Detection and characterization of Sp1 binding activity in human chondrocytes and its alterations during chondrocyte dedifferentiation.

Rita M. Dharmavaram  
*Thomas Jefferson University*

Gang Liu  
*Thomas Jefferson University*

Sheryl D. Mowers  
*Thomas Jefferson University*

Sergio A. Jimenez  
*Thomas Jefferson University*

Follow this and additional works at: https://jdc.jefferson.edu/medfp

Part of the Rheumatology Commons

Let us know how access to this document benefits you

**Recommended Citation**

Dharmavaram, Rita M.; Liu, Gang; Mowers, Sheryl D.; and Jimenez, Sergio A., "Detection and characterization of Sp1 binding activity in human chondrocytes and its alterations during chondrocyte dedifferentiation." (1997). *Department of Medicine Faculty Papers*. Paper 191. https://jdc.jefferson.edu/medfp/191
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)

Rita M. Dharmavaram, Gang Liu, Sheryl D. Mowers, and Sergio A. Jimenez‡

From the Division of Rheumatology, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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 2-8-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 unlabelled 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 Sp1 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.

‡ To whom correspondence and reprint requests should be addressed: Room 509, Bluemle Life Sciences Bldg., 233 S. 10th St., Philadelphia, PA 19107. Tel.: 215-503-5042; Fax: 215-925-4649.

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 non-denaturing 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 transcription factors in the regulation of expres-
sion of COL2A1. However, studies of the changes in DNA-
binding proteins that may occur during chondrocyte dediffer-
entiation 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-a(1) collagen gene
transcription (18). In this study we investigated the changes in
the levels and activity of the transcriptional factor Sp1 occur-
ing during the process of chondrocyte dedifferentiation.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Human Chondrocytes—Human fetal epiph-
yseal cartilage was removed under sterile conditions from femoral
heads, knee condyles, and tibial plateaua from spontaneous abortions.
The tissues were obtained from the International Institute for the
Advancement of Medicine (Philadelphia, PA), following protocols re-
viewed 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 Hank's medium
containing trypsin and bacterial collagenase (2 mg/ml each) for 1 h at
37 °C. The digest was discarded, and the tissue fragments were
minced and digested overnight at 37 °C in Dulbecco's minimum essen-
tial medium with 4.5 g/liter glucose containing 10% fetal bovine serum
and 0.5 mg/ml bacterial collagenase. The cells released by the enzym-
matic 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 × g for
5 min, resuspended, and washed four times with collagenase-free me-
dium. The average yield was 3.0 ± 0.4 × 10⁶ chondrocytes/g wet
weight, of cartilage.

The isolated chondrocytes were cultured at a density of 5 × 10⁶ cells
in 60-mm plastic dishes previously coated with 0.9 ml of a 10% (v/v)
of polyHEMA (19, 20) (PolySciences Inc. Malvern, PA) following a proce-
dure modified from that described by Folkman and Moscona (21) as
described previously (12). For coating the culture dishes with poly-
HEMA, 0.9 ml of a 10% (v/v) solution of polyHEMA in 95% ethanol was
prepared according to the procedures of Dignam et al. (23). The cells
were heated to 100 °C for 5 min in 1% SDS, 50 mM DTT, and 1%
water, of cartilage.

They were resuspended and then centrifuged at 3,000 rpm for 5 min and washed twice with phosphate-buffered saline.

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.

The isolated nuclei were prepared and used in the procedures of Dignam et al. (23). The cells were pooled and washed in phosphate-buffered saline (10 mM phos-
phate 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 MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonlic fluo-
rade, 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 MgCl₂, 0.02 mM EDTA, 0.2 mM phenyl-
methylsulfonlic fluoride, and 0.5 mM DTT). This was folowed 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, 50% glycerol,
1.5 mM MgCl₂, 1.2 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonlic
fluoride, 0.5 mM DTT) and centrifugation at 14,500 rpm for 30 min. The
supernatant was next dialyzed against approximately 50 volumes of
diisulfate buffer (20 mM HEPES, pH 7.9, at 4 °C, 20% glycerol, 100 mM
KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonlic 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 car-
ried out as follows. 150 ng of a(1)I1 procollagen minigene, which con-
tains the entire COL2A1 promoter region (24), was utilized as a tem-
plate. The primer spanned nucleotides 1116 (5′) to 1278 (3′) (P5/P6; 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 Revzin (25), as described previously (26). The
PCR products containing specific regions of COL2A1 prepared as de-
scribed above were end-labeled by kinasing the 5′-primer with T₇
polymerase kinase and [γ-³²P]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 × 10⁶ 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% poly-
acrylamide gels in 40 mM Tris acetate, 1 mM EDTA buffer and visual-
ized by autoradiography.

Determination of the Specificity of Binding—The specificity of bind-
ing of putative regulatory elements within the PCR fragments was
confirmed by competition with unlabeled specific oligonucleotides (con-
sensus AP-1, and AP-2, and AP-1 and AP-2) (Promega Corp., Madison,
Wisconsin) (24). Since the binding was determined to be specific, an
anti-Sp1 antibody (Santa Cruz Biotechnology) was utilized. Electro-
phoretic mobility shift assays were carried out as outlined above. Nu-
clear 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 quanti-
tated 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 al-
lowed 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 main-
tained 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
complexes was determined. 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.

Drosophila Schneider Line 2 Cell Culture and Transfections—Dros-
ophila 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%
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 Perfection 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 E0.7-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 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 MgCl2 was added to the binding reaction, and when equal concentrations of EDTA and MgCl2 were present (40 mM each) in the

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.
binding reaction no inhibition of Sp1 binding was observed. The inhibition of Sp1 binding by EDTA was not abrogated by the addition of ZnCl2. Thus, the formation of Sp1 protein complex requires the presence of Mg2+.

**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. 9A, four consensus Sp1 binding sites (GGGGCG) 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 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-
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 completely in the binding of nuclear extracts isolated from chondrocytes dedifferentiated into fibroblast-like cells. Since the binding of 88 nM of Sp1 oligonucleotide was saturated at a concentration of 264 nM, all complexes formed were detected (Fig. 11, 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).

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. 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.

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. F, 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.

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.”

---

[Graph and figure references omitted for brevity]
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).

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 E0.7, 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 pSV0-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 E0.7 was transfected alone or was co-transfected with pSV0-CAT into the *Drosophila* cells, no CAT activity was observed. However, when E0.7 was co-transfected with pPacSp1, the Sp1 expression plasmid, substantial
**Sp1 and Chondrocyte Dedifferentiation**

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 cell 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 μm chondrocyte nuclear protein was bound to the consensus Sp1 oligonucleotide as compared with a 23.2 μm 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 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-

---

**FIG. 12. Effect of Sp1 expression on transcriptional activity of human COL2A1 promoter constructs transfected into Drosophila Schneider line L2 cells.** *Drosophila* 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 82Z 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 pSV2-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 pSV2, E/0.2, and E/0.7. Results from three separate experiments are shown.
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/E0.7 and E/E0.2; 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.

REFERENCES
1. Eyre, D. R., Wu, J-J., Woods, P. (1992) in Articular Cartilage and Osteoarthritis (Kuhn, K., and Krieg, T., eds) pp. 272–315, Karger, Basel
2. Von der Mark, K. (1986) in Connective Tissue: Biological and Clinical Aspects (Kuettner, K. E., Schleyerbach, R., Perryrun, J. G., Haecall, V. C., eds) pp. 119–151, Raven Press, New York
3. Von der Mark, K., Gaus, V., Von der Mark, H., and Muller, P. (1977) Nature 267, 531–532
4. Benya, P. D., Paudle, R. B., and Nimni, M. E. (1978) Cell 15, 1313–1321
5. Benya, P. D., and Nimni, M. E. (1979) Arch. Biochim. Biophys. 192, 327–335
6. Benya, P. D., and Shaffer, J. D. (1982) Cell 30, 215–225
7. Kuettner, K. E., Menoli, V. A., Pauli, B. U., Wrobel, N. C., Thonar E. J. M., and Daniel, J. C. (1982) J. Cell. Biol. 93, 751–757
8. Archer, C. W., McDowel, J., Baylis, M. T., Stephens, M. D., and Bentley, G. (1990) J. Cell Sci. 97, 361–371
9. Malein-Gerin, F., Ruggiero, F., and Garene, G. (1986) Differentiation 43, 204–211
10. Bonaventure, J., Kadhom, N., Cohen-Solal, L., Ng, K. H., Borguignon, J., Lasselin, C., and Freisinger, P. (1994) Exp. Cell Res. 212, 97–104
11. Hauselmann, H. J., Fernandes, R. J., Mok, S. S., Schmidt, T. M., Block, J. A., Aylott, M. B., Kuettner, K. E., and Thonar, J. M. A. (1994) J. Cell Sci. 107, 17–27
12. Reginato, A. M., Iozzo, R. V., and Jimenez, S. A., (1994) Arthritis Rheum. 37, 1338–1349
13. Vikkula, M., Mestasanta, M., Syvanen, A-C., Ala-Kokko, L., Vuorio, E., and Peltonen, L. (1992) Biochem. J. 285, 287–294
14. Horton, W., Miyashita, T., Kohn, K., Hasell, J. R., and Yamada, Y. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8864–8868
15. Wang, L. Q., Balakir, R., and Horton, W. E., Jr. (1991) J. Biol. Chem. 266, 19878–19881
16. Savagner, P., Kresbach, P. H., Hatano, O., Miyashita, T., Liebman, J., and Yamada, Y. (1995) DNA Cell Biol. 14, 501–510
17. Mukhopadhyay, K., Lelebrve, V., Zhou, G., Guorafalo, S., Kimura, J. H., and de Crombrugge, B. (1995) J. Biol. Chem. 270, 27711–27719
18. Matta, A., Glumoff, V., Paakkonen, P., Liski, D., Pinttinen, P. K., and Elima, K. (1993) Biochem. J. 294, 365–371
19. Wichterle, O., and Lim, D. (1990) Nature 345, 117–118
20. Reboja, M. P., and Yasuda, H. (1965) J. Appl. Polymer Sci. 9, 2425–2435
21. Folkman, J., and Moesena, A. (1965) Nature 273, 345–349
22. Jimenez, S. A., Ala-Kokko, L., and Prockop, D. J., Merryman, C. F., Shepard, N., and Dodge, G. R. (1991) Matrix Biol. 16, 29–39
23. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
24. Sieron, A. L., Fertala, A., Ala-Kokko, L., Prockop, D. J. (1995) J. Biol. Chem. 269, 21232–21237
25. Garner, M. M., and Revzia, A., (1981) Nucleic Acids Res. 9, 3047–3060
26. Jimenez, S. A., Varga, J., Olsen, A., Li, L., Diaz, A., and Herhal, J., and Koch, J. (1994) J. Biol. Chem. 269, 12684–12691
27. Rigg, A. B., Suzuki, H., Bourgeois, S. (1970) J. Mol. Biol. 48, 67–83
28. Schneider, I. (1972) J. Embryol. Exp. Morphol. 27, 333–365
29. Courey, A. J., and Tjian, R. (1968) Cell 55, 887–898
30. Jianping, Y., Zhang, X., and Dong, Z. (1996) Mol. Cell. Biol. 16, 157–167
31. Saffer, J. D., Jackson, S. P., and Annarella, M. B. (1991) Mol. Cell. Biol. 11, 2189–2199
32. Nehls, M. C., Rippe, R. A., Velego, L., and Brenner, D. A. (1991) Mol. Cell. Biol. 11, 4065–4071
33. Liye Li, Artlett, C. M., Jimenez, S. A., Hall, D. J., and Varga, J. (1995) Gene 164, 229–234
34. Briggs, M. R., Kadonaga, J. T., Bell, S. P., and Tjian, R. (1986) Science 234, 47–52
35. Inagaki, Y., Truter, S., and Ramirez, F. (1994) J. Biol. Chem. 269, 14828–14834
36. Inagaki, Y., Truter, S., Tana, S., Di Liberto, M., and Ramirez, F. (1995) J. Biol. Chem. 270, 3535–3538
Detection and Characterization of Sp1 Binding Activity in Human Chondrocytes and Its Alterations during Chondrocyte Dedifferentiation

Rita M. Dharmavaram, Gang Liu, Sheryl D. Mowers and Sergio A. Jimenez

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

Access the most updated version of this article at http://www.jbc.org/content/272/43/26918

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 18 of which can be accessed free at http://www.jbc.org/content/272/43/26918.full.html#ref-list-1