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

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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 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.

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). 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 protein 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

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the importance of transacting factors in the regulation of ex-
pression 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 transcrip-
tion (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 epiph-
yseal 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 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 medium was then replaced with a fresh equal volume of
culture medium and the dish was digested overnight at 37 °C. The
digested fragments were then passed through a nylon membrane into a vessel
containing fresh medium. The released chondrocytes were washed three
 times and cultured in minimum essential medium containing 4.5 g/liter glucose,
10% fetal bovine serum, 1000 units/ml penicillin, 1 mg/ml streptomycin, 2 mM
Dulbecco's modified Eagle's medium supplemented with 12% heat-inactivated serum and 1%
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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 DNA 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 E0.7-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 corrected for variations in transfection efficiencies. The transfected cells 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 following the addition of labeled consensus Sp1 oligonucleotide for 1 h at room temperature. The results obtained from chondrocytes that were allowed to become fibroblast-like by culture for 40 days on plastic were similar to those obtained from freshly isolated chondrocytes (data not shown).

**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'-ATTCGATCGGGGCGGCGA-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 EDTA 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
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 mg. However, when 15 mg 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 (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-
plexes with nuclear proteins from human chondrocytes was essentially complete at a concentration of 264 nM, similar to 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 oligonucleotide, 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. 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.](image)

![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.](image)

![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.](image)
set of standard dilutions of free probe demonstrated that 23.2
nM of nuclear protein isolated from chondrocytes dedifferenti-
ated into fibroblast-like cells was present in the reaction (Fig.
11F). 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 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, respec-
tively. 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-trans-
fered with pPacSp1, the Sp1 expression plasmid, substantial

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**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 probe 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.
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 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 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 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 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.

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