10-24-1997

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 43, Issue of October 24, pp. 26918–26925, 1997

Printed in U.S.A.

Detection and Characterization of Sp1 Binding Activity in Human Chondrocytes and Its Alterations during Chondrocyte Dedifferentiation*

(Received for publication, December 18, 1996, and in revised form, June 27, 1997)

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We have detected DNA binding activity for a synthetic oligonucleotide containing an Sp1 consensus sequence in nuclear extracts from human chondrocytes. Changes in the levels of Sp1 oligonucleotide binding activity were examined in nuclear extracts from freshly isolated human chondrocytes, from chondrocytes that had been cultured under conditions that allowed the maintenance of a chondrocyte-specific phenotype on plastic dishes coated with the hydrogel poly(2-hydroxyethyl methacrylate), and from chondrocytes induced to dedifferentiate into fibroblast-like cells by passage in monolayer culture on plastic substrata. It was observed that Sp1 binding was 2–3-fold greater in nuclear extracts from dedifferentiated chondrocytes than in nuclear extracts from either freshly isolated chondrocytes or from cells cultured in suspension. The Sp1 binding activity was specific, since it was competed by unlabeled Sp1 but not by AP1 or AP2. The addition of a polyclonal antibody against Sp1 to nuclear extracts from freshly isolated chondrocytes or to extracts isolated from chondrocytes cultured in monolayer decreased the binding of Sp1 by ~85%. However, when the same experiment was carried out with nuclear extracts prepared from cells cultured on poly(2-hydroxyethyl methacrylate)-coated plates, only a very slight inhibition of Sp1 binding was observed. When fragments of the *COL2A1* promoter containing putative Sp1 binding sites amplified by polymerase chain reaction were examined, it was found that the amounts of DNA-protein complex formed with nuclear extracts from dedifferentiated chondrocytes were 2–3-fold greater than the amounts formed with nuclear extracts from freshly isolated chondrocytes or from cells cultured in suspension. Quantitation of DNA binding activity by titration experiments demonstrated that nuclear extracts from fibroblast-like cells contained approximately 2-fold greater Sp-1 specific binding activity than nuclear extracts from chondrocytes. The direct role of Sp1 in type II collagen gene transcription was demonstrated by co-transfection experiments of *COL2A1* promoter-CAT constructs in *Drosophila* Schneider line L2 cells that lack Sp1 homologs. This is the first demonstration of Sp1 binding activity in human chondrocytes and of differences in Sp1 DNA binding activity between differentiated and dedifferentiated chondrocytes.

* This work was supported by National Institutes of Health Program Project Grant AR39740 (to S. A. J.) and Training Grant AR07583 (to G. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The extracellular matrix of articular cartilage consists of a large number of tissue-specific macromolecules including type II, IX, and XI collagens and the large aggregating proteoglycan, aggrecan (1). These extracellular matrix components are produced by chondrocytes, highly differentiated cells responsible for the maintenance of the structural integrity of the tissue through a precisely regulated balance between the synthesis and the degradation of these cartilage-specific macromolecules. The biosynthetic program of chondrocytes is determined by the highly conserved expression of a set of cartilage-specific genes (type II, IX, and XI collagens and the proteoglycan aggrecan), which is maintained during complex biological processes such as cartilage development, differentiation, and repair (2).

Most of the studies that examined the stability of the chondrocyte phenotype have consistently shown that culture of these cells in monolayers on plastic substrata for prolonged periods or upon repeated passages leads to the loss of their spherical shape and to the acquisition of an elongated fibroblast-like morphology (3–10). These morphologic alterations are accompanied by profound biochemical changes including the loss of production of cartilage-specific macromolecules, initiation of synthesis of the interstitial collagens (types I, III, and V), and an increase in the synthesis of fibroblast-type proteoglycans (versican) at the expense of aggrecan (3–12). The chondrocyte-specific phenotype can be reexpressed when these cells are cultured in agarose or alginate matrices (6, 10, 11) or, as shown in our recent studies, by culture on a hydrogel (12).

Few studies have been performed to characterize the transcriptional activity and regulation of the promoter of the cartilage-specific type II procollagen gene (*COL2A1*) despite the crucial role that its encoded product plays in the maintenance of the structure and function of articular cartilage. Structural and functional analyses of the promoter regions of *COL2A1* have revealed multiple putative regulatory elements (13–17). Electrophoretic mobility shift assays employing bp −977 to −30 of the *COL2A1* promoter and nuclear extracts from chick embryonic chondrocytes indicated the involvement of an Sp1-like factor in the cartilage-specific expression of the gene, since the addition of anti-Sp1 antibodies to the binding reaction caused a supershift of the DNA-protein complex (16). Moreover, short mutations in the Sp1 binding sites abolished the formation of the DNA-protein complex (16). DNase I footprint analysis indicated that a sequence between bp −132 and −101 of the *COL2A1* promoter bound nuclear proteins isolated from chick embryonic chondrocytes (16). Western/Southwestern analyses showed that a protein complex that included Sp1 could bind to the *COL2A1* promoter and enhancer under nondenaturing conditions and was dissociated under denaturing conditions. These results suggested the formation of a DNA loop structure between the *COL2A1* promoter and enhancer that is mediated by nuclear proteins (16) and clearly indicated

the importance of transacting factors in the regulation of expression of *COL2A1*. However, studies of the changes in DNA-binding proteins that may occur during chondrocyte dedifferentiation have not been examined in detail, although one study showed that chondrocyte dedifferentiation was associated with the induction of nuclear factor binding activity for an AP-1 site and with a concomitant activation of pro-α1(I) collagen gene transcription (18). In this study we investigated the changes in the levels and activity of the transcriptional factor Sp1 occurring during the process of chondrocyte dedifferentiation.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Human Chondrocytes—Human fetal epiphyseal cartilage was removed under sterile conditions from femoral heads, knee condyles, and tibial plateaus from spontaneous abortions. The tissues were obtained from the International Institute for the Advancement of Medicine (Philadelphia, PA), following protocols reviewed and approved by the National Institutes of Health and the Institutional Review Committee in accordance with the National Organ Transplant Act and the Pennsylvania Organ Transplant Act. To remove adherent fibrous tissues, the cartilage was incubated in Hanks' medium containing trypsin and bacterial collagenase (2 mg/ml each) for 1 h at 37 °C. The medium was discarded, and the tissue fragments were minced and digested overnight at 37 °C in Dulbecco's minimum essential medium with 4.5 g/liter glucose containing 10% fetal bovine serum and 0.5 mg/ml bacterial collagenase. The cells released by the enzymatic digestion were filtered through a nylon membrane into a vessel containing fresh Dulbecco's minimum essential medium and 10% fetal bovine serum. The cells were collected by centrifugation at 250 $\times$ *g* for 5 min, resuspended, and washed four times with collagenase-free medium. The average yield was 3.0 $\pm$ 0.4 $\times$ 10^8 chondrocytes/g, wet weight, of cartilage.

The isolated chondrocytes were cultured at a density of 5 $\times$ 10^6 cells in 60-mm plastic dishes previously coated with 0.9 ml of a 10% (v/v) of polyHEMA[1] (19, 20) (PolySciences Inc. Malvern, PA) following a procedure modified from that described by Folkman and Moscona (21) as described previously (12). For coating the culture dishes with polyHEMA, 0.9 ml of a 10% (v/v) solution of polyHEMA in 95% ethanol was layered onto 60-mm Falcon bacterial culture dishes and was allowed to dry overnight under a tissue culture hood. The polyHEMA-coated dishes were sterilized by exposure to bactericidal ultraviolet light for 30 min. The culture medium employed for these studies was Dulbecco's minimum essential medium containing 4.5 g/liter glucose, 10% fetal bovine serum, 1000 units/ml penicillin, 1 mg/ml streptomycin, 2 mM glutamine, 1% vitamin supplements, 2.5 μg/ml fungizone, and 50 μg/ml ascorbic acid. The medium was replaced every 3–4 days. The differentiated chondrocytes were those cultured on poly(HEMA) dishes. The dedifferentiated chondrocytes were those that were plated at a density of 2.5 $\times$ 10^6 cells on T75 flasks so that they would grow as attached cells in the same medium as that used for growing cells on poly(HEMA)-coated dishes and passaged twice at 20-day intervals.

Western Blot Analysis—For Western blot analysis, freshly isolated chondrocytes, chondrocytes that had been cultured on poly(HEMA)-coated plates, and fibroblast-like chondrocytes that were passaged on plastic were utilized. The cells were suspended and then centrifuged at 3,000 rpm for 5 min and washed twice with phosphate-buffered saline. They were heated to 100 °C for 5 min in 1% SDS, 50 mM DTT, and 1% (v/v) glycerol, and the cell-associated proteins were separated by electrophoresis in 6% polyacrylamide gels on a minigel apparatus at 125 V for 90 min. The proteins were electroblotted at 40 V for 90 min from the polyacrylamide gel onto a supported nitrocellulose membrane, and the transferred proteins were reacted for 30 min with a 1:500 dilution (v/v) of SJ 441 antibody, which is a polyclonal antibody specific for the COOH-terminal telopeptide of human type II collagen (22), or with an anti-type I human collagen polyclonal antibody purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). The proteins on the filter were detected utilizing the ECL Western blotting detection reagent (Amersham Corp.).

Preparation of Nuclear Extracts—Chondrocyte nuclear extracts were prepared according to the procedures of Dignam *et al.* (23). The cells were pooled and washed in phosphate-buffered saline (10 mM phosphate buffer, pH 7.4, 137 mM NaCl, and 2.7 mM KCl). The resulting cell pellets were resuspended in a hypotonic buffer (10 mM HEPES, pH 7.9, at 4 °C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonic fluoride, and 0.5 mM DTT) approximately 5 times the packed cell volume and centrifuged at 3000 rpm for 5 min. The pellet was resuspended in 3 times the original packed cell volume in hypotonic buffer and incubated for 10 min on ice. Next, the cells were homogenized slowly with 10 strokes in a Dounce homogenizer, and the nuclei were collected by centrifugation at 4000 rpm for 15 min. The nuclei were resuspended in one-half packed nuclear volume of low salt buffer (20 mM HEPES, pH 7.9, at 4 °C, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonic fluoride, 0.5 mM DTT). This was followed by dropwise addition with continuous stirring of one-half packed nuclear volume of high salt buffer (20 mM HEPES, pH 7.9, at 4 °C, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonic fluoride, 0.5 mM DTT) and centrifugation at 14,500 rpm for 30 min. The supernatant was next dialyzed against approximately 50 volumes of dialysis buffer (20 mM HEPES, pH 7.9, at 4 °C, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonic fluoride, 0.5 mM DTT) for 5 h and then stored in aliquots at −70 °C. The resulting nuclear extracts were utilized for electrophoretic mobility shift analysis.

Amplification of COL2A1 Promoter by PCR—The reaction was carried out as follows. 150 ng of α1(II) procollagen minigene, which contains the entire *COL2A1* promoter region (24), was utilized as a template and was amplified with 10 pmol of each primer set (P1/P2, P3/P4, P5/P6, P7/P8; see Fig. 9) under the following conditions: 95 °C for 30 s and 60 °C for 1 min for 40 cycles followed by a final extension at 72 °C for 7 min in a Gene Amp 480 thermocycler (Perkin-Elmer).

Electrophoretic Mobility Shift Analysis of DNA-binding Proteins—Electrophoretic mobility shift analyses were performed according to the procedure of Garner and Revzia (25), as described previously (26). The PCR products containing specific regions of *COL2A1* prepared as described above were end-labeled by kinasing the 5′-primer with T_4 polynucleotide kinase and [γ-^{32}P]ATP and then carrying out the PCR reaction. The binding reaction was 20 mM Tris, pH 7.5, 10 mM sodium acetate, 0.5 mM EDTA, 5% glycerol, and 10–15 μg of nuclear extract in a final volume of 20 μl. Next, 3–5 $\times$ 10^3 cpm of end-labeled probe was added, and the incubation was carried out for 1 h at room temperature. DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide gels in 40 mM Tris acetate, 1 mM EDTA buffer and visualized by autoradiography.

Determination of the Specificity of Binding—The specificity of binding of putative regulatory elements within the PCR fragments was confirmed by competition with unlabeled specific oligonucleotides (consensus Sp1, AP1, and AP2 oligonucleotides, Santa Cruz Biotechnology, Santabury, CA). Once the binding was determined to be specific, an anti-Sp1 antibody (Santa Cruz Biotechnology) was utilized. Electrophoretic mobility shift assays were carried out as outlined above. Nuclear extracts were preincubated with increasing concentrations of the anti-Sp1 antibody for 60 min at 20 °C before the binding reaction was carried out.

Quantitation of Sp1-binding Proteins in Chondrocytes—The level of Sp1-binding protein in nuclear extracts of chondrocytes was quantitated by protein titration experiments analyzed by gel shift analysis as described by Riggs *et al.* (27). One set of binding reactions contained a constant amount of labeled Sp1 and an increasing concentration of protein. The amounts of bound and free probe were quantitated by scintillation counting of excised regions of the gel. In the plateau region with excess protein, it was possible to determine the amount of protein required to reach equilibrium with the DNA probe. This reaction allowed determination of the proportion of DNA probe bound to protein, since under some conditions binding of probe is not complete even if there is excess protein present. The second set of titration experiments was performed under identical conditions except that the amount of protein determined from the first set of binding reactions was maintained constant while the amount of Sp1 oligonucleotide was increased. At plateau, all of the protein would be expected to be saturated with the Sp1 oligonucleotide. The counts per minute at plateau correspond to the amount of probe required to bind all of the active protein involved in DNA binding. By comparing the amount of probe in the DNA-protein complex to a set of standard dilutions of free probe included on a gel electrophoresed in parallel, the number of moles of protein in the reaction can be calculated assuming a one-to-one binding, since the specific activity and the molarity of probe was known.

Drosophilia Schneider Line 2 Cell Culture and Transfections—*Drosophila* Schneider line 2 cells (28), which lack Sp1 homologs, were cultured in Schneider *Drosophila* medium (Life Technologies, Inc., Rockville, MD) supplemented with 12% heat-inactivated serum and 1%

[1] The abbreviations used are: polyHEMA, poly(2-hydroxyethyl methacrylate); DTT, dithiothreitol; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; bp, base pair(s).

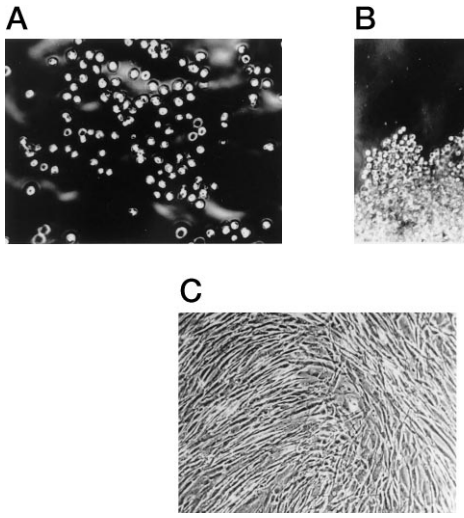

FIG. 1. **Phase contrast micrographs of cultured human fetal chondrocytes.** The cells were isolated as described under "Experimental Procedures." *A*, freshly isolated chondrocytes. *B*, freshly isolated chondrocytes 12 days following plating on polyHEMA. *C*, freshly isolated chondrocytes passaged twice at 20-day intervals on plastic.

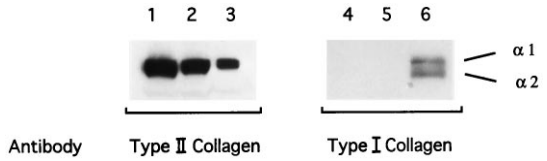

FIG. 2. **Western blot analysis of collagens synthesized by cultured human fetal chondrocytes.** The cell-associated proteins synthesized by chondrocytes cultured under the various conditions were extracted as described under "Experimental Procedures" and subjected to Western blot analysis utilizing either a polyclonal antibody specific for the type II procollagen telopeptide (22) or a polyclonal antibody specific for type I collagen. *Lanes 1* and *4*, freshly isolated chondrocytes. *Lanes 2* and *5*, chondrocytes cultured on polyHEMA. *Lanes 3* and *6*, chondrocytes dedifferentiated into fibroblast-like cells following passage on plastic. The position of migration of the α1 and α2 chains of type I collagen is shown.

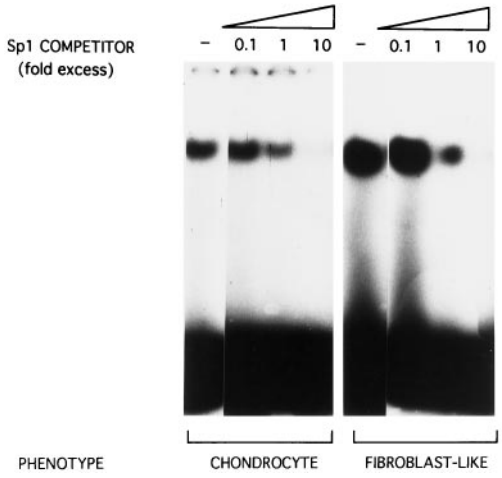

FIG. 3. **Binding of nuclear extracts from freshly isolated chondrocytes and from fibroblast-like cells.** Nuclear extracts were prepared from freshly isolated chondrocytes or from chondrocytes that were allowed to become fibroblast-like by culture for 40 days on plastic and were examined for binding to consensus Sp1 oligonucleotide and competition analysis with unlabeled Sp1. The nuclear extracts were preincubated with the indicated amounts of unlabeled consensus Sp1 oligonucleotide at room temperature for 30 min followed by the addition of labeled consensus Sp1 oligonucleotide for 1 h at room temperature.

penicillin/streptomycin at 25 °C. The cells were seeded at a density of 1×10^6 cells/60-mm dish 16 h prior to transfection. Transient transfections were performed by the calcium phosphate precipitation method with the Profection mammalian transfection system (Promega, Madison, WI). The transfections were performed using 100 μg of either pPacSp1 plasmid, which contains a 2.1-kilobase pair Sp1 cDNA insert or the insertless plasmid pPac0 (29) and 5 μg of either E/0.7-CAT, a human *COL2A1* promoter construct spanning bp −577 to +63 linked to the CAT reporter gene or E/0.2-CAT, a human *COL2A1* promoter construct spanning bp −131 to +63 linked to the CAT reporter gene. All of the reactions contained 0.5 μg of phsp82LacZ, a plasmid containing the *Drosophila* heat shock protein 82 promoter fused to the *lacZ* gene to correct for variations in transfection efficiencies. The transfected cells were harvested following a 48-h incubation, and CAT activity was determined from equal amounts of cell extracts as described previously (26).

RESULTS

Detection of Sp1 Binding in Chondrocyte Nuclear Extracts—Nuclear extracts were prepared from freshly isolated chondrocytes, from chondrocytes cultured on polyHEMA for 12 days under conditions that allow the preservation of the cartilage-specific phenotype, and from chondrocytes that lost their phenotype and became morphologically fibroblast-like by passage in monolayer culture on plastic for 40 days. The phase-contrast morphology of the three cell types is shown in Fig. 1. Western blot analysis of cell extracts isolated from the three different cell types indicated that culture of chondrocytes on polyHEMA allows preservation of the cartilage-specific phenotype as they continue to express type II collagen and not type I collagen (Fig. 2). In contrast, culture and passage of chondrocytes in monolayer on plastic leads to loss of their chondrocyte-specific phenotype as these cells express markedly lower levels of type II collagen and initiate production of large amounts of fibroblast-specific type I collagen (Fig. 2).

For detection of Sp1 binding activity, the consensus Sp1 oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) was radiolabeled and used as a probe in the binding assays. As shown in Fig. 3, there was binding of nuclear proteins from freshly isolated chondrocytes and from fibroblast-like cells to consensus Sp1 oligonucleotide. However, the binding of nuclear extracts from dedifferentiated chondrocytes to Sp1 was 2–3-fold greater than the binding of nuclear extracts from freshly isolated chondrocytes. The results obtained from chondrocytes that were cultured on polyHEMA-coated plates in suspension were similar to those obtained from freshly isolated chondrocytes (data not shown).

Specificity of Sp1 Binding—The binding of labeled Sp1 to nuclear extracts from both chondrocytes and fibroblast-like cells was competed away completely when a 10-fold excess of unlabeled Sp1 was added (Fig. 3). However, when unlabeled AP1 or AP2 was added to the reaction, the binding of labeled Sp1 was not competed away, indicating that the DNA-protein complexes were specific for Sp1 (Figs. 4 and 5). Moreover, when the binding of recombinant Sp1 was carried out with the consensus Sp1 oligonucleotide, a DNA-protein complex of the same size as that observed upon binding of Sp1 to chondrocyte or fibroblast-like nuclear proteins was observed (not shown). The DNA-protein complex formed between recombinant Sp1 and consensus Sp1 oligonucleotide had the same pattern of migration as the complex formed by the consensus Sp1 oligonucleotide with nuclear proteins from either chondrocytes or fibroblast-like cells. The Sp1 binding with extracts from both chondrocytes and fibroblast-like cells was enhanced by the addition of increasing amounts of KCl as shown in Fig. 6. Moreover, the addition of increasing amounts of EDTA inhibited the formation of the DNA-protein complex (Fig. 7). The inhibition of Sp1 binding by EDTA was abrogated when $MgCl_2$ was added to the binding reaction, and when equal concentrations of EDTA and $MgCl_2$ were present (40 mM each) in the

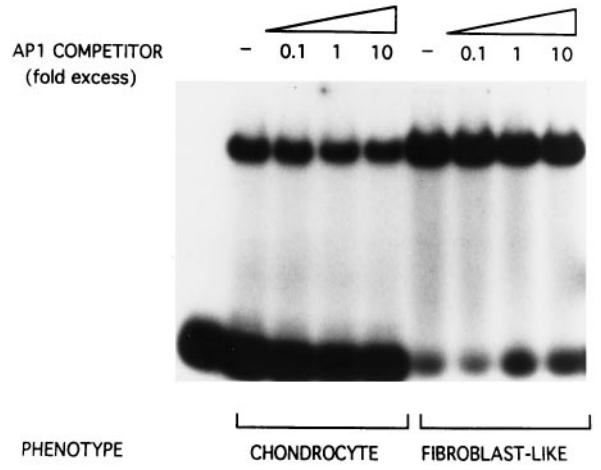

FIG. 4. **Competition analysis with unlabeled AP-1.** Binding of nuclear extracts with ^{32}P-labeled consensus Sp1 was carried out in the presence of increasing amounts of AP-1. The nuclear extracts were preincubated with the indicated amounts of unlabeled consensus AP1 oligonucleotide at room temperature for 30 min followed by the addition of labeled consensus Sp1 oligonucleotide for 1 h at room temperature.

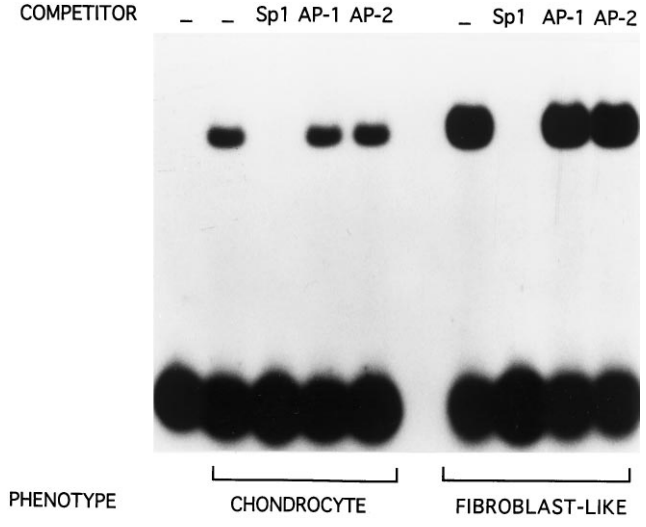

FIG. 5. **Competition analysis with unlabeled Sp1, AP-1, and AP-2.** Binding of nuclear extracts with ^{32}P-labeled consensus Sp1 was carried out in the presence of a 10-fold excess of unlabeled Sp1, AP-1, or AP-2. The nuclear extracts were preincubated with unlabeled consensus Sp1, AP-1, or AP-2 at room temperature for 30 min followed by the addition of labeled consensus Sp1 oligonucleotide for 1 h at room temperature.

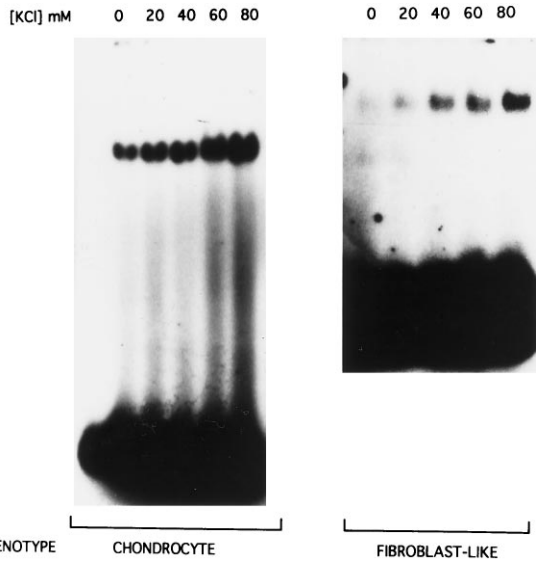

FIG. 6. **Effect of increasing concentrations of KCl on binding of nuclear extracts isolated from chondrocytes and fibroblast-like cells to consensus Sp1 oligonucleotide.** Binding reactions were performed as described under "Experimental Procedures." The nuclear protein utilized for each experiment was 15 μg for chondrocytes and 5 μg for fibroblast-like cells. The indicated concentrations of KCl were present in each binding reaction.

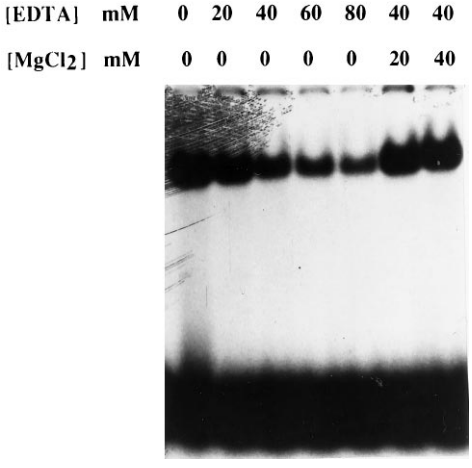

FIG. 7. **Effects of increasing concentrations of EDTA and $MgCl_2$ on binding of chondrocyte nuclear extracts to consensus Sp1 oligonucleotide.** The indicated amounts of EDTA and $MgCl_2$ were present in the binding reaction that was performed as described under "Experimental Procedures."

binding reaction no inhibition of Sp1 binding was observed. The inhibition of Sp1 binding by EDTA was not abrogated by the addition of $ZnCl_2$. Thus, the formation of Sp1 protein complex requires the presence of Mg^{2+}.

Effect of the Addition of a Polyclonal Sp1 Antibody to Nuclear Extracts—When a polyclonal anti-Sp1 antibody was preincubated with the chondrocyte nuclear extracts prior to the binding reaction, only a weak inhibition of the protein-Sp1 complex was observed even at an antibody concentration of 15 μg. However, when 15 μg of anti-Sp1 was added to the fibroblast-like nuclear extract, greater than 80% inhibition of the DNA-protein complex was observed (Fig. 8).

Amplification of COL2A1 Promoter by PCR—As illustrated in Fig. 9*A*, four consensus Sp1 binding sites (GGGCGG) at nucleotides −80 to −75, −115 to −110, −119 to −114, and −198 to −193 have been identified in the human *COL2A1* promoter. Three of these (at nucleotides −80, −119, and −198) are found at identical locations in the human, mouse, and rat *COL2A1* promoters. The extremely high conservation of these sequences coupled with the observation that the CCAAT box is absent in the human *COL2A1* promoter suggests that these Sp1 sites may play a major role in determining the activity of the *COL2A1* promoter. We amplified the region encompassing of the *COL2A1* promoter nucleotides −391 to −40 in four fragments designated P1/P2, P3/P4, P5/P6, and P7/P8 as shown in Fig. 9 (*A* and *B*).

Detection of Nuclear Proteins from Freshly Isolated Chondrocytes and from Chondrocytes Dedifferentiated into Fibroblast-like Cells That Bind to Sp1 Binding Sites in the COL2A1 Promoter—As shown in Fig. 10, we detected binding of chondrocyte nuclear proteins to *COL2A1* promoter fragments spanning bp −226 to −148 (fragment P5/P6; Fig. 10, *lane 1*) and bp −169 to −40 (fragment P7/P8; Fig. 10, *lane 7*) from the initiation of transcription site. Since these fragments contain putative Sp1 sites, we examined the effects of added unlabeled Sp1 to the binding reaction. The formation of DNA-protein com-

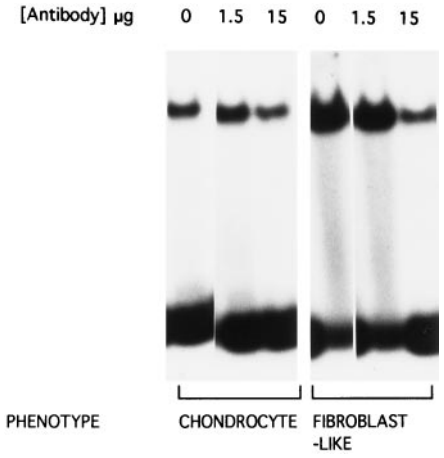

FIG. 8. **Effect of preincubation with specific anti-Sp1 antibody on the binding of nuclear extracts isolated from chondrocytes and fibroblast-like cells to Sp1 oligonucleotide.** The nuclear extracts were preincubated with the indicated amounts of a polyclonal anti-Sp1 antibody at room temperature for 30 min before the binding reaction, which was performed as described under "Experimental Procedures."

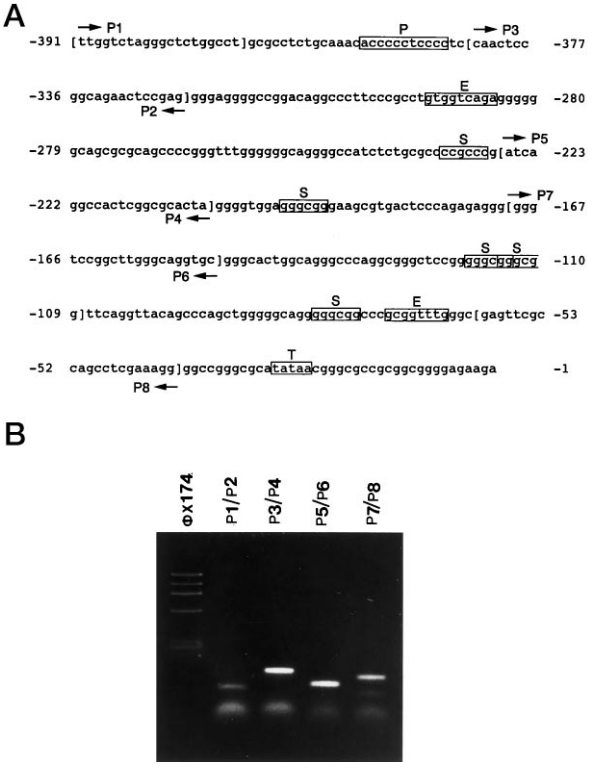

FIG. 9. **PCR amplification of the region encompassing nucleotides −391 to −40 of the *COL2A1* promoter.** *Panel A*, sequence of the *COL2A1* promoter showing the consensus regulatory sequences and the position of the primers employed. The putative regulatory elements are *boxed*. *P*, pyrimidine-rich sequence; *E*, enhancer core elements; *S*, Sp1 binding sites; *T*, TATAAA box. *Panel B*, agarose gel electrophoresis of the amplified PCR products utilizing the indicated primers.

plexes with nuclear proteins from human chondrocytes was completely competed by a 10-fold excess of unlabeled Sp1, indicating that the Sp1 sites on P5/P6 and P7/P8 are involved in binding (Fig. 10, *lanes 2* and *8*). The DNA-protein complex formed by each *COL2A1* promoter fragment was also competed by a 10-fold excess of corresponding unlabeled *COL2A1* promoter fragment as shown in Fig. 10, *lanes 3* and *9*. In addition, P5/P6 competed the complex formed with P7/P8 in nuclear extracts isolated from chondrocytes and chondrocytes dedifferentiated into fibroblast-like cells (results not shown). Similar results were observed when nuclear proteins were isolated from chondrocytes dedifferentiated into fibroblast-like cells except that there was a 2–3-fold greater binding in nuclear extracts from dedifferentiated chondrocytes cultured in monolayer as compared with that from fresh chondrocytes (Fig. 10, *lanes 4*, *5*, *6*, *10*, *11*, and *12*).

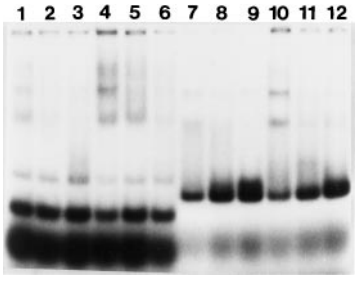

FIG. 10. **Binding of nuclear extracts from freshly isolated chondrocytes and from chondrocytes dedifferentiated into fibroblast-like cells to PCR-amplified *COL2A1* promoter fragments.** 10 μg of each nuclear extract from freshly isolated chondrocytes or from chondrocytes allowed to become fibroblast-like by culture on plastic for 40 days was utilized and electrophoresed on a 6% polyacrylamide gel for DNA binding. The *COL2A1* promoter fragments examined were P5/P6 (bp −226 to −148, *lanes 1–6*) and P7/P8 (bp −169 to −40, *lanes 7–12*). *Lanes 1–3* and *7–9*, binding with chondrocyte nuclear protein; *lanes 4–6* and *10–12*, binding with fibroblast-like nuclear protein; *lanes 2*, *5*, and *11*, competition with 10-fold excess cold Sp1; *lanes 3*, *6*, *9*, and *12*, competition with a 10-fold excess of the oligonucleotide used for binding.

Quantitation of Sp1-binding Proteins in Chondrocytes—The amounts of nuclear proteins from chondrocyte and fibroblast-like cells that bind to the consensus Sp1 oligonucleotide were quantitatively determined by DNA-binding protein titration experiments. The first set of binding reactions contained a constant amount of labeled Sp1 oligonucleotide (88 nM) and increasing concentrations of nuclear protein. When the chondrocyte nuclear protein concentration was increased from 1 to 25 μg/reaction, an increase in the amount of DNA-protein complex formed was detected (Fig. 11, *A* and *B*). Increasing the concentration of nuclear proteins in the reaction above 20 μg/reaction did not result in a further increase in binding, indicating that at this concentration equilibrium was reached with the labeled Sp1 oligonucleotide. Therefore, in the next set of reactions, the concentration of nuclear proteins was maintained constant at 20 μg/reaction, and the amount of Sp1 oligonucleotide was increased from 22 to 352 nM/reaction. As shown in Fig. 11, *C* and *D*, the binding was essentially complete at a concentration of 264 nM. Thus, at the plateau of 264 nM, all of the DNA-binding protein was saturated with Sp1. Next, a set of standard dilutions of the free oligonucleotide ranging from 22 to 352 nM was electrophoresed, and the radioactivity of the free probe was determined (Fig. 11, *E* and *F*). Comparison of the amount of oligonucleotide in the DNA-protein complex with a set of standard dilutions of free oligonucleotide demonstrated that 11.88 nM of chondrocyte nuclear protein was bound to the Sp1 oligonucleotide in the reaction, assuming a one-to-one binding of the probe and the Sp1 oligonucleotide. Similar experiments were performed with nuclear extracts isolated from chondrocytes dedifferentiated into fibroblast-like cells. Since the binding of 88 nM of Sp1 oligonucleotide was saturated at a protein concentration of 20 μg (Fig. 11, *A* and *B*) in the next set of reactions, 20 μg of protein was used per reaction, and the concentration of Sp1 oligonucleotide was increased from 22 to 352 nM/reaction. As shown in Fig. 11, *C* and *D*, the binding was essentially complete at a concentration of 264 nM, similar to that observed for chondrocyte nuclear protein. Comparison of the amount of oligonucleotide in the DNA protein complex to a

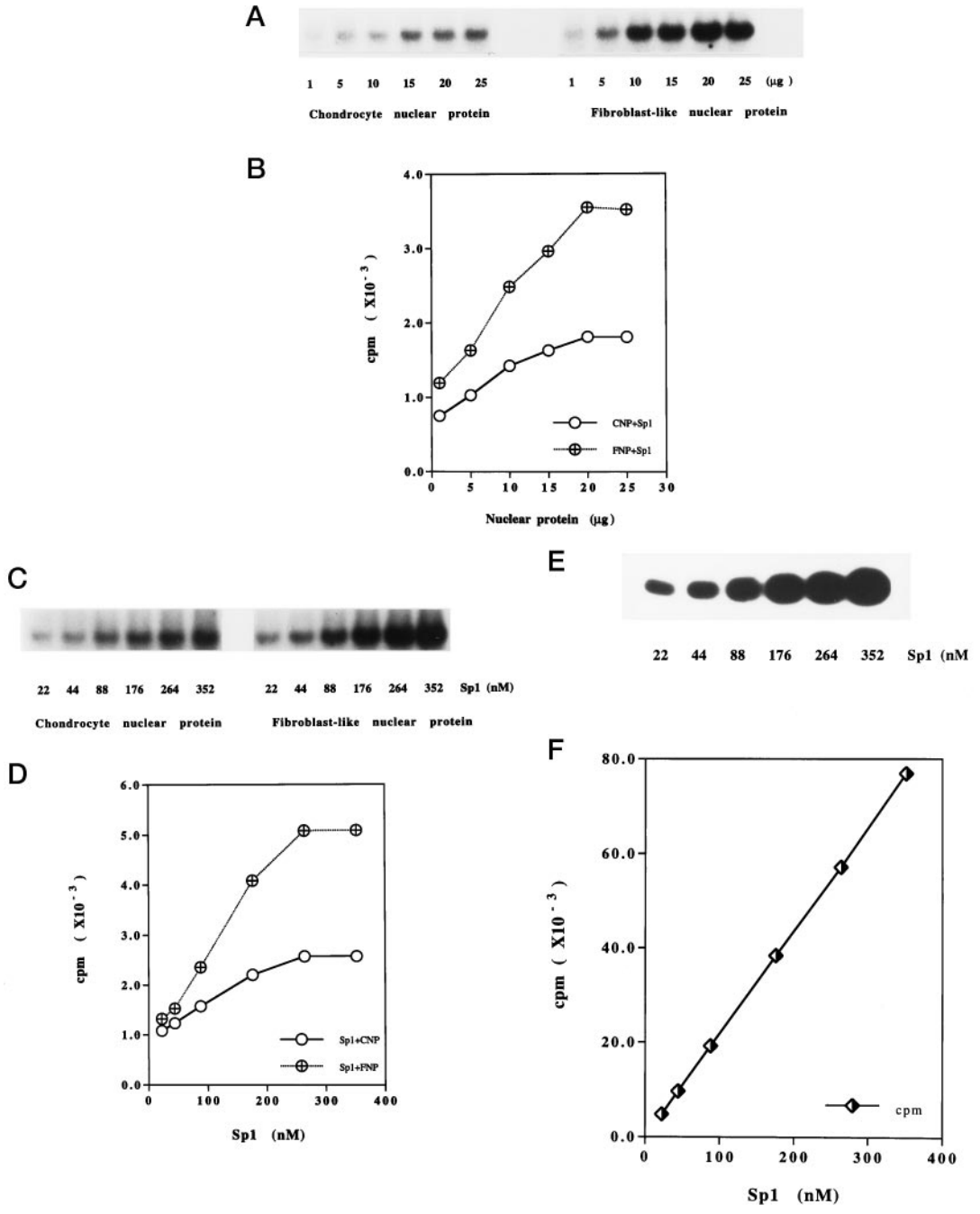

FIG. 11. **Protein titration analyzed by gel shift analysis.** *A*, increasing concentrations of nuclear proteins (chondrocyte nuclear protein (*CNP*) and fibroblast-like cell nuclear protein (*FNP*)) were incubated with a constant amount of consensus Sp1 radiolabeled oligonucleotide (88 nM) *B*, graph showing the radioactivity in DNA-protein complexes shown in *A* plotted as a function of protein concentration. *C*, titration of DNA-protein binding with increasing concentrations of consensus Sp1 radiolabeled probed with 20 μg of chondrocyte nuclear protein or fibroblast-like nuclear protein. *D*, graph showing the radioactivity in DNA-protein complexes shown in *C* plotted as a function of Sp1 oligonucleotide concentration. *E*, autoradiogram of a set of standard dilutions of free Sp1 oligonucleotide analyzed by gel shift analysis. *F*, plot of radioactivity as a function of Sp1 oligonucleotide concentration shown in *E*.

set of standard dilutions of free probe demonstrated that 23.2 nM of nuclear protein isolated from chondrocytes dedifferentiated into fibroblast-like cells was present in the reaction (Fig. 11*F*). Therefore, the amount of nuclear proteins binding to the Sp1 oligonucleotide was about 2-fold higher in nuclear extracts isolated from chondrocytes dedifferentiated into fibroblast-like cells as compared with that of nuclear extracts isolated from chondrocytes.

Sp1 Activation of the Transcription of COL2A1 Promoter-CAT Constructs in Drosophila Schneider Cells—To provide direct evidence of the role of Sp1 in *COL2A1* transcription, cotransfection experiments were performed with *Drosophila* Schneider line L2 cells that lack homologs of Sp1. The construct E/0.7, which spans bp −577 to +63 of the *COL2A1* promoter, was co-transfected with either the Sp1 expression plasmid pPacSp1 or the insertless plasmid pPac0, an identical plasmid lacking the Sp1 cDNA. The plasmid pSV_2-CAT, which contains the SV40 promoter linked to the CAT reporter gene, or the plasmid pSV0-CAT, an identical plasmid but lacking the SV40 promoter, was used as positive or negative control, respectively. As shown in Fig. 12, when E/0.7 was transfected alone or was co-transfected with pSV0-CAT into the *Drosophila* cells, no CAT activity was observed. However, when E/0.7 was co-transfected with pPacSp1, the Sp1 expression plasmid, substantial

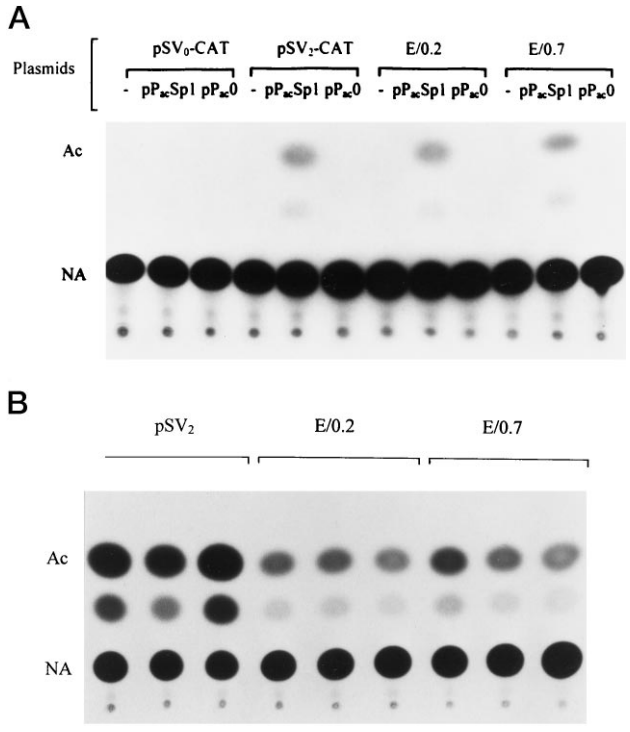

FIG. 12. **Effect of Sp1 expression on transcriptional activity of human *COL2A1* promoter constructs transfected into *Drosophila* Schneider line L2 cells.** *Drosophilia* Schneider line L2 cells were cultured and transfected by the calcium phosphate precipitation method using either the pPacSp1 expression plasmid or the insertless pPac0 and were co-transfected with 5 μg of either E/0.7, a human *COL2A1* promoter construct spanning bp −577 to +63 linked to the CAT reporter gene or E/0.2, a human *COL2A1* promoter construct spanning bp −131 to +63 linked to the CAT reporter gene. All reactions contained 0.5 μg of the phsp82LacZ, a plasmid containing the *Drosophila* heat shock protein 82 promoter fused to the *lacZ* gene to normalize for variations in transfection efficiencies. The transfected cells were harvested and CAT activity determined. *A*, autoradiogram of a representative CAT assay. *Drosophila* Schneider L2 cells were co-transfected with either pPacSp1 expression plasmid or the insertless plasmid pPac0 and *COL2A1*-CAT E/0.2 and E/0.7 as described under "Experimental Procedures." The plasmids pSV_2-CAT, a plasmid containing the SV40 early promoter and pSV0-CAT, a plasmid lacking the SV40 early promoter were used as positive and negative controls, respectively. *Ac*, acetylated chloramphenicol; *NA*, non-acetylated chloramphenicol. *B*, autoradiogram of co-transfection of *Drosophila* Schneider line L2 cells with pPacSp1 expression plasmid, and pSV_2, E/0.2, and E/0.7. Results from three separate experiments are shown.

CAT activity was detected. When the construct E/0.2, which spans bp −131 to +63 was utilized in experiments similar to those with E/0.7, substantial CAT activity was obtained when it was co-transfected with the Sp1 expression plasmid pPacSp1. However, the CAT activity obtained with E/0.2 was >43% lower than that obtained with E/0.7. The higher CAT activity produced when E/0.7 was employed may be due to the presence of two additional Sp1 binding sites in the construct E/0.7 in comparison with E/0.2.

DISCUSSION

The results reported here revealed qualitative and quantitative alterations in Sp1 binding activity during chondrocyte dedifferentiation. This conclusion is based on studies carried out with consensus Sp1 oligonucleotide showing that (i) Sp1 binding activity was present in nuclear extracts from all three cell types studied; (ii) Sp1 binding was 2–3-fold greater in nuclear extracts from chondrocytes dedifferentiated into fibroblast-like cells by passage in monolayer culture on plastic substrate than in freshly isolated chondrocytes or in chondrocytes allowed to maintain their phenotype by culture on polyHEMA-coated dishes; (iii) Sp1 binding was specific, since it was competed by unlabeled Sp1 and not by AP1; (iv) Sp1 binding was enhanced by KCl and inhibited by the addition of EDTA; (v) A polyclonal antibody against Sp1 decreased the binding of Sp1 by 85% in chondrocytes dedifferentiated into fibroblast-like cells but caused only a very slight inhibition in freshly isolated chondrocytes or in chondrocytes cultured in suspension on polyHEMA. Inhibition of Sp1 binding by this polyclonal antibody has been previously reported in the human granulocyte-macrophage colony-stimulating factor gene promoter (30).

We also observed that culture of chondrocytes under conditions that result in the acquisition of fibroblast-like morphology resulted in an increase in DNA binding activity to *COL2A1* promoter fragments containing Sp1 sites. The increase in binding to the consensus Sp1 oligonucleotide or to the *COL2A1* promoter fragment could be due to several reasons. First, there may be an increased expression of the Sp1 gene. Increase in Sp1 mRNA has been observed in several organs during mouse embryo development (31). Second, there may be post-translational mechanisms that are involved. These could be *O*-linked glycosylation, protein kinase phosphorylation, or formation of multimers on single or multiple GC elements. The differential antibody response observed in the two morphologically different cells types may indicate that the DNA-binding proteins are different in the two cell types although they have the same apparent molecular mass. Alternatively, it is possible that there are subtle differences in the binding of Sp1 oligonucleotide to the same nuclear protein in the two different cell types that are reflected in the differential antibody response. Quantitative analysis of the amounts of binding proteins employing DNA-binding protein titration assays demonstrated that 11.88 nM chondrocyte nuclear protein was bound to the consensus Sp1 oligonucleotide as compared with a 23.2 nM concentration of fibroblast-like cell nuclear protein. *Drosophila* Schneider L2 cells that lack homologs of Sp1 have previously been utilized in co-transfection experiments with the Sp1 expression plasmid and COL1A1 promoter-CAT constructs to demonstrate a direct role of Sp1 in the transcription of this gene (32, 33). Higher stimulation of the COL1A1 promoter activity with CAT constructs containing progressively greater number of Sp1 sites has previously been reported (33). The activity of the *COL2A1* promoter was also significantly increased by Sp1 expressed in *Drosophila* Schneider line L2 cells. Higher stimulation of promoter activity was observed when a *COL2A1* construct containing a greater number of Sp1 sites was utilized. These results indicated a direct role of Sp1 in regulation of activity of the *COL2A1* promoter.

Although Sp1 is a ubiquitous transcription factor that is present in all mammalian cells that have been examined (34), it has been demonstrated that its binding affinity and transcriptional properties can be altered by different cytokines via indirect action with co-factors. The role of Sp1 in regulation of the α2(I) procollagen gene expression has been extensively studied (35–36). Transforming growth factor-β stimulates the expression of the α2(I) procollagen gene by increasing the affinity of an Sp1-containing transcriptional complex that is bound to a sequence in the promoter termed the transforming growth factor-β-responsive element (35). The same element also mediates the transcriptional signal of the cytokine tumor necrosis factor-α that inhibits α2(I) procollagen gene expression (34). Therefore, it is very likely that Sp1 along with other co-factors may be involved in the regulation of expression of *COL2A1* in chondrocytes.

Our observations of reduced DNA binding of Sp1 in differentiated chondrocytes in comparison with fibroblast-like dedifferentiated chondrocytes can be proposed as a molecular mecha-

nism that contributes to alterations in expression of *COL2A1* and possibly other genes that are differentially regulated in the two cell types. Further studies to examine the specific sequences within the *COL2A1* promoter that interact with Sp1 and to identify the precise mechanism of Sp1 binding will further our understanding of the mechanisms responsible for the profound changes in the expression of this gene occurring during the process of chondrocyte dedifferentiation or in diseases such as osteoarthritis.

Acknowledgments—We are grateful to Dr. M. B. Goldring for the gift of plasmids E/0.7 and E/0.2; to Dr. John Varga for pPacSp1, pPac0, and hsp82 *lacZ* plasmids; to Dr. Jim Jaynes for *Drosophila* Schneider line L2; and to Dr. Elena Hitraya for helpful discussions.

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Detection and Characterization of Sp1 Binding Activity in Human Chondrocytes and Its Alterations during Chondrocyte Dedifferentiation

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J. Biol. Chem. 1997, 272:26918-26925.
doi: 10.1074/jbc.272.43.26918