A Composite Element Binding the Vitamin D Receptor and the Retinoic X Receptor α Mediates the Transforming Growth Factor-β Inhibition of Decorin Gene Expression in Articular Chondrocytes

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Decorin, a small leucine-rich proteoglycan may play an important role in the attempt of cartilage repair initiated by chondrocytes in early stages of osteoarthritis, through its ability to bind collagen fibrils and growth factors such as transforming growth factor-β (TGF-β). We previously demonstrated that TGF-β decreased decorin mRNA steady state levels in articular chondrocytes (Demoor, M., Rédini, F., Boittin, M., and Pujol, J.-P. (1998) Biochim. Biophys. Acta 1398, 179–191). Here, we investigated the effect of TGF-β on decorin gene expression in both primary cultures of articular chondrocytes and chondrocytes dedifferentiated by serial passages. Transient transfection of cells with plasmid constructs of the decorin promoter linked to the luciferase reporter gene revealed transcriptional repression by TGF-β, in fully differentiated as well as dedifferentiated chondrocytes. Experiments with 5′-deleted constructs allowed characterization of a TGF-β-responsive element in the shortest construct (base pairs (bp) −155/+289). DNase I footprinting analysis delineated a negative TGF-β-responsive region between −140 and −111 bp in the decorin proximal promoter. Gel retardation assays demonstrated that TGF-β modulates decorin gene expression through transcription factors, the nature and mode of action of which depend on the differentiation state of the chondrocytes; two DNA-protein complexes were formed in the region −144/−127 bp with nuclear extracts from primary chondrocytes, whereas a higher mobility complex was observed in the −127/−111 bp region for dedifferentiated cells. Antibodies against vitamin D and retinoic acid receptors used in supershift experiments showed that these nuclear receptors are involved in the regulation of decorin gene expression in articular chondrocytes.

The mechanical properties of articular cartilage are determined by the composition of its extracellular matrix and the biological interactions of its constituents. Tenasa strength is provided by a framework of collagen fibers, whereas viscoelasticity of the tissue relies on a hydrated gel of high molecular weight proteoglycans (PGs), the aggrecans. In cartilage fibrils, collagens II, IX, and XI form a heterotypic aggregate that is essential for the control of fibril dimensions and surface properties (1, 2). Other participants in the fibril assemblages are collagen-binding glycoproteins, such as decorin and fibromodulin (3), but their functions are not completely understood.

Decorin belongs to the small leucine-rich proteoglycan family that also includes biglycan and epiphycan and the kera- tan sulfate proteoglycans fibromodulin, lumican, and keratocan (reviewed in Ref. 4). The core proteins of these molecules are composed of repeated motifs with conserved leucine residues (3). As an ubiquitous component of extracellular matrices, decorin is synthesized by the majority of mesenchymal cells (3, 5) and is found preferentially in association with collagen fibrils (6, 7). However, it also interacts with a variety of other components such as fibronectin (8), thrombospondin (9), transforming growth factor-β (TGF-β) (10), and a receptor required for its endocytosis (11). These binding properties, mainly mediated by the core protein, imply an important role in the structural organization of the extracellular matrix (5, 12). Because of its high affinity for TGF-β1, -β2, and -β3 isoforms (13), decorin functions as a matrix reservoir for these growth factors. However, controversial data have reported an inhibitory (14) or enhancing (15) effect of decorin on TGF-β activity, depending on the cell type studied.

In addition to its structural role, decorin can also affect cell growth. Its levels are greatly increased in quiescent cells, whereas its expression is generally absent in transformed cells and in most tumorigenic cell lines. De novo expression of decorin induces cell cycle arrest by activating the cyclin-kinase inhibitor p21 (16, 17). Recently, it has been shown that decorin interacts with the epidermal growth factor receptor, inducing activation of the mitogen-activated protein kinase pathway, mobilizes intracellular calcium, up-regulates p21, causes growth arrest (18), and blocks the growth of tumor xenografts (19). It has also been shown that decorin causes a functional inactivation of the oncogenic ErbB2 protein in breast carcinomas (20), leading to growth suppression and cytодifferentiation of mammary carcinoma cells.

Although decorin plays a crucial role in regulating assembly of collagen molecules and controlling cell proliferation, the role of this proteoglycan in cartilage has not yet been clearly identi-
tified; nor have been the mechanisms controlling its expression in normal and osteoarthritic joint tissues. In cartilage, differentially glycanated forms of decorin have been described during life and under pathological conditions (21–24).

Culture of chondrocytes in monolayers for prolonged periods or upon repeated passages leads to cell dedifferentiation, reflected by a decreased production of cartilage-specific macromolecules (collagen type II, aggrecan) (25–27), synthesis of the interstitial collagenas (types I, III, and V), and fibroblast-type PG versican at the expense of aggrecan (28–31). Similarly, the osteoarthritic process is associated with a progressive phenotypic modulation of the chondrocytes as a response to inflammatory cytokines, growth factors, and fragments of matrix molecules, not normally present in their microenvironment (32–34). In this context, no study has been yet performed to characterize the transcriptional activity and regulation of the decorin promoter in articular chondrocytes despite the crucial role that this proteoglycan plays in the maintenance of the collagen fibrillar structure and TGF-β binding. Recent cloning of the human (35) and murine (36) genes and the development of various decorin promoter/chloramphenicol acetyltransferase reporter gene constructs (37) make this goal now achievable.

The human decorin gene contains two leader exons in its 5'-untranslated region (35). However, no functional activity for the region spanning exon Ia was found (37), whereas strong basal promoter activity was detected using the 1-kb region 5' to exon Ib (35). This promoter can be divided into two main regions: a proximal promoter of −188 bp and a distal promoter of −800 bp (Fig. 1). The proximal promoter contains two functional TATA boxes and a CAAT box (38). Moreover, it contains two tumor necrosis factor-α (TNF-α)-responsive elements that mediate the transcriptional repression of the decorin gene by this cytokine (39). The proximal promoter region also contains a canonical and a functional activator protein 1 (AP1)-binding site, a bimodal regulator of decorin gene expression, which allows both repression by TNF-α and induction by interleukin-1 (40). The distal promoter of decorin harbors a number of cognate cis-acting factors including AP1, AP5, and NF-kB, several direct repeats, and a TGF-β-negative element (38).

Since TGF-β has been earlier characterized as a cartilage-inducing factor (41) and it is expressed in both articular cartilage and isolated chondrocytes (42, 43), it is of interest to determine its potential role in differentiation and repair process of the tissue. In this regard, TGF-β has been shown to stimulate the biosynthesis of matrix components (collagens and PGs) in several tissues and cell types, including chondrocytes (44, 45). It also inhibits the production of metalloproteases and enhances the expression of their tissue inhibitors, the TIMPs (46). Therefore, it appears as a good candidate for cartilage repair, particularly in joint diseases such as rheumatoid arthritis and osteoarthritis.

We previously showed that TGF-β inhibited decorin mRNA expression in both primary and passaged cultures of rabbit articular chondrocytes (47). Using the same culture model, we demonstrate here that a 30-bp region of the decorin promoter (−111/−140 bp), harboring a vitamin D receptor (VDR)-retinoid X receptor (RXR) motif, is responsible for the TGF-β down-regulation of the decorin gene expression in both differentiated and dedifferentiated chondrocytes. Our results raise the possibility that the VDR-RXR motif could be implicated as a regulator of decorin expression in articular chondrocytes.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Articular Chondrocytes—**Articular cartilage slices were taken from the shoulders and the knees of 3-week-old male rabbits. Chondrocytes were isolated by sequential digestion, as previously described (48) and cultured in Dulbecco’s modified Eagle’s medium supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (0.25 μg/ml) and 10% heat-inactivated fetal calf serum (Life Technologies, Inc.). The cells were grown at 37 °C in a 5% CO₂, 95% atmosphere with medium change every 2–3 days. After reaching confluency (6–7 days), the primary cultures were used for experiments in order to avoid dedifferentiation of the chondrocytes. Such primary cultures were previously shown to produce both collagen type II and PGs specific to cartilage (28, 48). To study the influence of cell differentiation on the decorin gene expression, chondrocyte cultures were passaged up to five times using trypsinization of the monolayers (0.25% trypsin in phosphate-buffered saline; Life Technologies).

**Preparation of Nuclear Extracts—** All the buffers used for the preparation of nuclear extracts contained the following protease inhibitors: 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin, and pepstatin (10 μg/ml each). Cultures of rabbit articular chondrocytes were harvested at 80% confluency, and nuclear extracts were prepared according to the procedures of Dignam et al. (49) or, when mentioned, by the miniprep/protocol of Andrews and Fuller (50).

**Decorin-luciferase Constructs and Transient Cell Transfection Assays—** Transient transfection experiments were performed using various 5'-deletion constructs derived from pUC DEC-10/CAT (37). pUC DEC-10/CAT contained a 1.43-kb fragment of the human decorin gene, corresponding to approximately 1 kb of decorin promoter, and exon Ib, linked to the chloramphenicol acetyltransferase reporter gene cloned into the pUC-CAT vector. The promoter region between the SalI (−1004) and EcoRII (+269) sites was subcloned upstream of the luciferase reporter gene (pGL2, Promega) to generate the pGL2–1.273 kb plasmid. Other constructs were generated from pGL2–1.273 kb as follows. A 955-bp PvuII fragment of the decorin gene (−805/−150 bp) was cloned into the BglII site of pGL2-basic, which were made blunt. Plasmid pGL2–948 bp corresponds to the −679/−269 DNA fragment of the decorin gene cloned into the HindIII, EcoRII sites of pGL2-basic. The last plasmid, pGL2–424 bp (−155/−269 bp), was generated after digestion with SmaI and vector fusion.

Plasmid pGL2-TK wt was obtained by subcloning three copies of the 33-bp wild-type DEC oligonucleotide (−143/−111 bp) into the thymidine kinase (TK)-luciferase expression vector with HindIII and BglII linkers. Similarly, the mutant plasmid pGL2-TK mut2 was generated with the mutation directed against the −127/−109 bp region corresponding to the VDR-RXR binding site (see “Results”).

For transient transfection assays, chondrocytes were seeded at a density of 10⁵ cells/100 mm-culture plate in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. At 80% confluency, transfections were performed by the calcium phosphate/DNA co-precipitation method, using HEPES (51). Briefly, the cells were transfected with 15 μg of decorin promoter-luciferase vector DNA, together with an expressing pSV-40 β-galactosidase expression vector (4 μg) that was used as an internal control of transfection efficiency (52), mixed with 50 μl of 2.5 mM CaCl₂. Five hundred μl of 2xEHEPS buffer were then slowly added. After 20 min of incubation at room temperature, the DNA solution was added to the chondrocytes, and the cultures were incubated at 37 °C. Fifteen to 20 h after transfection, the medium was replaced by a fresh medium in the presence or absence of 2 ng/ml TGF-β1 (R&D Systems). The cells were harvested 48 h after the addition of DNA and after 72 h for chondrocytes subcultured three or five times (P3 and P5, respectively). The cells were then washed twice with phosphate-buffered saline and extracted with lysis buffer (0.25 M Tris-HCl, pH 7.9). Luciferase activity was assayed on total cell extracts using d-luciferin substrate (Promega). Total light emission during the
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FIG. 2. Activity of deleted decorin promoter constructs in TGF-β1-treated rabbit articular chondrocytes in fully differentiated (primary cultures) (A) or dedifferentiated chondrocytes (fifth passage cultures) (B). Fifteen μg of each construct were transfected by the calcium phosphate precipitation method. Four μg of pSV40-β-Gal were co-transfected in order to control the efficiency of transfection. After a night recovery, the medium was discarded, and the cells were cultured for another 24-h (primary chondrocytes) or 48-h (dedifferentiated) period in the presence or absence of 2 ng/ml TGF-β1. Luciferase activities from three averaged experiments were measured and standardized for the activity in the internal control and the protein level. The effect of TGF-β1 is indicated as the percentage of respective control plasmids.

RESULTS
Identification of a 155-bp Sequence in the Decorin Promoter That Mediates TGF-β1 Inhibitory Effect—We previously demonstrated that TGF-β decreases decorin mRNA steady-state levels in rabbit articular chondrocytes, both in primary culture and after dedifferentiation by serial passages (47). To further investigate the mechanism responsible for this down-regulation of decorin gene expression, we performed transient cell transfection experiments with several constructs that contain deleted 5′-sequences of human decorin promoter linked to the luciferase reporter gene, as indicated in Fig. 2. Preconfluent rabbit articular chondrocytes were transfected with different constructs for 20 h, and the cultures were then incubated with 2 ng/ml TGF-β1, a concentration previously shown to reduce decorin mRNA levels by about 80% (47). Assay of luciferase activity after 24 h of incubation indicated that TGF-β1 induced a decrease of the promoter activity of the pGL2-1.273 construct, which contains the entire promoter of the decorin gene (83% reduction compared with control; Fig. 2A). The inhibition was of a similar extent as that previously observed at the mRNA level (47), suggesting that the TGF-β1 down-regulation of decorin gene expression is mediated by transcriptional mechanisms.

To characterize the TGF-β-responsive sequences of the decorin promoter, a series of 5′-deletions of the decorin promoter linked to the luciferase reporter gene was tested. Deletion of the 5′-sequences from position −1904 to −155 did not alter the response to TGF-β1, although the basal activity of the promoter was also reduced. Specifically, TGF-β1 reduced the promoter activity by 70–80% in all of the constructs tested (Fig. 2A), including the shortest one (−155/+269 bp). This suggested that TGF-β-responsive elements are present in a 155-bp sequence upstream of the transcription start site.

In dedifferentiated chondrocytes, the inhibitory effect of TGF-β1 was still observed with the four constructs studied. TGF-β responsiveness was minimally affected after five passages, with 49–75% reduction of decorin gene transcription compared with controls (Fig. 2B). Since the inhibitory effect of TGF-β1 was observed with the −155 bp construct, we concluded that the TGF-β-responsive elements present in the −155 bp were still active when chondrocytes had lost their phenotype.
The protected region is represented diagrammatically on the lanes 5 (treated by 2 ng/ml TGF-β1 primary culture or dedifferentiated by five passages, treated or not (0.5, 30, 45, and 60 s, respectively); extracts from differentiated chondrocytes treated (269) was labeled at the ing. The probe (11002/11001) was added in lanes 5–8, incubation with nuclear extracts from differentiated chondrocytes treated (lanes 7 and 8) or not (lanes 5 and 6) by TGF-β1; lanes 9 and 10, incubation with nuclear extracts of dedifferentiated chondrocytes treated (lane 10) or not (lane 9) by TGF-β1. B, schematic localization of the protected region D1 in the decorin promoter.

DNase I Footprinting and EMSA Delineate a TGF-β-responsive Region of 30 bp—A portion of the TGF-β-responsive region of the decorin promoter identified in transient cell transfection assays was used as a probe in DNase I footprinting experiments (−155+/−269 bp). Nuclear proteins from the crude extracts of primary cultures of rabbit articular chondrocytes protected a region of the decorin promoter named D1 from DNase I digestion (Fig. 3A, lanes 5 and 6). When compared with data from the A + G sequencing reaction, this region was shown to correspond to the sequence −140/−111 of the decorin promoter (Fig. 3A, lanes 5 and 6 compared with lane 1). When nuclear extracts from TGF-β-treated chondrocytes were used (lanes 7 and 8), the protection was reduced, as compared with nuclear proteins from control chondrocytes (lanes 5 and 6).

The results demonstrate that one or several nuclear factors extracted from primary cultures of chondrocytes bind to a 30-bp region of the decorin promoter encompassing the sequence −140/−111 bp. In the presence of TGF-β, the binding is decreased, as a consequence of a decreased level or reduced binding activity of these nuclear factors. The DNase I protection was also reduced for nuclear extracts derived from dedifferentiated chondrocytes (subcultured three and five times, P3 and P5, respectively), a new complex of higher mo-

Fig. 3. DNase I footprint of the decorin promoter. A, nuclear extracts (25 μg) were prepared from rabbit articular chondrocytes in primary culture or dedifferentiated by five passages, treated or not treated by 2 ng/ml TGF-β1. They were analyzed by DNase I footprinting. The probe (−155+/−269) was labeled at the SmaI site on the coding strand. The protected region is represented diagrammatically on the left. Lane 1, A + G ladder; lanes 2–4, free probe digested by DNase I (0.5×, 30, 45, and 60 s, respectively); lanes 5–8, incubation with nuclear extracts from differentiated chondrocytes treated (lanes 7 and 8) or not (lanes 5 and 6) by TGF-β1; lanes 9 and 10, incubation with nuclear extracts of dedifferentiated chondrocytes treated (lane 10) or not (lane 9) by TGF-β1. B, schematic localization of the protected region D1 in the decorin promoter.

Fig. 4. Binding of rabbit articular chondrocytes nuclear proteins to the −143/−111 domain of the decorin gene promoter. A, EMSAs were performed with nuclear extracts from primary chondrocytes (lanes 2 and 3) and chondrocytes dedifferentiated by three (lanes 4 and 5) and five (lanes 6 and 7) passages in the presence of the radiolabeled DEC probe (−143/−111 bp). Unlabeled competitor (−143/−111; 25×) was added in lanes 3, 5, and 7. The specific protein-DNA complexes are indicated by an arrow and named C1, C2, and C3. B, EMSAs were performed in the presence of nuclear extracts from primary chondrocytes and 25× unlabeled competitor corresponding to the region −188/−140 bp of the decorin promoter (lane 3) or to the DEC sequence (lane 4). C, direct binding between nuclear extracts from primary chondrocytes and five probes encompassing other regions of the decorin promoter. Lanes 2–6, as compared with the DEC probe (−143/−111 bp, lane 1).
EMSA was performed with nuclear proteins extracted from three passages (primary chondrocytes and from chondrocytes dedifferentiated by \( \text{TGF-}\beta_1 \)) with the radiolabeled \( \text{DEC} \) probe in the presence of increasing amounts (5–30 \( \mu \)g) of nuclear extracts from primary chondrocytes (A) and from chondrocytes dedifferentiated by three passages (B). The arrows point to the complexes C1, C2, and C3 that are generated.

To further confirm the specificity of binding between the DEC sequence and nuclear extracts from articular chondrocytes, additive competition experiments were performed in the presence of competitors that did not correspond to the DEC sequence (–188/–140 bp). In this case, the formation of both complexes was not altered, as compared with the competition with unlabeled DEC probe (Fig. 4B, lanes 3 and 4). Furthermore, direct binding experiments were realized with five different probes, corresponding to a sequence in the decorin promoter that is not involved in TGF-\( \beta_1 \) effect (i.e., –188/–140 bp). The results show patterns of binding that are different from those obtained with the DEC probe (Fig. 4C, lanes 2–6 compared with lane 1).

Additive EMSA experiments were carried out with increasing amounts of nuclear extracts from differentiated and dedifferentiated chondrocytes (Fig. 5, A and B). The results presented in Fig. 5A show that a third complex progressively appeared, becoming predominant in the presence of 30 \( \mu \)g of nuclear extracts from primary chondrocytes (Fig. 5A, lanes 4 and 5 compared with lanes 2 and 3). This complex seems to be identical to C3, already seen with nuclear extracts from differentiated chondrocytes (Fig. 4A, lane 4). We can therefore hypothesize that low concentrations of transcription factors preferentially bind to higher affinity binding sites, forming the two complexes C1 and C2. When the concentration increased, the factors probably bind to lower affinity sites that are present in higher quantity at the cell surface, leading to the formation of the higher mobility complex C3. In the presence of nuclear extracts from dedifferentiated chondrocytes, C3 was the only complex observed, whatever the concentration used (Fig. 5B, lanes 2–5).

**TGF-\( \beta_1 \) Inhibits Formation of the Transcription Complexes**—EMSAs were performed with nuclear proteins extracted from chondrocytes cultured in the presence of TGF-\( \beta_1 \) (2 ng/ml), in order to determine the involvement of this factor in the formation of the DNA-protein complexes previously observed. The presence of TGF-\( \beta_1 \) inhibited the formation of both complexes C1 and C2 (Fig. 6A, lanes 4, 7, 9, and 11, compared with lanes 2 and 3, 5 and 6, 8, and 10, respectively). Similarly, the binding of the transcription factors corresponding to the formation of C3 was reduced in the presence of nuclear extracts from TGF-\( \beta_1 \)-treated dedifferentiated chondrocytes (Fig. 6B, lane 13 compared with lane 12). The overall results indicated that TGF-\( \beta_1 \) decreased the binding of transcription factors to the sequence –140/–111 bp, leading to the inhibition of decorin expression, as previously revealed by transient transfection assays (Fig. 2) and RNA quantification (47).

**Different Cis-acting Elements Are Involved in the Regulation of Decorin Expression during the Dedifferentiation Process**—Nuclear extracts of primary chondrocytes (PO) were incubated with the labeled probe DEC, corresponding to the –143/–111 bp DNA fragment of the decorin promoter, in the presence of five unlabeled oligonucleotides (oligonucleotides I–V overlapping the region –143/–108 bp), used as competitors (Table I). The resulting complexes were analyzed by EMSA (Figs. 7 and 8). In the presence of nuclear proteins from differentiated chondrocytes, the two complexes C1 and C2 were both specifically competed by an excess of unlabeled DEC probe (Fig. 7, lanes 2–4 compared with lane 1, and lanes 15–17 compared with lane 14) and by the addition of unlabeled –144/–127 bp probe (named probe I; lanes 5–7 compared with lane 1), but not by an excess of probes II, III, IV, and V, corresponding, respectively, to –133/–120 (lanes 8–10), –127/–109 (lanes 11−13), –123/–108 (lanes 18−20), and –136/–120 (lanes 21–23). These results indicate that both complexes C1 and C2 contain transcription factors that bind specifically to the region comprised between positions –144 and –127 bp of the decorin promoter. Competitive EMSAs were performed in parallel with nuclear extracts from dedifferentiated cells. When the total DEC probe (–143/–111 bp) was incubated with those extracts, only the C3 complex was detected (Fig. 8A, lane 1), as previously shown. This binding complex was specifically competed by unlabeled DEC probe (–143/–111, lanes 2 and 3), by unlabeled probe III (–127/–109, lanes 8 and 9), and to a lesser extent by unlabeled probes I and V (–144/–127 and –136/–120, respectively; lanes 4 and 5 and lanes 12 and 13) but not by unlabeled probes II and...
IV (–133/–120 and –123/–108, respectively; lanes 6 and 7 and lanes 10 and 11).

Since the oligonucleotide –127/–109 (probe III) was highly competitive, we searched for nuclear factors that could bind to specific sites in that sequence. Myc-max and VDR/RXR appeared as good candidates, since their consensus sequences display less than two mismatches with the corresponding decorin sequence. Nucleotides corresponding to binding sites for Myc-max and VDR/RXR were therefore synthesized (Table I) and used as competitors in EMSAs (as 10-, 25-, and 50-fold excess). The specific protein-DNA complexes C1 and C2 are indicated by an arrow.

To identify the proteins implicated in the formation of the three complexes C1, C2, and C3, antibodies directed against VDR and RXR were used to interfere with the protein-DNA complexes in EMSAs. Mutations at the DEC 5′-end (mut1; mutations of Tst-1/Oct-6 binding site) diminished the formation of both complexes C1 and C2 in the presence of nuclear extracts from primary chondrocytes while that of the complex C3 appeared (Fig. 9, lane 6 compared with lane 2). In the presence of nuclear extracts from dedifferentiated chondrocytes, the formation of the C3 complex was still observed (lanes 7 and 8 compared with lanes 3 and 4). The second mutation, mut2, which was directed against the VDR-DR3 binding site, did not affect the formation of C1 and C2 (lane 10 compared with lane 2) but diminished that of C3, corresponding to the binding of transcription factors to VDRE (lanes 11 and 12 compared with lanes 3 and 4). Finally, when mutations were directed against both the Tst-1/Oct-6 and the VDR-RRX binding sites (mut3), the formation of the three complexes was tremendously diminished (lanes 14–16 compared with lanes 2–4).

The mutational analyses confirmed that the crucial residues for the binding specificity of C1 and C2 in differentiated chondrocytes are located between positions –144 and –127 bp and between positions –127 and –109 for the formation of C3 in dedifferentiated chondrocytes.

Identification of VDR/RXR as the Transcription Factors Involved in Decorin Gene Expression in Articular Chondrocytes—To identify the proteins implicated in the formation of the three complexes C1, C2, and C3, antibodies directed against VDR and RXR were used to interfere with the protein-
DNA interaction. Results presented in Fig. 10 clearly demonstrate that anti-VDR, anti-RXR antibodies alone or in combination were able to specifically inhibit C1 and C2 complex formation in the presence of nuclear extracts from differentiated chondrocytes (lanes 2–4, respectively, compared with lane 1), and diminished the formation of C3 in dedifferentiated cells (lanes 6–8 compared with lane 5). These results thus confirm the involvement of both nuclear receptors in the regulation of decorin expression in articular chondrocytes.

A Single Complex Is Formed in the Presence of Nuclear Extracts from Human Articular Chondrocytes—The species specificity was also studied by EMSAs in the presence of the DEC probe and nuclear extracts from human chondrocytes compared with primary and dedifferentiated rabbit articular chondrocytes. The results presented in Fig. 11 revealed that only one complex was formed in the presence of nuclear proteins from human chondrocytes, migrating at the same distance as C3, observed with dedifferentiated rabbit chondrocytes (lane 3 compared with lane 2). Competitive EMSAs showed that the formation of this complex is almost totally abolished by a molar excess of cold DEC probe, probe I (−144/−127) and probe III (−127/−109) (Fig. 11, lanes 4–6 compared with lane 3).

The decoVDRE Is a Functional Element—We explored the effect of the mutation directed against the VDR-RXR binding site on decorin gene expression by using transient transfection assays in rabbit articular chondrocytes. We subcloned three copies of decoVDRE in right (WT+) or wrong (WT−) orientation or synthetic oligonucleotide corresponding to the mutation mut2 upstream of the TK minimal promoter driving the luciferase reporter gene. Fig. 12 shows that the TGF-β1-induced inhibitory effect on decorin expression was lost when mut2 was subcloned instead of decoVDRE (Fig. 12A), demonstrating that

FIG. 8. Competition experiments with oligonucleotides from the −143/−108 sequence. Experimental conditions were the same as those described in the legend to Fig. 7, except that nuclear extracts from dedifferentiated chondrocytes were used in A and B. Competitions were made with a 25- and 50-fold excess of unlabeled oligonucleotides I–V (A) and with a 50-fold excess of unlabeled consensus Myc-max and VDR-RXR sequences (B). The specific protein-DNA complex C3 is indicated by an arrow. C, competitions were performed with nuclear extracts from differentiated chondrocytes in the presence of a 50-fold excess of unlabelled consensus Myc-max and VDR-RXR sequences.

FIG. 9. Cooperativity between the 5′- and the 3′-end of the −144/−111 bp sequence of the decorin promoter for the binding of VDR/RXR. EMSAs were performed with the radiolabeled −143/−111 bp sequence of decorin promoter wild type (Dec, lanes 1–4) or mutated in its 5′-end (mut1, lanes 5–8), in its 3′-end (mut2, lanes 9–12), or in both ends (mut3, lanes 13–16) in the presence of nuclear extracts from primary chondrocytes (lanes 2, 6, 10, and 14) and dedifferentiated by three passages (lanes 3, 7, 11, and 15) and by five passages (lanes 4, 8, 12, and 16). The specific protein-DNA complexes C1, C2, and C3 are indicated by arrows.

FIG. 10. The nuclear receptors VDR and RXR are involved in the formation of the three DNA-protein complexes. Supershift experiments were performed with the radiolabeled −143/−111 bp sequence of the decorin promoter in the presence of nuclear extracts from primary chondrocytes (lanes 1–4) or chondrocytes dedifferentiated by five passages (lanes 5–8) and antibodies directed against VDR (1:10; lanes 2 and 6), RXR (1:10; lanes 3 and 7), or both (lanes 4 and 8).
the sequence decVDRE is necessary for TGF-β responsiveness of the decorin promoter. The experiments performed in parallel with pGL2-TK wt (−) revealed that the inhibitory effect of TGF-β was no more significant, as compared with pGL2-TK wt (+) (Fig. 12B).

**DISCUSSION**

Studies on the regulation of decorin gene expression are motivated by the role played by this gene product in the regulation of TGF-β bioactivity and matrix assembly. Since TGF-β is an important mediator of extracellular matrix remodeling, it could play a pivotal role in the attempt of cartilage repair in the early stages of OA. We have previously shown that decorin expression was related to the quiescent state of rabbit articular chondrocytes and that TGF-β decreased decorin messenger RNA expression in this cell system (47). Down-regulation of decorin expression by TGF-β at the protein and RNA levels had been well documented in a variety of cell types including fibroblasts (55); however, studies performed at the transcriptional level are quite few. Therefore, we have extended our study and demonstrated here that TGF-β is also a potent down-regulator of decorin gene expression in articular chondrocytes.

Among potential binding sites present in the promoter of decorin gene exon Ib, a TGF-β-negative element was found at position −685 (35). This motif conforms to the consensus sequence GnnTTGGtGa that has been found in the promoter region of the proteinase stromelysin (56). This putative TGF-β-negative element could function as a transcriptional silencer and could suppress decorin activity in TGF-β-sensitive cells. In our experimental model, 5′-end progressive deletions of the promoter used in transient transfection assays showed that a strong basal activity of the promoter was detected with the longest construct (−1004/−269), and then the activity diminished with the PspI construct (−805/−150) and increased with the EcoRV construct (−679/−269). These results suggest that a silencer could be present in the promoter of exon Ib between positions −805 and −679, corresponding to the position of the TGF-β-negative element (−685). Similar results were obtained with fibroblasts, where a decrease in functional activity of the promoter was observed when the construct including the TGF-β-negative element was used (56).

To our knowledge, only one study described the direct effect of TGF-β on decorin gene expression (39). This work reported that TGF-β, together with TNF-α, exerted an inhibitory effect on decorin gene expression in fibroblasts. However, whereas TNF-α effect required promoter sequences between positions −188 and −140 bp relative to the transcription start site, 1 kb of decorin promoter sequences were not sufficient to mediate TGF-β response.

In the present study, we demonstrated for the first time that a region of the first 155 bp upstream of the initiation site in the promoter of exon Ib is responsible for the inhibitory effect exerted by TGF-β on decorin gene expression in rabbit articular chondrocytes. The region involved in TGF-β effect was more precisely defined with DNase I protection assays, which revealed a 30-bp region between residues −140 and −111 rela-
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Fig. 13. Hypothetical DNA conformation of the sequence −144/−111 bp of the decorin gene promoter.

Fig. 14. Comparison of the putative decVDRE with the consensus and other identified VDREs. The consensus VDRE was suggested as a direct repeat with a 3-nucleotide spacer (DR3). The bases depicted in darker type are those homologous to the randomly selected, highest affinity VDRE. *, consensus VDRE determined by binding of VDR-RXR heterodimers to randomly synthesized oligonucleotide sequences.

Fig. 15. Possible functional cross-talks between decorin, vitamin D, TGF-β, and p21.

formation of the DNA strand in that region may implicate the 3′-end even for the binding of VDR/RXR in the region −140/−127 bp. A hairpin-like conformation of DNA can be therefore suggested in this region, where transcription factors of the VDR/RXR family bind at the same time to the VDRE sequence (−123/−111 bp) and to the 5′-end of the probe (−140/−127 bp) (Fig. 13). Surprisingly, no VDRE motif is described in the latter, but a sequence sharing high homology (93.3%) with the factor Tst-1/Oct-6 is present. However, Oct-6 can be induced by retinoic acid, and the nuclear receptor RXR has been reported to bind to Tst-1 (59). In the presence of TGF-β, the formation of both complexes C1 and C2 is decreased, in parallel with a diminution of decorin expression. Therefore, the factors involved in regulation of decorin expression can be described as transactivating ones.

When the chondrocytes are dedifferentiated, the factor involved binds to the 3′-end of the DEC sequence, more precisely to the −123/−111 bp region. This time, a typical VDRE motif is present, consisting of two hexanuclear repeats separated by a 3-bp spacer named DR3. VDR is shown to bind to the 3′-end of the sequence, whereas RXR binds to the 5′-end (60). Sequence comparison between the cis-binding element present in the −125/−108 bp region of decorin promoter and consVDRE revealed 13 identical bases out of 15 nucleotides (Fig. 14). When compared with other identified VDREs, hdecVDRE shows high homology with VDRE present in the human and rat osteocalcin genes (61, 62).

Cooperative action of TGF-β and vitamin D, and the phenotype of VDR knock-out mice indicate that there may be cross-talk between the two signaling pathways (63). Interestingly, it has been recently reported that Smad 3, one of the Smad proteins downstream in the TGF-β signaling pathway, was found to act as a coactivator specific for ligand-induced transactivation of VDR (64). Looking at the role of Smad 6 and Smad 7, which are negative regulators of the TGF-β/BMP signaling pathway, the same group showed that Smad 7, but not Smad 6, inhibited the formation of the VDR-Smad 3 complex, whereas Smad 6 had no effect (65). Taken together, the results from the literature revealed that the interplay between TGF-β and the vitamin D signaling pathway is, in part, mediated by both classes of Smad proteins, which modulate VDR transactivation positively and negatively. We may therefore suggest that in our experimental model, regulation of decorin expression by TGF-β is mediated by the formation of VDR-Smad 3 or VDR-Smad 7 complex, leading to the inhibition of the gene expression. Therefore, a regulatory loop can be suggested, involving decorin, TGF-β, and vitamin D. These interplays are even more complex when considering the presence of a VDRE motif in the promoters of TGF-β2 and p21 (66, 67). Furthermore, decorin has been widely described as the product of a quiescent gene (39), and its down-regulation by TGF-β in chondrocytes may contribute to make them start to proliferate as it occurs in the
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A Composite Element Binding the Vitamin D Receptor and the Retinoic X Receptor α Mediates the Transforming Growth Factor-β Inhibition of Decorin Gene Expression in Articular Chondrocytes
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