagen protein expression in primary RACs. 15 μ g of protein extracts from RAC, treated or not by IL-6, the soluble receptor, or both (25 ng/ml), were subjected to Western blotting. The membranes were immunoblotted with polyclonal antibodies directed against type II collagen (1/1000 dilution) and β -actin as a control of loading (1/300 dilution). After washing with TBST, polyvinylidene difluoride membranes were incubated with the appropriate secondary antibody and proteins were revealed with the SuperSignal West-pico detection kit. Histograms represent the relative expression of type II procollagen normalized to β -actin protein signal. Results are means of three independent experiments performed on different rabbits \pm S.E. C, control; **, $p < 0.01$; ***, $p < 0.001$.

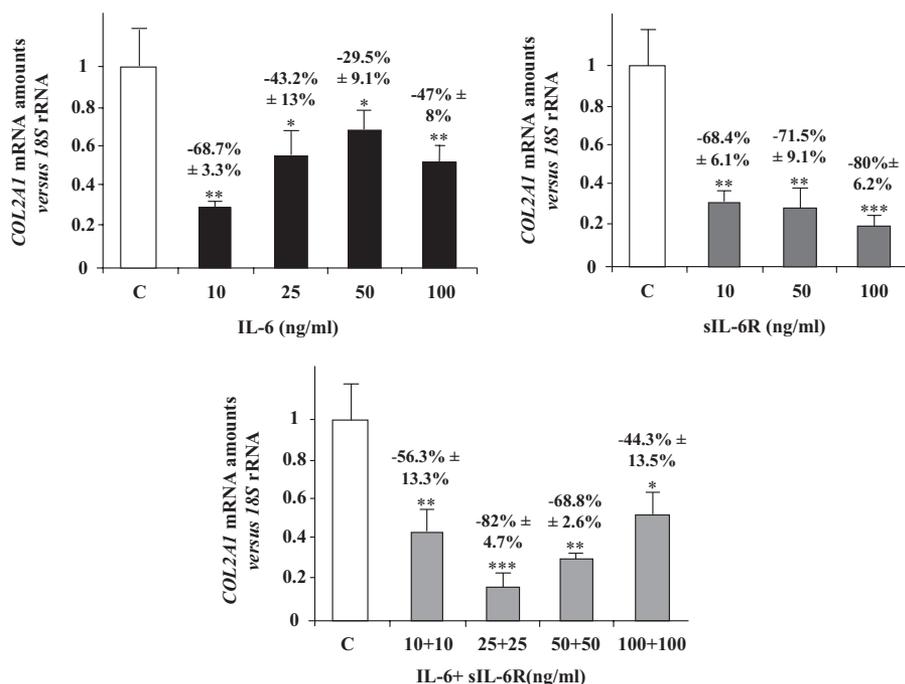


FIGURE 3. Effect of IL-6, sIL-6R, or both on the steady-state levels of COL2A1 mRNA in cultured chondrocytes. Total RNAs (2 μ g) from RACs treated or not with IL-6, sIL-6R, or both at concentrations ranging from 10 to 100 ng/ml, were reverse-transcribed into cDNA. The resulting products were diluted (1/100) and analyzed by real-time RT-PCR using specific primers for COL2A1 mRNA and 18S rRNA. Expression of COL2A1 transcripts was normalized to 18S rRNA and presented graphically as relative mRNA levels of COL2A1. Results represent mean \pm S.D. of triplicate samples. C, control; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

lysed according to the manufacturer's instructions. The DNA was then sheared using enzymatic digestion, and specific protein-DNA complexes were immunoprecipitated using either anti-Sp1, anti-Sp3, or anti-STAT3 antibodies (Santa Cruz Biotechnology).

Following immunoprecipitation, cross-linking was reversed, the proteins were removed by treatment with proteinase K, and the DNA was purified and used as a template in PCR. ChIP primers (Table 1) were used to amplify a 171-bp fragment corresponding to the core COL2A1 gene promoter.

Calculation and Statistical Analysis—Each experiment was repeated at least three times with similar results. Results are expressed as mean \pm S.D. of triplicate determinations. Statistical significance was assessed by using the Student's *t* test.

RESULTS

IL-6 and/or sIL-6R Induce Inhibition of Type II Collagen Production in Chondrocytes—We first determined the effect of increasing concentrations of IL-6, sIL-6R, alone or in combination, on collagen neosynthesis in RAC primary cultures. Newly synthesized collagens were assayed after [3 H]proline labeling of the chondrocytes. IL-6 treatment was found to reduce total collagen neosynthesis (Fig. 1), essentially formed of type II collagen (\sim 80% of total collagen) (8, 36, 42, 43). Similar data were observed with sIL-6R and the combination of IL-6+sIL-6R, indicating that there is no synergistic action (Fig. 1). The inhibitory effect of the cytokine and/or its soluble receptor was dose-independent in the concentration range used, and this action was mainly observed in the cell-layer fraction compared with culture medium associated fraction (data not shown).

Then, we specifically determined the IL-6 inhibitory effect on type II collagen synthesis using Western blotting on cell extracts and type II procollagen antibody. By comparison to untreated RAC, treatment with IL-6, sIL-6R, or both significantly reduced type II collagen protein amounts by \sim 40–60% (Fig. 2).

IL-6 and/or sIL-6R Decrease the COL2A1 mRNA Steady-state Levels in Chondrocytes—To determine whether the effect of IL-6 and sIL-6R was exerted at the transcriptional level, the steady-state levels of COL2A1 mRNA were estimated by real-time RT-PCR performed on total RNA extracts of primary RAC treated or not with increasing concentrations of IL-6, sIL-6R, or both. As shown in Fig. 3, the steady-state levels of COL2A1 mRNA were decreased under incubation with IL-6, sIL-6R, or a combination of both, independently of the dose in the concentration range of the cytokine and/or its soluble receptor used.

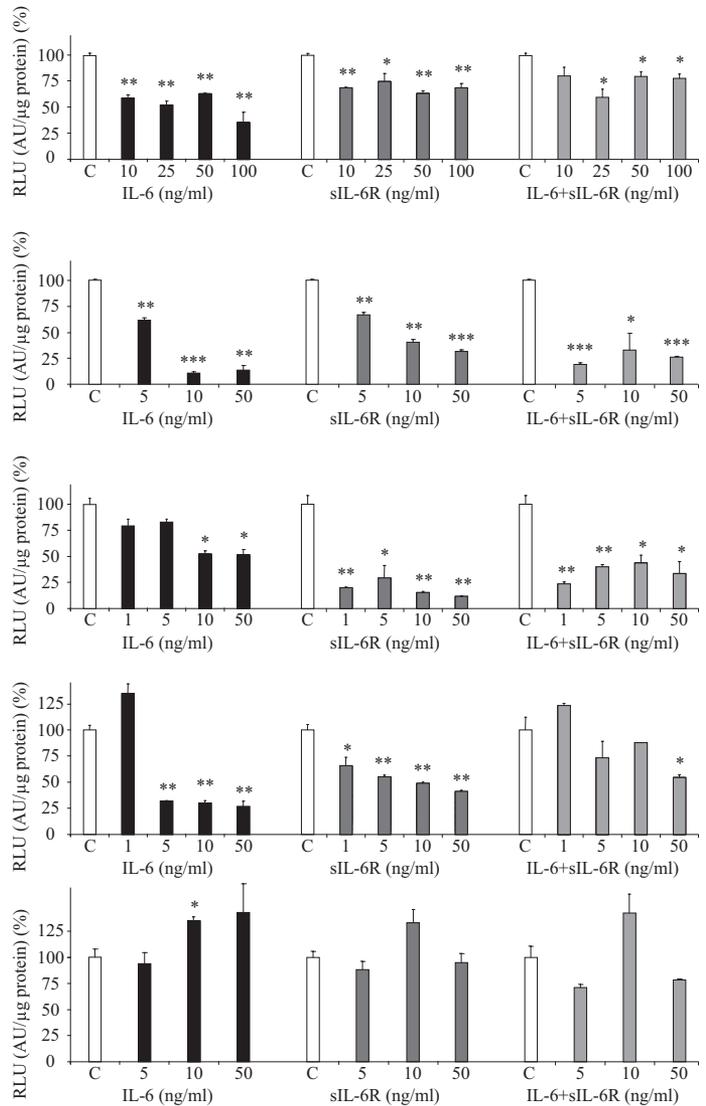
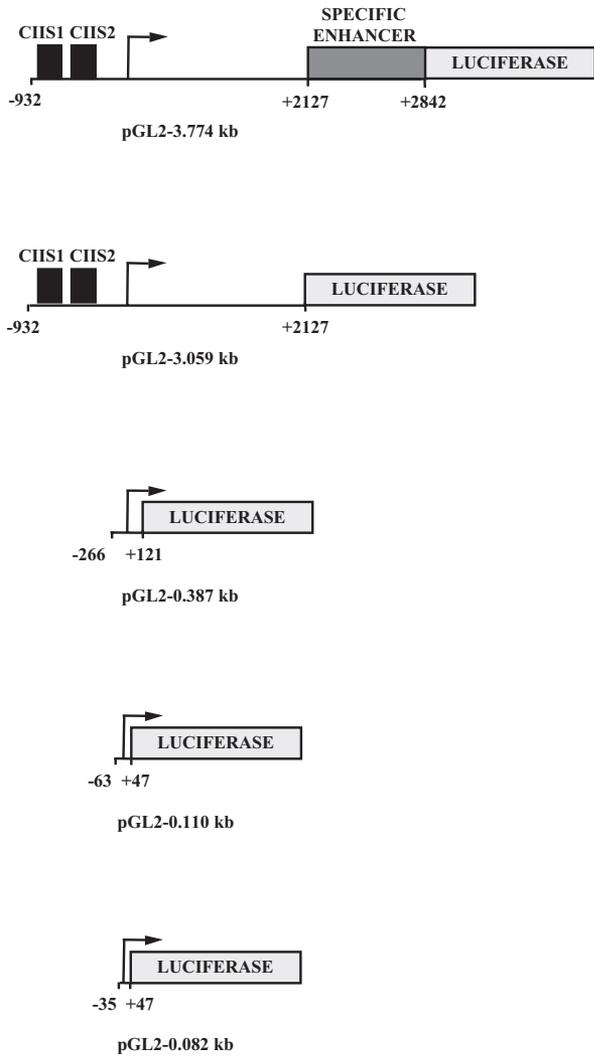


FIGURE 4. Effect of IL-6, sIL-6R, or both on the transcriptional activity of the human COL2A1 gene in primary chondrocytes. RACs (80% confluency) were transiently transfected with 10 μg of different COL2A1 reporter plasmids, together with the expression vector pSV40-βgal (2 μg). After overnight transfection, the medium was changed and the cells were incubated with or without IL-6, sIL-6R, or both at concentrations ranging from 1 to 100 ng/ml, in DMEM containing 2% FCS. 24 h later, the samples were harvested, and RLUs were determined. Each series of transfections was performed in triplicate. Transcriptional activity of each construct was expressed as relative luciferase activity, after correction for protein amounts. The data are expressed as % of IL-6, sIL-6R, or both effects versus respective control. Values are the means of triplicate samples ± S.D. CHS1 and CHS2: silencers 1 and 2 of COL2A1 gene, respectively; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

A -63/-35-bp Minimal Region of the COL2A1 Gene Mediates the Inhibitory Effect of IL-6 and/or Its Soluble Receptor—To better understand the mechanisms that mediate the down-regulation of COL2A1 gene transcription by IL-6·sIL-6R, primary RACs were transfected with several constructs of the 5'- and 3'-flanking region of the COL2A1 gene surrounding the transcription start site, linked to the luciferase reporter gene, and incubated the cells with varying concentrations of IL-6 and/or sIL-6R. The expression of the reporter gene was always found to be reduced in the presence of IL-6 or sIL-6R, compared with respective controls, whatever the construct size, except for the shortest (-35 bp) (Fig. 4). Combination of both IL-6 and sIL-6R did not induce a more pronounced inhibitory effect, suggesting that the cytokine and its soluble receptor do not exert a synergistic action. Therefore, it is likely that the IL-6- and/or sIL-6R-induced

inhibition of COL2A1 gene transcription is mediated by the -63/-35-bp sequence of the minimal promoter.

IL-6·sIL-6R Decrease the DNA Binding Activity of Sp1 and Sp3 to the -63/-35-bp Sequence of COL2A1—As shown in Fig. 4, IL-6 and/or its soluble receptor inhibit transcriptional activity of COL2A1 gene by the -63/-35 region. In previous studies, we extensively characterized this region, which was found responsible for the down-regulation of COL2A1 gene transcription induced by two other cytokines, TGF-β1 and IL-1β (8, 36). Two retarded complexes called **a** and **b** were detected with the -67/-30 and -50/+1 wt probes, centered by a Sp1-like cis-element located between -41 and -33 bp. Only the DNA binding activity of a complex in which are present both Sp1 and Sp3 was found to be reduced under TGF-β1 and IL-1β treatment of the RAC, whereas the binding of **b** complex was not modulated by both cytokines, suggesting that **b** bound nonspecifically (8, 36).

IL-6·sIL-6R Repress COL2A1 Gene Transcription

To determine if IL-6 and/or its soluble receptor proceed by a similar mechanism of action as TGF- β 1 and IL-1 β , an EMSA analysis was performed using the -50/-26 wt probe and nuclear extracts from RAC treated or not by the cytokine and/or its soluble receptor. As shown in Fig. 5A, complex **a** is

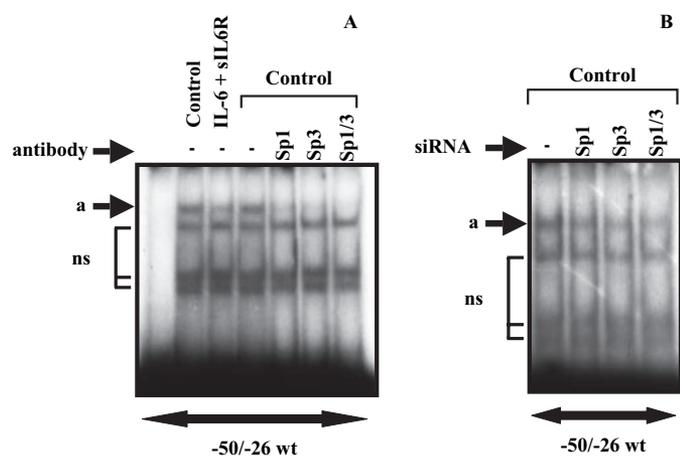


FIGURE 5. IL-6 and/or sIL-6R decrease the binding of Sp1·Sp3 to the -50/-26-bp responsive element. DNA-protein complexes were analyzed by EMSA. *A*, a labeled double-stranded -50/-26 wt probe was incubated with 10 μ g of nuclear extracts from primary RACs, treated or not with both IL-6 and sIL-6R (25 ng/ml). Antibody interference assays using specific antibodies were performed as indicated under "Experimental Procedures." Protein-DNA complexes **a** and **ns** (nonspecific) are indicated by an arrow. *B*, in this experiment, nuclear protein were extracted from primary RACs transfected at 80% confluency with 2 μ g of control siRNAs or siRNAs directed against Sp1 and/or Sp3. 10 μ g of these nuclear extracts was then incubated with the -50/-26 wt probe.

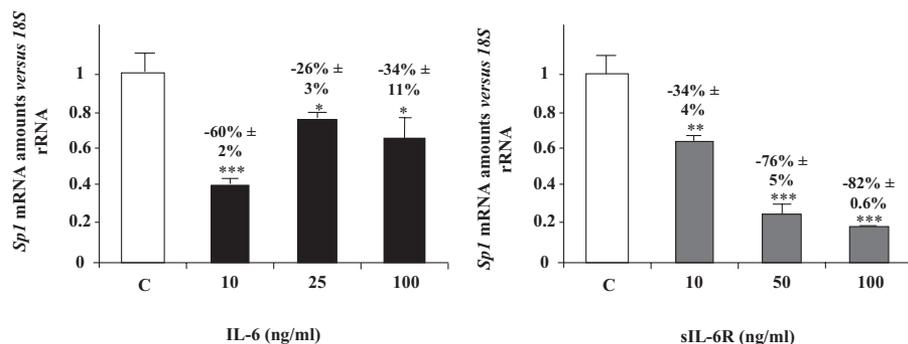


FIGURE 6. Effect of IL-6 and sIL-6R on the steady-state levels of Sp1 mRNAs in cultured chondrocytes. The same diluted cDNA as in Fig. 3 was analyzed by real-time RT-PCR using specific primers for Sp1 mRNA and 18S rRNA. Results, normalized to 18S rRNA and presented graphically as relative levels of Sp1 mRNA, represent means \pm S.D. of triplicate samples. C, control; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

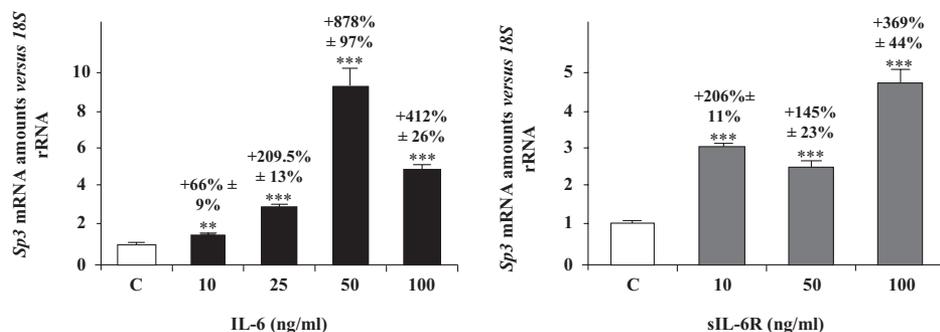


FIGURE 7. Effect of IL-6 and sIL-6R on the steady-state levels of Sp3 mRNAs in cultured chondrocytes. The same diluted cDNA as in Fig. 3, were analyzed by real-time RT-PCR using specific primers for Sp3 mRNA and 18S rRNA. Results are expressed as in Fig. 6. C, control; **, $p < 0.01$; ***, $p < 0.001$.

formed upon the incubation of the probe with primary RAC nuclear extracts. The DNA-binding activity of Sp1 and Sp3 involved in the formation of complex **a** was found to be decreased by IL-6 and sIL-6R. The cytokine or its soluble receptor alone decreases the **a** complex formation (data not shown). The binding activity of the transcription factors involved in the higher migrating complexes is not modulated by the cytokine and/or its soluble receptor, confirming its nonspecific binding. Similar data were obtained when the -50/+1 sequence was used as a probe (data not shown).

The implication of Sp1 and Sp3 in the formation of complex **a** is demonstrated by the use of antibodies directed against these two transcription factors. Addition of a Sp1 or a Sp3 antibody or both was found, at least in part, to prevent the **a** complex formation (Fig. 5A). Another approach has been used to demonstrate that complex **a** involved these Sp factors. For that purpose, chondrocytes were transfected with a control siRNA or with siRNAs directed against Sp1 and Sp3, and nuclear extracts were assayed in EMSA. As shown in Fig. 5B, transfection of the Sp1 or Sp3 or both siRNAs decreased the binding of **a** complex. In conclusion, these experiments indicate that IL-6 and/or sIL-6R decrease transcriptional activity of COL2A1 gene by reducing the binding of Sp1 and Sp3 to the -41/-33 bp sequence of the promoter.

IL-6 and/or sIL-6R Modulate the Expression of Sp1 and Sp3 in Chondrocytes—Next, we searched for a potential effect of IL-6·sIL-6R on the expression of Sp1 and Sp3. Real-time RT-PCR analysis was performed using total RNA extracted from

RAC treated or not by the cytokine and/or its soluble receptor. The steady-state levels of Sp1 were found to be decreased by IL-6, sIL-6R, or their combination (Fig. 6 and data not shown), whereas the steady-state levels of Sp3 mRNA were elevated by the cytokine and/or its soluble receptor (Fig. 7). Strictly similar data as the ones obtained with IL-6 alone were observed when the chondrocytes are incubated with both IL-6 and sIL-6R (data not shown). Again, the effect of IL-6 and/or its soluble receptor were not dependent on the dose in the concentration range used, and a concentration of 10 ng/ml produced significant effects.

Western blotting was also performed to determine if there was a correlation with the respective amounts of Sp1·Sp3 proteins present in nuclear and cytosolic extracts from RAC. As shown in Fig. 8, IL-6 and/or sIL-6R reduced significantly the amounts of Sp1 protein present in nuclear extracts by at least 2-fold. Similarly, the cytokine and/or its

IL-6·sIL-6R Repress COL2A1 Gene Transcription

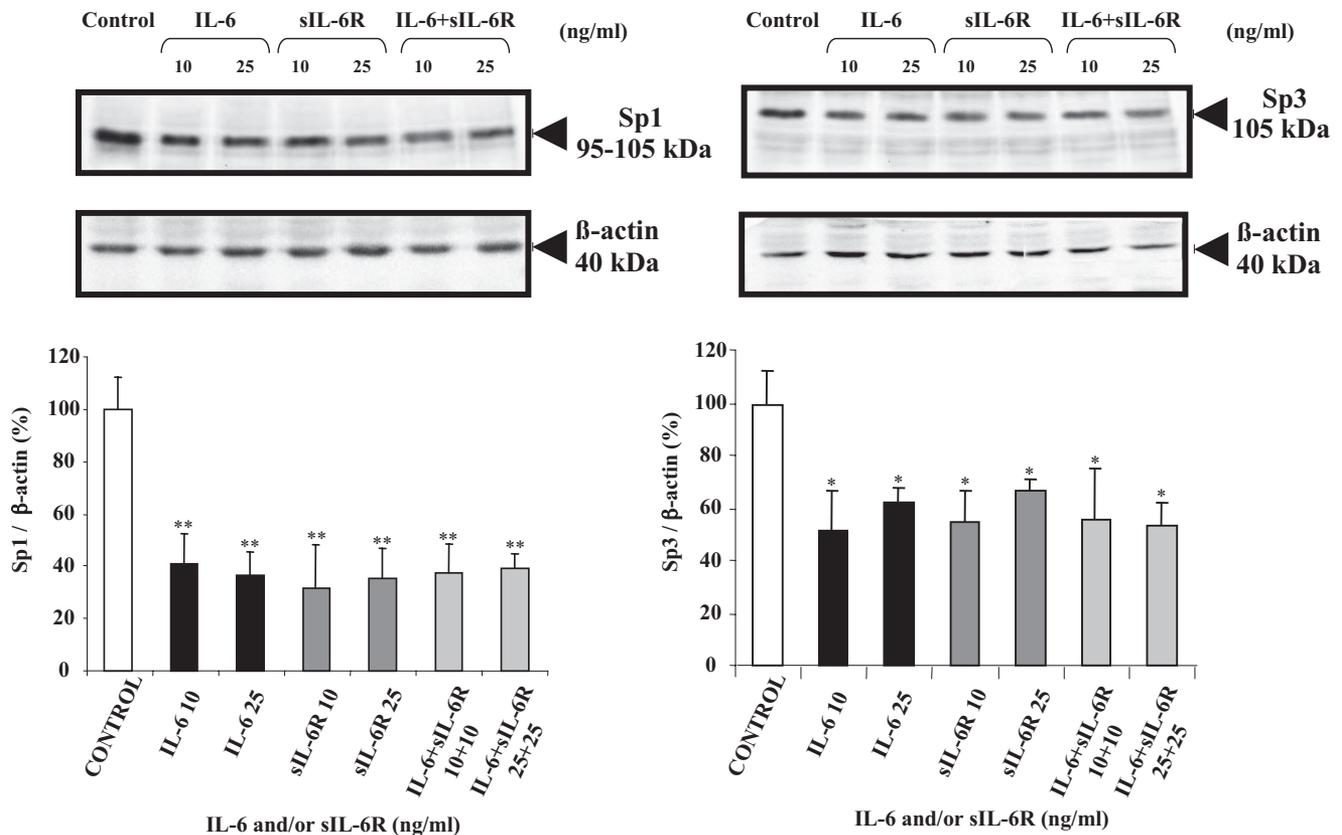


FIGURE 8. Effect of IL-6, sIL-6R, or both on Sp1 and Sp3 nuclear protein expressions in primary RAC. 15 μ g of RAC nuclear extracts, treated or not by IL-6, the soluble receptor or both (10 or 25 ng/ml), were subjected to Western blot. The membranes were immunoblotted with polyclonal antibodies directed against Sp1 or Sp3 (1/1000 dilution), and β -actin as a loading control (1/300 dilution). Then, the membranes were processed and quantified as described under "Experimental Procedures." Histograms represent expression of Sp1 and Sp3 normalized to β -actin protein signal. Results represent the means of three experiments \pm S.E. *, $p < 0.05$; **, $p < 0.01$.

soluble receptor produce a less depressing effect on Sp3 expression. Because the inhibitory effect of IL-6 and/or sIL-6R in Western blot experiments is more pronounced on Sp1 compared with Sp3, the cytokine and its soluble receptor are able to decrease the Sp1·Sp3 ratio.

To determine if there was a difference in the effects of IL-6 and/or sIL-6R on the expression of Sp1 and Sp3 in the cytosol and nuclear compartments, Western blotting was also performed on cytosolic extracts. Data showed that cytosolic expression of Sp1 and Sp3 is similarly affected by the cytokine and/or its soluble receptor compared with the effects observed on nuclear translocated Sp1 and Sp3, suggesting that IL-6 and/or sIL-6R probably exert a control on the translation rate of Sp3 (data not shown). Again, the decrease of Sp3 expression was less pronounced compared with Sp1 (data not shown).

We concluded that IL-6 and/or sIL-6R regulate the transcription of Sp1 without affecting its translocation. The mechanism of action of the cytokine and/or its soluble receptor on Sp3 expression seems to be more complex. They could induce a decrease in the translation rate or an increase in its protein degradation rate, because the steady-state levels of Sp3 mRNAs are increased, whereas both the protein level and binding activity of Sp3 are decreased.

A -41/-33-bp COL2A1 Sp Oligonucleotide, Used as a Decoy, Prevents IL-6- and/or sIL-6R-induced Inhibition of Type

II Collagen Expression Mediated by the Short 63-bp COL2A1 Promoter—Decoy experiments were performed to further delineate the involvement of Sp1 and Sp3 in IL-6·sIL-6R-induced down-regulation of *COL2A1* gene transcription through the 63-bp promoter. RACs were cotransfected with the pGL2-0.367kb and pGL2-0.110kb reporter plasmids in the presence of wild-type or mutated double-stranded oligonucleotides harboring the -41/-33 Sp-*cis*-acting element present in *COL2A1* promoter. The luciferase activity of reporter constructs was determined 24 h after the overnight transfection period following incubation or not with IL-6 and/or sIL-6R in the presence of decoy oligonucleotides. The data presented in Fig. 9 demonstrated that the expression of both reporter constructs was decreased under the treatment with the cytokine and/or its soluble receptor, in the absence of the decoy oligonucleotides. When chondrocytes were transfected and incubated in the presence of the -50/+1mc wt oligonucleotide, the decoy oligonucleotide prevented IL-6·sIL-6R-down-regulation of *COL2A1* transcription. By contrast, when the -50/+1 region was mutated in the -41/-33-bp sequence containing the Sp1·Sp3 binding site susceptible to mediate the IL-6·sIL-6R inhibitory effect and used as a decoy oligonucleotide, the inhibitory effect on the reporter construct was always effective. These data clearly demonstrate that IL-6 and/or its soluble receptor inhibit *COL2A1* gene transcription by the -41/-33-bp GC box, which binds Sp1 and Sp3, both of the latter

IL-6-sIL-6R Repress COL2A1 Gene Transcription

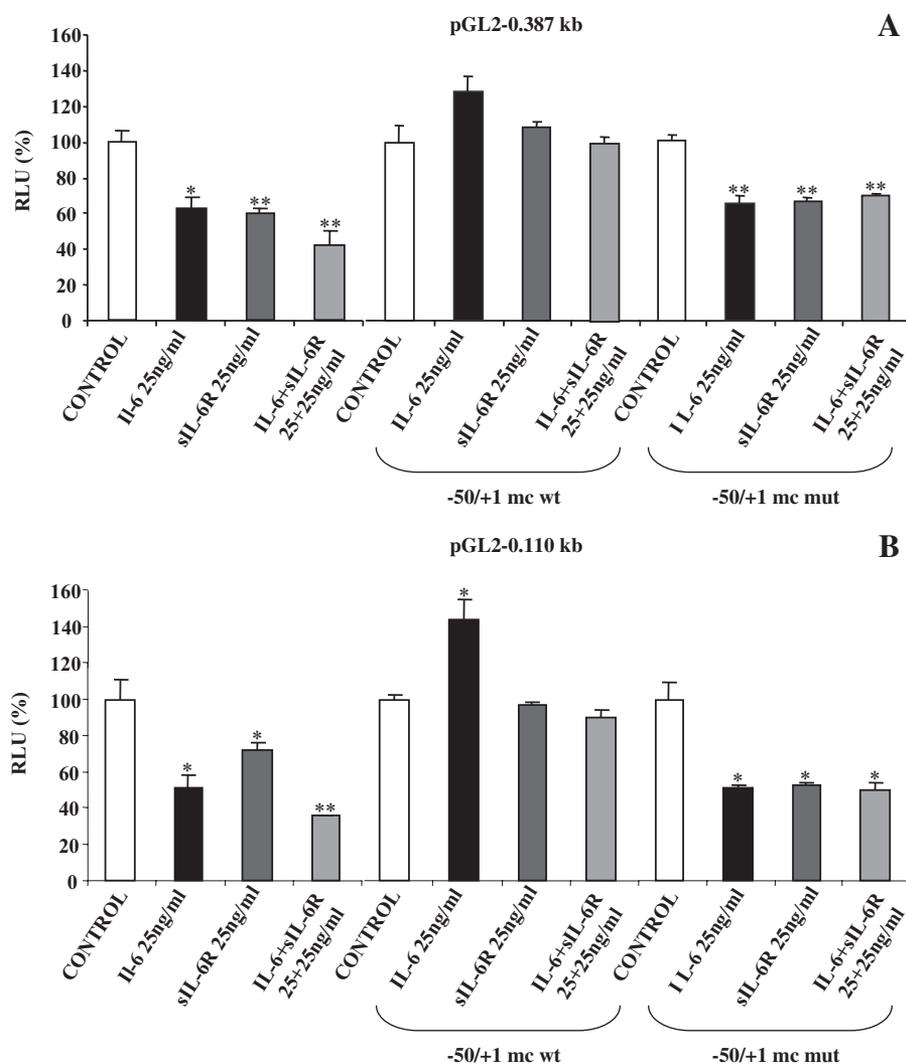


FIGURE 9. Involvement of the $-41/-33$ Sp sequence in IL-6-sIL-6R-induced inhibition of type II collagen gene expression. RAC cultures in 9.6-cm² dishes were transfected with 10 μ g of pGL2-0.387 kb (A) or pGL2-0.110 kb (B) constructs, pSV40 β -gal (2 μ g) and with 20 μ g of a multicopy (two copies) of the $-50/+1$ wild-type ($-50/+1$ mc wt) sequence found in *COL2A1* promoter or mutant counterpart ($-50/+1$ mc mut). The $-50/+1$ mc mut bears a mutation in the $-41/-33$ bp sequence centered by Sp1 DNA-binding site. After the transfection period, cells were incubated for 24 h in DMEM plus 2% FCS containing or not IL-6 and/or sIL-6R (25 ng/ml). During the incubation period, the decoy oligonucleotides were also added to the culture medium. 24 h later, the RLU were determined and expressed in % of IL-6-sIL-6R effects versus respective control and represent the mean \pm S.D. of three independent samples. *, $p < 0.05$; **, $p < 0.01$.

displaying a reduced binding activity to this *cis*-element. This mechanism leads to a decreased transcription activity.

Effects of Sp1 and Sp3 on the Transcriptional Activity of the 63-bp Promoter Mediating IL-6 and/or sIL-6R Inhibition of COL2A1 Gene Expression—To further investigate the transcriptional functions displayed by Sp1 and Sp3 in the IL-6 and sIL-6R down-regulation of human *COL2A1* gene transcription, cotransfections of RACs were performed with Sp1 and Sp3 expression vectors, together with the different *COL2A1* reporter plasmids, including pGL2-0.110 kb luciferase vector mediating the IL-6-sIL-6R-induced inhibition of transcription. We have previously demonstrated that Sp1 is a strong activator of the *COL2A1* promoter (8). By contrast, upon Sp3 overexpression, which was at that time encoded by a partial cDNA lacking the 5'-end, no transcriptional effect on *COL2A1* transcription was detected (8). Nevertheless, Sp3 was able to coun-

teract the Sp1 *trans*-activation of *COL2A1* gene. These effects were mediated by a short promoter containing a 266-bp region upstream the transcription start site of the human *COL2A1* gene (8).

Therefore, in the present study, we wanted to determine the function of forced expression of Sp3 encoded by a full-length cDNA on the expression of *COL2A1*. As shown in Fig. 10, we confirmed that Sp1 overexpression strongly activated the transcription of the pGL2-3.774 kb, pGL2-0.387 kb, and pGL2-0.110 kb constructs. The overexpression of Sp3 encoded by a full-length cDNA was shown for the first time to decrease the transcription of all the reporter constructs and of pGL2-0.110 kb. Moreover, forced Sp3 expression blocked the Sp1 induction of the *COL2A1* promoter activity, confirming our previous data (8).

Taken that Sp3 is capable of antagonizing Sp1 *trans*-activation and that Sp1-Sp3 binding activities are decreased, the present data further demonstrated that Sp1 and Sp3 proteins are responsible for mediating IL-6-sIL-6R-induced inhibition of the human *COL2A1* expression in primary RAC through the increase in Sp3-Sp1 ratio.

Sp1 and/or Sp3 siRNAs Prevent IL-6 and/or sIL-6R-induced Inhibition of Type II Collagen Expression—To finally demonstrate that Sp1-Sp3 decreased expression and binding activities are responsible for down-regulation of type II collagen

expression induced by IL-6 and/or sIL-6R, experiments with siRNAs directed against these two zing fingers factors were carried out. We first determined the efficiency of these siRNAs to block Sp1 and Sp3 expression by real-time RT-PCR assays. As shown in Fig. 11A, the reduced steady-state levels of Sp1 mRNAs were still observed under IL-6 and/or sIL-6R exposure of the RACs transfected with a control siRNA. Transfection of the cells with a Sp1 siRNA produced a decrease of $\sim 70\%$ on the levels of Sp1 mRNA in the control samples. In these conditions, IL-6 and/or sIL-6R did not reduce the amounts of Sp1 mRNA anymore (Fig. 11A). In parallel experiments, induction of Sp3 mRNA was observed under the effect of IL-6 and/or sIL-6R in RAC transfected with a control siRNA (Fig. 11B). Transfection of a Sp3 siRNA effectively reduced Sp3 mRNA levels by $\sim 60\%$. The induction of Sp3 mRNA expression caused by IL-6 and/or sIL-6R was not observed when a Sp3 siRNA was transfected in

the RAC (Fig. 11B). Having demonstrated the efficiency of the siRNAs directed against Sp1 and Sp3, the effect of Sp1·Sp3 expression blockade on IL-6 and/or sIL-6R down-regulation of type II collagen expression was then evaluated. We found that the transcription of the pGL2-0.110kb construct was decreased when a Sp1 siRNA was used, whereas it was increased when a Sp3 siRNA was transfected in the cells (Fig. 12A). These data validate the transcriptional functions of the two Sp factors.

Additionally, the transcription of the reporter construct was still reduced by IL-6 and/or sIL-6R treatment when a control siRNA was transfected (Fig. 12, B and C). In contrast, transfection of a siRNA directed against Sp1 alone and/or Sp3 was able to prevent the IL-6·sIL-6R-induced inhibition of COL2A1 transcription, suggesting that a heterotypic complex involving Sp1 and Sp3 is required to inhibit the transcription by the cytokine and its soluble receptor (Fig. 12, B and C).

This antisense strategy was also applied to estimate the observed effects of the cytokine and/or its soluble receptor at the level of COL2A1 mRNA amounts. Fig. 13A shows that a Sp1 siRNA decreased the steady-state levels of COL2A1 mRNA, whereas an Sp3 siRNA enhances the amounts of type II collagen mRNA. As shown in Fig. 13 (B–D), IL-6 and/or sIL-6R decreased the steady-state levels of COL2A1 mRNA when chondrocytes were transfected by a control siRNA. Once these cells were transfected with a Sp1 siRNA alone (Fig. 13B), or a Sp3 siRNA (Fig. 13C), or both (Fig. 13D), the inhibitory effect of the cytokine and/or its soluble receptor was no more observed.

To further demonstrate that a Sp1·Sp3 heteromeric could be involved in the inhibition of type II collagen expression by IL-6 and/or sIL-6R, immunoprecipitation experiments were performed using anti-Sp1 and anti-Sp3 antibodies.

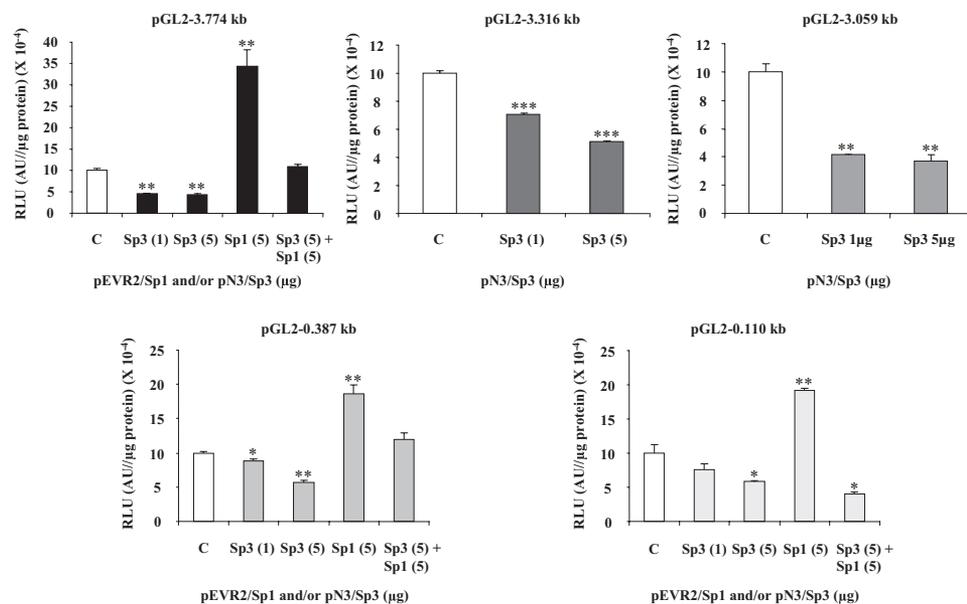


FIGURE 10. Sp3 represses COL2A1 gene transcription through the 63-bp promoter region responsive to IL-6, sIL-6R, or both and prevents Sp1 trans-activation. Primary RAC in 9.6-cm² dishes were cotransfected with 10 μg of different reporter constructs and the indicated amounts of Sp1 and/or Sp3 insertless expression vectors (pEV2 and pN3, respectively) and/or pEV2/Sp1 and/or pN3/Sp3. After overnight transfection, the medium was changed and 24 h later, the samples were harvested. The RLU values represent the mean ± S.D. of three independent samples. C, control; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

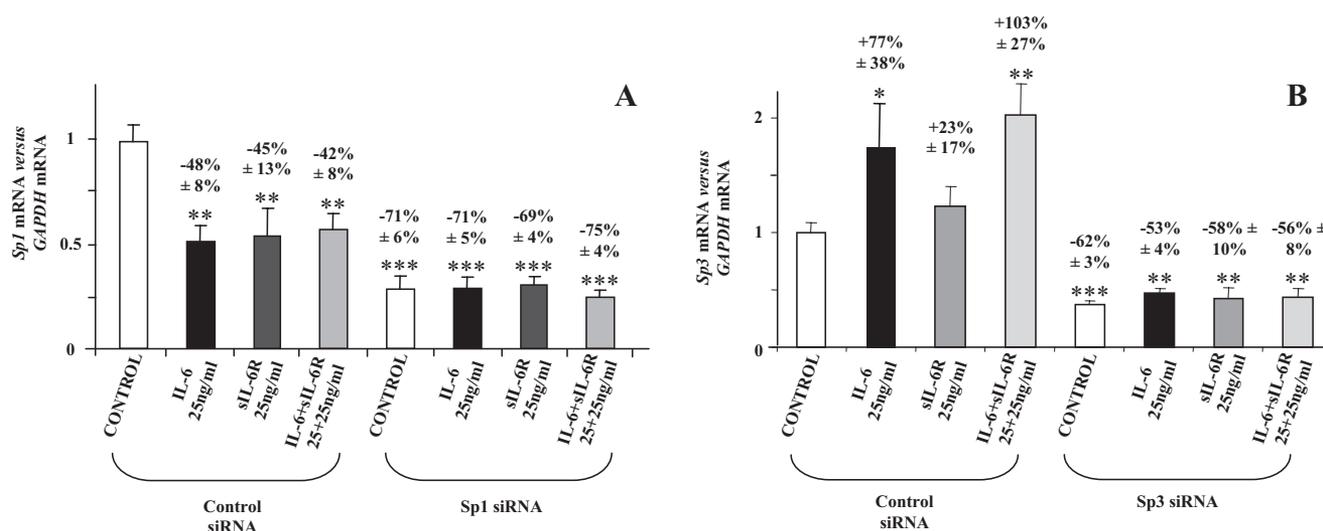


FIGURE 11. Effect of Sp1 and Sp3 siRNAs on IL-6·sIL-6R modulation of Sp1 and Sp3 expressions. RAC in 9.6-cm² dishes were transfected at 80% confluency with 2 μg of control siRNAs or siRNAs directed against Sp1 and Sp3. After overnight transfection, the medium was changed and the cells were incubated for 24 h in the presence of IL-6, the soluble receptor or both (25 ng/ml). At the end of the experiment, the cells were harvested and subjected to total RNA extraction. Total RNAs (2 μg) were reverse-transcribed into cDNA. The resulting products were diluted (1/100) and analyzed by real-time RT-PCR using specific primers for Sp1 mRNA, Sp3 mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Results, normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA and presented graphically as relative levels of Sp1 (A) and Sp3 (B) mRNAs, represent means ± S.D. of triplicate samples. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

IL-6-sIL-6R Repress COL2A1 Gene Transcription

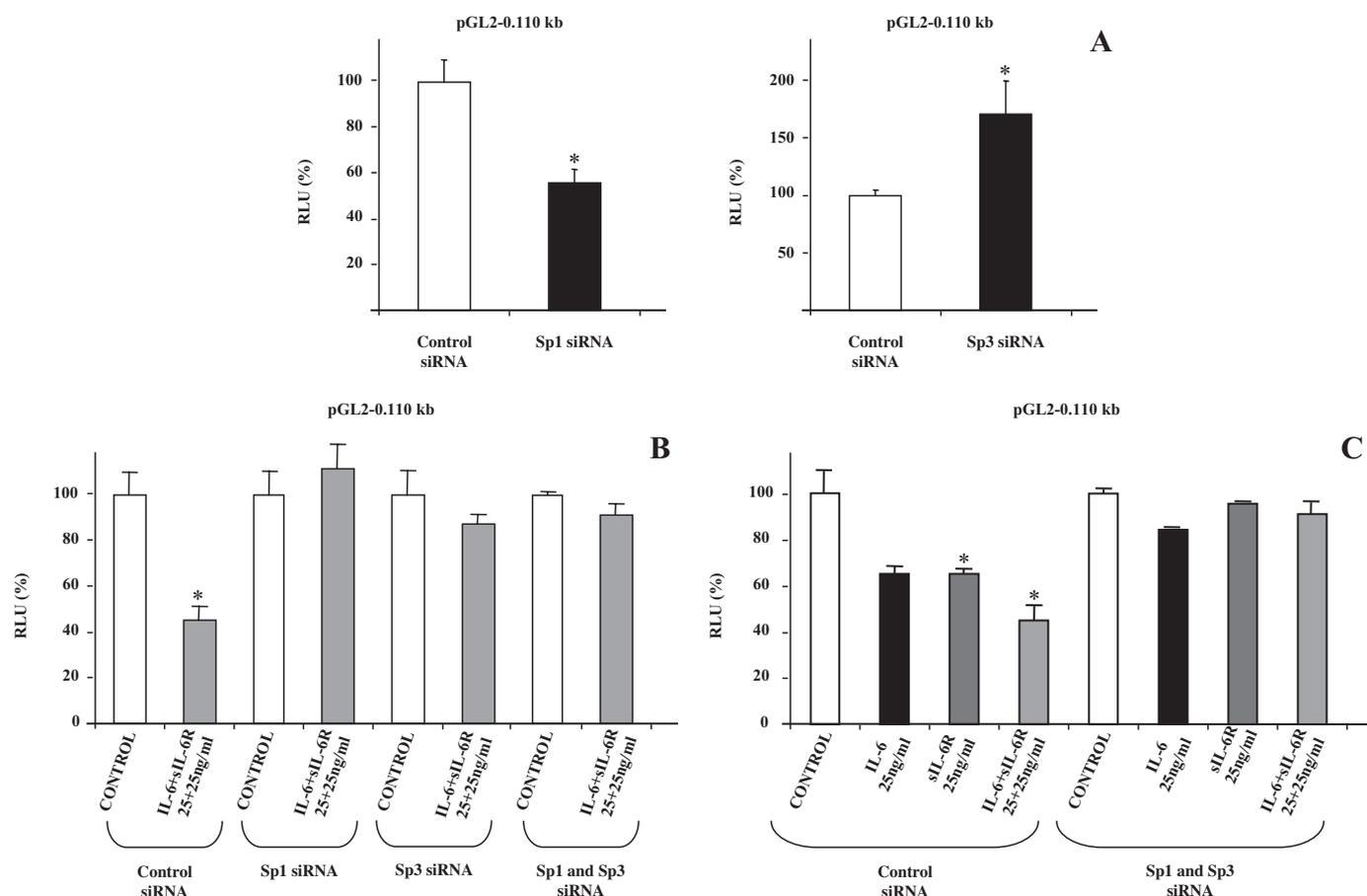


FIGURE 12. **Effect of Sp1 and Sp3 siRNAs on IL-6-sIL-6R-induced repression of the COL2A1 gene transcription.** RAC in 9.6-cm² dishes were cotransfected at 80% confluency with pGL2-0.110 kb construct, pSV40β-gal (2 μg), and 2 μg of control or siRNAs directed against Sp1 and/or Sp3. After overnight transfection, cell cultures were processed as described in Fig. 10. 24 h later, RLU were determined and are expressed in % versus control siRNAs (A) or versus each respective control (B and C) and represent the mean ± S.D. of three independent samples. *, $p < 0.05$.

Moreover, because HDACs have been associated to transcriptional repression, an immunoprecipitation was carried out with an anti-HDAC1 antibody. Then, immunoprecipitated proteins were subjected to Western blotting, and the membranes were incubated with Sp1, Sp3, and HDAC1 antibodies. As shown in Fig. 14, Sp3 and Sp1 were found to physically interact (Fig. 14, *ii*). Moreover, Sp1 co-immunoprecipitates with HDAC1 (Fig. 14, *i* and *iii*). By contrast, Sp3 was not able to interact with HDAC1 (Fig. 14, *ii* and *iii*).

All together, these findings strongly suggest that a heteromeric Sp1-Sp3 complex is necessary for fulfilling the inhibitory effect of IL-6 and/or sIL-6R on type II collagen expression; and that HDAC1 is associated with this transcription inhibition complex.

Sp1 and Sp3 Bind to the COL2A1 Promoter in Vivo—To determine if Sp1 and Sp3 are effectively involved in the modulation of COL2A1 gene by IL-6 and its soluble receptor, and to validate our *in vitro* experimental data, ChIP assay was performed. As shown in Fig. 15, approximately similar amount of Sp1 and Sp3 are recruited onto the promoter region of COL2A1 in control conditions. When the chondrocytes are incubated in the presence of IL-6-sIL-6R, although Sp1 was still bound to the COL2A1 promoter but in lower amounts, higher amounts of Sp3 interact with this sequence compared with control sample indicating that the Sp3:Sp1 ratio is increased.

ChIP experiments performed on this region of COL2A1 gene using an STAT3 antibody failed to demonstrate any interaction of this transcription factor with the promoter in basal conditions, as well as any involvement of STAT3 in the down-regulation of COL2A1 gene expression induced by IL-6 and its soluble receptor (data not shown).

DISCUSSION

During the degenerative pathologies of cartilage, pro-inflammatory cytokines such as IL-1β and tumor necrosis factor-α exert a deleterious effect on the components of the cartilage ECM by altering the chondrocyte metabolic phenotype. However, IL-6 may also participate in these pathological processes, as a synergistic partner whose expression is induced by IL-1β.

The levels of IL-6 in the synovial fluid and serum from patients affected by rheumatoid arthritis or by osteoarthritis are significantly more elevated compared with healthy subjects (21, 44). Furthermore, it is established that IL-6, LIF, and oncostatin M contribute directly to the degradation of ECM. The work performed by Qing Li *et al.* (45) underlines the stimulating effect of oncostatin M on MMP types 1, 3, and 13 in proliferating cultures of bovine chondrocytes. Similarly, LIF has been shown to stimulate the catabolism of proteoglycans in goat cartilage explants (46).

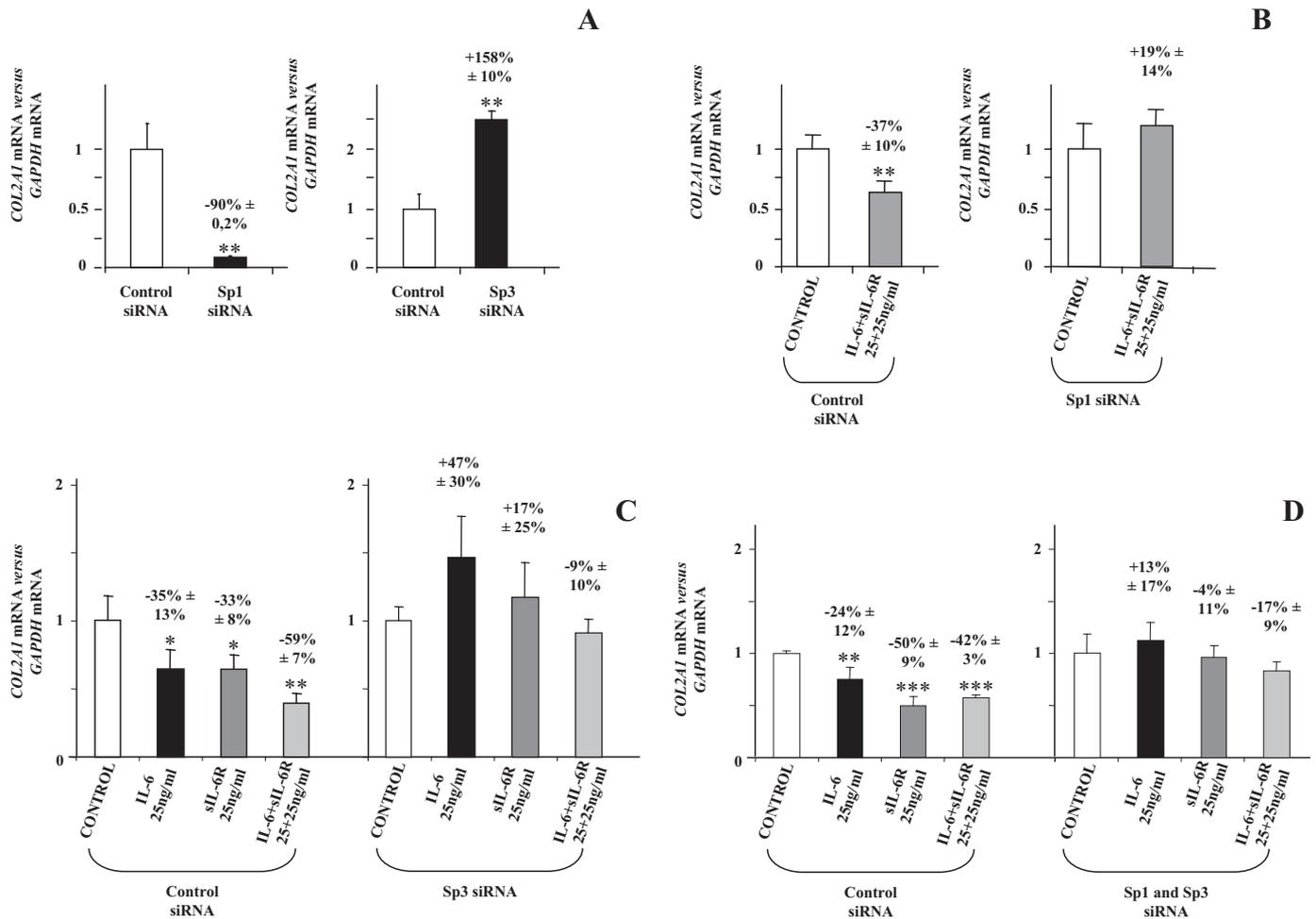


FIGURE 13. Effect of Sp1 and Sp3 siRNAs on IL-6-sIL-6R-induced decrease of the steady-state levels of COL2A1 mRNA. RAC in 9.6-cm² dishes were transfected and processed as described in Fig. 11. At the end of the experiment, the cells were harvested and subjected to total RNA extraction followed by real-time RT-PCR. Expression of COL2A1 transcripts was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and presented graphically as relative mRNA levels of COL2A1 versus respective control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. A, effect of Sp1 and Sp3 siRNAs on COL2A1 mRNA amounts in control conditions. B, effect of Sp1 siRNAs on COL2A1 mRNA amounts in the presence of both IL-6-sIL-6R. C, effect of Sp3 siRNAs on COL2A1 mRNA amounts in the presence of IL-6, sIL-6R, or both. D, effect of combined action of Sp1 and Sp3 siRNAs on COL2A1 mRNA amounts in the presence of IL-6, sIL-6R, or both.

For these reasons, we investigated the effects of IL-6 and its soluble receptor on the expression of type II collagen, at three levels: type II collagen protein expression, COL2A1 mRNA amounts, and the activity of COL2A1 gene transcription. In view of the contradictory reports concerning the action of this cytokine and/or its soluble receptor on the ECM components, each partner was studied separately and in combination.

IL-6, but also sIL-6R, were found to induce a down-regulation of type II collagen expression, and the addition of sIL-6R is not required to observe the effect of the cytokine. A correlation was observed between type II collagen protein amounts and the related COL2A1 mRNA levels, indicating that the effect of IL-6, with or without its soluble receptor, resulted from a transcriptional control.

Our transfection experiments demonstrated that this inhibition is mediated by the -63/-35-bp sequence of the COL2A1 promoter. This effect requires HDAC activity as demonstrated by the use of trichostatin A (data not shown) and immunoprecipitation studies revealing an interaction between Sp1 and HDAC1.

In our functional assays, decoy oligonucleotides containing the wild-type -41/-33-bp cis-element transfected in the RACs were able to prevent IL-6- and/or sIL-6R-induced inhibition of COL2A1 gene transcription, whereas point mutations in this sequence did not eliminate IL-6/sIL-6 repression. Therefore, the function of the -41/-33-bp sequence is strongly dependent on Sp1-Sp3, as demonstrated in EMSA where the DNA-binding activity of these transcription factors was clearly decreased by the cytokine and/or its soluble receptor.

This finding is of interest, because other cytokines were shown to act through the same region to down-regulate transcription of COL2A1 gene. For example, Osaki *et al.* (47) have reported that repression of COL2A1 gene transcription by interferon- γ requires STAT1- α and implies a core promoter region spanning -45 to +11 bp, which contains the TATA-box and GC-rich sequences but no STAT-binding elements. They suggest indirect interaction of activated STAT1- α with the general transcriptional machinery that drives constitutive COL2A1 expression. Furthermore, the STAT inhibitor parthenolide was capable of abrogating the IL-6-sIL-6R decrease of COL2A1 mRNA (32). Arguing in this sense, it has been reported that

IL-6/sIL-6R Repress COL2A1 Gene Transcription

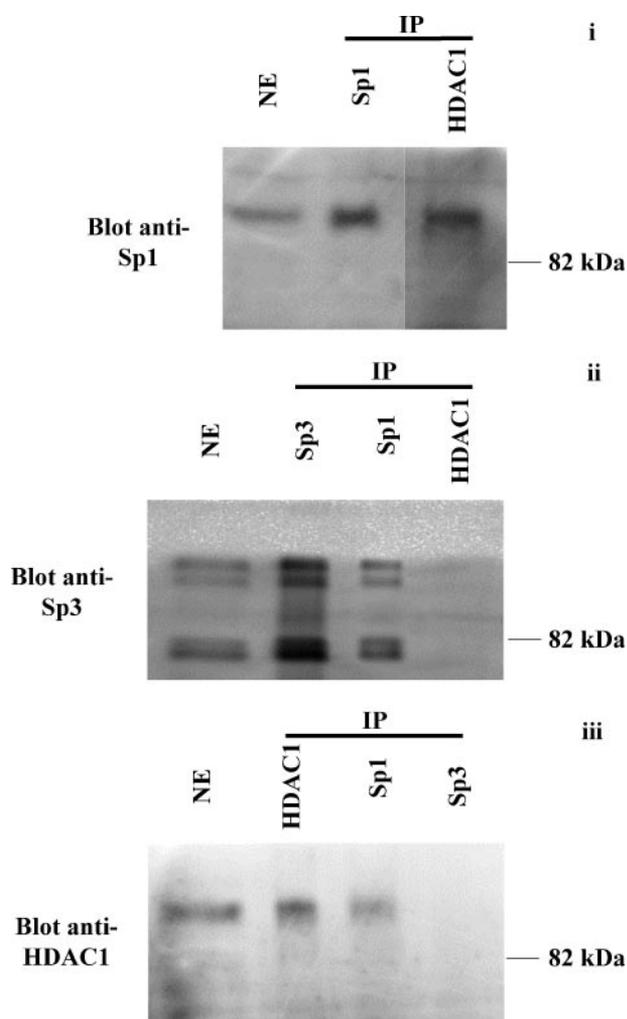


FIGURE 14. Sp1 interacts with Sp3 and HDAC1 in chondrocytes. After immunoprecipitations using either anti-Sp1, anti-Sp3, or anti-HDAC1 antibodies, nuclear extracts from control RAC cultures were subjected to Western blotting. The membranes were immunoblotted with antibodies directed against Sp1 (*i*), Sp3 (*ii*), or HDAC1 (*iii*) (1/1000 dilution). Then, the membranes were processed and quantified as described under "Experimental procedures." As positive control, 15 μ g of nuclear extracts (NE), which was not subjected to immunoprecipitation, were also loaded onto SDS-PAGE gels.

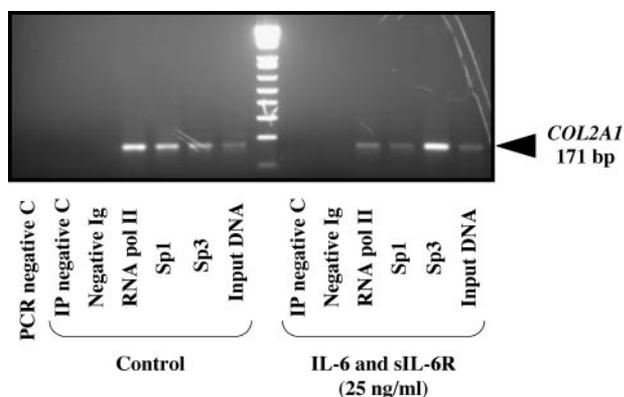


FIGURE 15. Sp1 and Sp3 bind to the COL2A1 gene promoter in ChIP assay. RAC treated or not with IL-6 and sIL-6R were fixed, digested, and immunoprecipitated with Sp1 and Sp3 antibodies, followed by PCR amplification with primers flanking the $-41/-33$ Sp-*cis*-acting element present in COL2A1 promoter. As positive control (*Input*), the same primers were used on the genomic DNA. Samples were also immunoprecipitated with anti-RNA pol II used as another positive control and with nonspecific immunoglobulin antibody (*Ig*, control *Ig*) used as a negative control. C, control.

STAT3 can physically interact with Sp1 in co-immunoprecipitation assays (48), leading to transcriptional cooperativity (49).

The study of Tan *et al.* (50) reported that another cytokine, IL-1 β , reduces COL2A1 gene transcription by increasing the expression of Egr-1, as well as its binding activity to the $-119/-112$ bp region of COL2A1 promoter. As a consequence, Sp1, which can also bind to this sequence, could no longer exert its transactivating effect. However, we previously found that IL-1 β and TGF- β 1 inhibited COL2A1 transcription via the 63-bp promoter region by decreasing the binding activity of Sp1 and Sp3 and the Sp1·Sp3 ratio (8, 36). Indeed, Sp3 antagonizes the transactivating effects of Sp1 by binding to the same *cis*-acting element localized between -41 and -33 bp. A similar mechanism appears to apply also to the IL-6/sIL-6R effect observed in the present study.

Another work from our laboratory reported a decrease of SOX9 expression associated with the parallel reduction in the amounts of COL2A1 mRNA under incubation in the presence of both IL-6/sIL-6R (32). However, we also found that either IL-6 or sIL-6R alone are able to decrease the steady-state levels of SOX9 mRNA by 55 and 69%, respectively (data not shown). Because several SOX9-interacting transcription factors or cofactors involved in tissue-specific expression of COL2A1 have been identified (3, 4), in parallel experiments, we demonstrated that IL-6, sIL-6R, or both decrease also the mRNA steady-state levels of some of these proteins, *i.e.* L-Sox5 ($\sim 25-60\%$), SOX6 ($\sim 42-55\%$), and p300 ($\sim 40-55\%$) (data not shown). Indeed, an Sp3 siRNA was found to increase the amounts of SOX9 mRNA by $\sim 70\%$ (data not shown). Given that transcription factors are known to act in concert, it is conceivable that IL-6/sIL-6R *in vivo* effect could involve Sp1·Sp3, the STATs, p300 and the SOXs, in a complex capable of maintaining an open chromatin network around the constitutive COL2A1 promoter. Therefore, we may postulate that the effect of IL-6/sIL-6R on COL2A1 transcription involves the two main regulatory regions of that gene, *i.e.* the proximal promoter and the first intron, that are responsible for high level and tissue-specific expression of the gene (4, 5, 51).

From the data obtained, we may hypothesize that IL-6 and sIL-6R also involve changes in the Sp1·Sp3 ratio to down-regulate type II collagen expression at the transcriptional level. Several approaches support this hypothesis. First, IL-6 and/or sIL-6R are capable of decreasing the expression of the β -galactosidase gene. This gene was cloned downstream to the SV40 promoter where several binding sites for the members of the Sp family are present (GC boxes). Although the variations of β -galactosidase activity reflect generally variations of transfection efficiency in transcriptional studies, we found a clear reduction of enzyme activity in all our assays, whatever the COL2A1 gene construct used. Then, Western blotting and EMSA indicated that IL-6 and/or sIL-6R exert a repressive effect on the Sp1 and Sp3 translocated into the nucleus, as well as on their DNA-binding activity to the $-41/-33$ -bp responsive element of COL2A1. Moreover, the inhibitory effect of IL-6 and/or sIL-6R in Western blot experiments on cytosolic and nuclear extracts is more pronounced on Sp1 compared with Sp3. Finally, Sp3 seems to be more efficiently recruited on the COL2A1 promoter in ChIP assays, when compared with Sp1. Overall, these

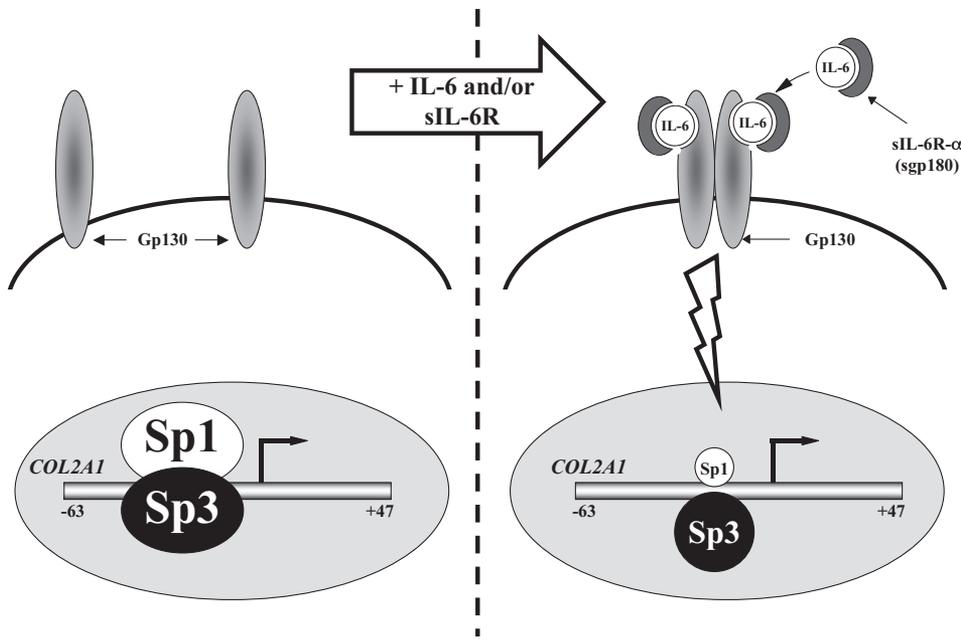


FIGURE 16. Diagram illustrating the structure of the proximal *COL2A1* promoter and the Sp1·Sp3 interactions under basal and IL-6/sIL6R culture conditions.

data justify that IL-6 and sIL-6R decrease the Sp1·Sp3 ratio as illustrated in Fig. 16.

Sp transcription factors have already been shown to mediate IL-6 effect on transcription of genes, including low density lipoprotein receptor gene and the *C/EBPδ* gene (49, 52). Despite their different transcriptional functions, Sp1 and Sp3, generally compete for the same DNA-binding sites. *In situ*, several studies have demonstrated that both factors are associated to DNA-binding sites of a variety of promoters (53–57). They form homotypic and heterotypic complexes with numerous proteins, including other Sp family members (58–60). These complexes determine the role of Sp proteins as either transcriptional activators or repressors. Some studies reported the existence of a Sp1·Sp3 complex in different cellular models (61, 62), whereas other demonstrated that Sp1 and Sp3 do not form a heteromeric complex (63). From our experiments with siRNAs directed against Sp1 and Sp3, we deduced that inhibition of either Sp1 or Sp3 alone is sufficient to prevent IL-6 and/or sIL-6R down-regulation of *COL2A1* gene transcription, suggesting that a heteromeric complex is required. An explanation for such effects would be that the **a** complex observed in EMSA with the wild-type –50/–26 bp probe (and –50/+1 probe, data not shown) is formed by a ternary complex in which a Sp1·Sp3 protein-protein interaction binding to the probe mediates the inhibitory effect of the cytokine and/or its soluble receptor, rather than representing two protein monomer·DNA complexes comigrating at the same place in the gel. Indeed, antibody interference assays in EMSA demonstrate that both factors are implicated in complex **a**. Therefore, it is likely that Sp3 alone, even in greater amounts in complex **a**, is not sufficient to mediate the cytokine/sIL-6R inhibition. A heteromeric Sp1·Sp3 complex is rather necessary for such an effect, and our immunoprecipitation experiments demonstrating that Sp1 and Sp3 co-immunoprecipitate corroborate this hypothesis. This is in agreement with the fact that IL-6 has been shown to stimu-

late low density lipoprotein receptor gene expression via binding activation of both Sp1 and Sp3 (52). The only difference with our data is that we deal with a down-regulation by the IL-6·sIL-6R and that the effect involves decreased binding activity of the two Sp factors.

Sp1 and Sp3 cooperativity has also been shown to be necessary for driving the expression of type X collagen gene in hypertrophic chondrocytes together with increased Sp3·Sp1 ratio (64). Dedifferentiation of the articular chondrocyte from the resting zone of cartilage as well as differentiation from pre-hypertrophic to hypertrophic phenotype, are characterized by the repression of type II collagen synthesis and, in the latter case, by an increase in type X collagen expression. These events suggest that the

increase in Sp3·Sp1 ratio could be a general mechanism affecting the expression of different collagen isotypes as a function of differentiation state of the cells (6, 7, 65).

Our studies have been performed with doses of sIL-6R in the range of the concentrations detected in the sera or synovial fluid of pathological and nonpathological donors (22, 65). sIL-6R acts in our system as an agonist of IL-6 functions, because it exerts inhibitory effects on type II collagen gene transcription of the same magnitude as the cytokine, and combination of both the cytokine and the soluble receptor does not produce a synergistic effect. The absence of a synergistic mechanism can also be explained by the endogenous synthesis of both IL-6 and sIL-6R by chondrocytes (22, 66). Enzyme-linked immunosorbent assay revealed that our chondrocyte model synthesizes IL-6 at a concentration of ~15 pg/ml culture medium (data not shown). Therefore, it cannot be excluded that the use of higher concentrations of exogenous IL-6 and/or sIL-6R should be necessary to observe a synergistic mechanism of action. Nevertheless, the overall effect of the cytokine and/or sIL-6R is clearly inhibitory on the expression of two of the phenotypic markers of chondrocytes, *i.e.* type II collagen and aggrecans (Refs. 30 and 32 and this study).

The present work, together with the data of Legendre *et al.* (33) demonstrating that IL-6·sIL-6R up-regulate MMP-1, -3, and -13 and ADAMTS-4 and -5/11 gene expression in chondrocytes, suggests a repressive and pro-catabolic role of IL-6·sIL-6R in osteoarthritis and rheumatoid arthritis, in association with tumor necrosis factor- α and IL-1 β , this latter being able to induce IL-6 and LIF expression in articular chondrocytes through the extracellular signal-regulated kinase signaling pathway (66).

Nevertheless, further research is required to determine the role of STAT1 and STAT3 in the down-regulation of type II collagen in chondrocytes, because previous data from our laboratory have shown that inhibition of the STAT pathway

IL-6-sIL-6R Repress COL2A1 Gene Transcription

suppressed the IL-6-negative effect on type II collagen, aggrecan core, and link protein synthesis (32). However, the main regulating regions of *COL2A1* (promoter and first intron) do not contain any STAT binding element. Nevertheless, a protein-protein type of interaction between the STATs and Sp1-Sp3 and/or with the complex of transcription initiation, via an association with the transcriptional cofactor p300/CBP, has been suggested (47). Furthermore, a regulation of Sp1 and/or Sp3 expressions by the STATs is also possible. In addition, IL-6 has been shown to induce vascular epidermal growth factor transcription and secretion in human glioblastoma cells through involvement of STAT3 (67). The promoter region of vascular epidermal growth factor responsive to the cytokine is centered by a GC box that binds Sp1-Sp3 but does not contain a STAT3 binding element. In that system, IL-6 induces a necessary physical interaction of STAT3 with Sp1, so that a novel transcriptional activation mechanism for STAT3 in the context of a STAT3 binding element-free promoter is possible (67). However, our ChIP experiments failed to demonstrate the interaction of STAT3 with the *COL2A1* proximal promoter, but such a mechanism is likely to occur in our chondrocyte model with other STATs, because we have shown that STATs are implicated in IL-6-sIL-6R down-regulation of the major cartilage-specific matrix genes (32). Work is in progress to determine if other STATs could interact with Sp1-Sp3 and/or the basal transcription machinery in our system.

In summary, our data further corroborate that the Sp3-Sp1 ratio is a critical parameter for *COL2A1* transcriptional regulation and targeting Sp3 in the future could be a key approach to allow the recovery of the differentiated cartilage phenotype in osteoarthritis chondrocytes.

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Interleukin-6 (IL-6) and/or Soluble IL-6 Receptor Down-regulation of Human Type II Collagen Gene Expression in Articular Chondrocytes Requires a Decrease of Sp1-Sp3 Ratio and of the Binding Activity of Both Factors to the *COL2A1* Promoter

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