Structural and Functional Characterization of the Human Perlecan Gene Promoter

TRANSCRIPTIONAL ACTIVATION BY TRANSFORMING GROWTH FACTOR-β VIA A NUCLEAR FACTOR 1-BINDING ELEMENT™

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Perlecan, a modular heparan sulfate proteoglycan of basement membranes and cell surfaces, plays a crucial role in regulating the assembly of extracellular matrices and the binding of nutrients and growth factors to target cells. To achieve a molecular understanding of perlecan gene regulation, we isolated the 5′-flanking region and investigated its functional promoter activity and its response to cytokines. Transient cell transfection assays, using plasmid constructs harboring the perlecan promoter linked to the chloramphenicol acetyltransferase reporter gene, demonstrated that the largest 2.5-kilobase construct contained maximal promoter activity. This promoter region was functionally active in a variety of cells of diverse histogenetic origin, thus corroborating the widespread expression of this gene product. Stepwise 5′ deletion analyses demonstrated that the 461-basepair (bp) proximal promoter retained ~90% of the total activity, and internal deletions confirmed that the most proximal sequence was essential for proper promoter activity. Nanomolar amounts of transforming growth factor-β induced 2–3-fold perlecan mRNA and protein core levels in normal human skin fibroblasts, and this induction was transcriptionally regulated; in contrast, tumor necrosis factor-α had no effect and was incapable of counteracting the effects of TGF-β. Using additional 5′ deletions and DNase footprinting analyses, we mapped the TGF-β responsive region to a sequence of 177 bp contained between –461 and –285. This region harbored a 14-bp element similar to a TGF-β-responsive element present in the promoters of collagen α1(I), α2(I), elastin, and growth hormone. Electrophoretic mobility shift assays and mutational analyses demonstrated that the perlecan TGF-β-responsive element bound specifically to TGF-β-inducible nuclear proteins with high affinity for NF-1 member(s) of transcription factors.

Perlecan (1), a modular proteoglycan carrying primarily heparan sulfate side chains, is present in virtually all vascularized tissues (2, 3). This proteoglycan is expressed either as an integral component of basement membranes or diffusely in connective tissues as diverse as ovarian stroma, skin, and cartilage (4, 5). Perlecan epitopes are distributed along the sinusoidal spaces of the liver, spleen, bone marrow, and lymphoreticular system including thymus, tonsils, and lymph nodes (4). The latter suggests that perlecan may be important for the normal development and function of the bone marrow and lymphoreticular system (6). Analysis of the primary structure of human perlecan reveals an assembly of five consecutive domains with homology to molecules involved in the control of cell proliferation, lipoprotein uptake, and adhesion (1, 7). This molecular architecture suggests that perlecan may play a variety of biological functions in different tissue locations (8). For example, perlecan can bind several growth factors via its heparan sulfate side chains (9, 10), while the protein core perlecan can bind nidogen (11) and various integrins (12, 13). Recently, we provided direct evidence that basic fibroblast growth factor binds to heparan sulfate chains attached to domain I and that the concerted action of heparanases and proteases could modulate the bioavailability of this growth factor in vivo (14). The perlecan-mediated induction of angiogenesis is supported by the observation that metastatic melanoma cells exhibit a marked induction of this gene, and this increase correlates with an abundant deposition of perlecan proteoglycan in the neovascularized tumor stroma (15). In addition, perlecan binds to fibronectin (16), γ-interferon (17), and TGF-β (18), and recent evidence indicates that perlecan can be adhesive for fibroblasts, endothelial cells (19), and chondrocytes (5) while being anti-adhesive for hematopoietic cells (19). These opposing functions are not surprising given the multipurpose structure of the protein core and the complexity of its potential post-translational modifications. The latter may result in heparan sulfate side chains either alone (20) or in combination with chondroitin sulfate (21), fatty acylation (22), or the total lack of glycanation of the protein core which can be produced as a glycoprotein (5, 23).

Perlecan expression is up-regulated by nanomolar concentrations of TGF-β in human colon carcinoma cells (24) and murine uterine epithelial cells (25). TGF-β also stimulates the

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The abbreviations used are: TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; CAT, chloramphenicol acetyltransferase; TGF-βRE, TGF-β-responsive element; kb, kilobase pair(s); bp, base pair(s); ATF, activating transcription factor; NF, nuclear factor; CTF, ciliary transcription factor.

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synthesis of basic fibroblast growth factor-binding heparan sulfate proteoglycans in mouse 3T3 cells (26). Moreover, it is quite evident that perlecan is a key regulator of the complex interactions that take place during neovascularization, and that the interplay amongst TGF-β, basic fibroblast growth factor, and perlecan is biologically relevant (3, 14).

We previously reported the complete genomic organization of the human perlecan HSPG2 gene, including 0.77 kb of the 5′-flanking region (27). In the current investigation we cloned and sequenced 2.5 kb of the upstream region, and, by using transient cell transfection assays and stepwise 5′ or internal deletions, we demonstrate functional promoter activity of the perlecan gene. The perlecan promoter was transcriptionally activated by TGF-β but was unresponsive to TNF-α. A TGF-β-responsive element was mapped to a 14 bp sequence located in the proximal promoter region by DNase footprinting and electrophoretic mobility shift assays. The perlecan TGF-β-responsive element bound specifically to TGF-β-inducible nuclear proteins which contained NF-1 members of transcription factors. Collectively our results indicate that perlecan is up-regulated by TGF-β via a transcriptionally mediated control of perlecan promoter and that these effects could be important in the modulation of this proteoglycan during angiogenesis, tissue remodeling, and tumor formation.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Cultures—**Radionucleotides [α-32P]dCTP, [α-32P]dATP, and [γ-32P]dATP (3000–5000 Ci/mmol, 1 Ci = 37 Gbq) and [14C]chloramphenicol (~100 mCi/mmol) were obtained from Amersham Corp. Restriction endonucleases were purchased from Promega (Madison, WI). Human recombinant TNF-α was purchased from Boehringer Mannheim. Human recombinant TGF-β2 was a generous gift from Dr. David Osen (Celtix Laboratories, CA). HeLa, WiDr colon carcinoma, Saos-2 osteosarcoma, HL-60 promyelocytic leukemia, HT1080 fibrosarcoma, and NIH-3T3 fibroblastic cells were obtained from the American Type Culture Collection. Mouse M2 melanoma cells were obtained from Dr. I. J. Fidler (Houston, TX). Human diploid fibroblast cultures, established from neonatal foreskins, were utilized at passages 3–8. The cell cultures were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mm glutamine, and 100 units/ml penicillin.

**Plasmid Construction—**Deletion constructs cloned upstream of the CAT reporter gene within the pUC or pBluescript (pBS) vector (Stratagene) were generated as described before (28). The 2.5-kb 5′-flanking region linked to oxon 1 (86 bp) was subcloned into the pBS vector and then subcloned into the respective sites in the pUC-CAT vector. Shorter constructs were generated by deletion of the 2.5 kb construct using various restriction endonucleases. Constructs (−1850 to −1500) were generated by digestion of the −2500 construct with HindIII and EcoRV, which deleted −1800 and −700 bp fragments between −2500 and −1800. The asymmetric distribution of SmaI sites in the −2500 construct allowed the utilization of partial digestion of the construct thereby generating various 5′ and internal deletion products. These constructs were subsequently self-ligated and sequenced to confirm the correct orientation.

**Transient Cell Transfection and CAT Assays—**Transient transfections of cell types were performed by the calcium phosphate procedure essentially as described before (28, 29). Briefly, subconfluent cells in 75-cm² flasks were trypanoyzed, washed, and co-transfected in suspension with 20–40 µg of perlecan promoter-CAT constructs and 10 µg of pSV-β-galactosidase plasmid to provide an internal standard for normalization of the values. The cells were incubated for 48 h, washed with Hanks’ balanced salt solution (Ca2+ and Mg2+ free), and incubated in Dulbecco’s modified Eagle’s medium for an additional 12 h. The cells were then washed again and assayed for β-galactosidase activity (29). CAT assays were performed as described before (30). The products were resolved on preloaded thin layer chromatography plates in a chloroform/methanol (65:35) mobile phase and subjected to autoradiography. To quantify the acetylated [14C]chloramphenicol, the autoradiograms were subjected to scanning laser densitometry and computer integration. To study the transcriptional regulation by TGF-β and TNF-α, minor variations to the protocols described above were made. Essentially, cells were treated as above except that the cells were placed in Dulbecco’s modified Eagle’s medium containing 1% fetal calf serum 4–5 h before the addition of growth factors. In experiments without growth factors, the cells were placed in DMEM containing 1% fetal calf serum. After 40 h of additional incubation, the cells were rinsed with phosphate-buffered saline and harvested. Aliquots corresponding to identical β-galactosidase activity were used for each CAT assay. After autoradiography, the plates were re-run and counted.

**Isolation of RNA, Northern Blotting, and Immunoblotting—**Total RNA was isolated using standard procedures (31) and analyzed by Northern blotting as described before (29). Human perlecan cDNA, clone HS1, encompassing domain III (32) was used as a probe, or the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for normalization of the values. Both probes were radiolabeled (~106 cpm/µg of DNA) by random priming (33) using [α-32P]dCTP. The [32P]dDNA-mRNA hybrids were visualized by autoradiography, and the steady-state levels of perlecan or glyceraldehyde-3-phosphate dehydrogenase were quantitated by laser scan densitometry and computer integration, using various exposures to guarantee a linear range. For immunoblotting, human skin fibroblasts were grown to confluence, washed and incubated for 48 h in serum-free medium in the presence or absence of either TGF-β or TNF-α. At the end of each incubation, aliquots of media were processed for slot immunoblotting (6). Briefly, scalar dilutions of media were blotted into nitrocellulose strips using a Minifold II (Schleicher and Schuell); the strips were blocked with 0.05% Tween 20, 4% nonfat dry milk and incubated with the monoclonal antibody 7B5 directed against domain III of human perlecan (34). Following several washes, the membranes were incubated with horseradish peroxidase-labeled goat anti-mouse IgG, washed again, and incubated with the chemiluminescence substrate reagent (15). The membranes were exposed to radiographic films for 0.5–1 min and quantified by laser scanning densitometry.

**Preparation of Nuclear Extracts and DNA Footprinting—**Nuclear extracts from fibroblasts treated with or without TGF-β (10 ng/ml) for 18–24 h or from HeLa cells were prepared essentially as described before (34). Protein concentration was determined by a colorimetric method (35). At the end of each purification, aliquots were snap-frozen in liquid nitrogen and stored at −80 °C until analyzed. DNA footprinting analysis was performed to map the nuclear protein binding sites in the proximal promoter region. To this end, the −461 promoter CAT construct was labeled with [32P]dCTP on the coding strand at the unique RsrII site, located at −118. Briefly, the RsrII-linearized plasmid was isolated by agarose gel electrophoresis, labeled to high specific activity with Klenow enzyme (New England Biolabs Inc.), purified by column chromatography, and heated at 65 °C to inactivate the enzyme. The fragments were purified and subjected to HindIII digestion. The sizes were released from the 5-kb vector by agarose gel electrophoresis. The HindIII-5R-R II fragment was identified by exposing the gels to an x-ray film, electrophoresed for 10 min on DEAE paper slotted in the gel, and eluted in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 7.2) overnight. The labeled DNA fragments (2–4 × 105 cpm) were first incubated for 15 min on ice with 5–10 µg of nuclear extracts in a buffer containing 1 µg of poly(dI-dC), 4 µg MgCl2, 1 mM CaCl2, 2 mM dithiothreitol, 1 µg of bovine serum albumin, and 0.1 mM KCl. The solutions were then subjected to DNA I digestion (100 milliunits), terminated by 3 volumes of a solution containing EDTA (10 mM) and yeast tRNA, precipitated with ethanol, resuspended in a denaturing buffer, and analyzed on a denaturing 6% gel (36). Radioactive DNA molecular mass markers were run in parallel (29).

**Electrophoretic Mobility Shift Assays—**Double stranded oligonucleotides were radiolabeled with [32P]P using the T4 polynucleotide kinase, purified by acrylamide gel electrophoresis, and eluted overnight. A series of oligonucleotides (listed in Table I) were tested in competition assays including the TGF-β responsive element (TβRE), a 14-bp sequence located between −301 and −314, four mutated TβREs, a 14-bp TGF-β-responsive sequence of the rat e11 collagen promoter, and consensus oligodeoxynucleotides for the NF-1 and AP-2 transcription factors. The labeled probes were incubated for 15 min in a buffer containing 100 mM Heps, pH 7.9, 1 mM EDTA, 0.2 mM NaCl, 40 mM MgCl2, 20 mM dithiothreitol, 20 mM spermidine, 1–2 µg of poly(dI-dC), and 4% glycerol (29), with or without molar excess of oligonucleotide competitors. Nuclear extracts (10–20 µg) from control or TGF-β-treated fibroblasts or HeLa cells mixed to the DNA for an additional 15 min. The protein-DNA complexes were separated on 6% polyacrylamide gels (20:1; acrylamide to bisacrylamide) in 22 mM Tris borate, 22 mM boric acid, and 0.5 mM EDTA (37). The gels were fixed, dried under vacuum, and exposed to x-ray films. Protein-DNA complexes were removed from the dried gels, and the radioactivity was measured in a scintillation counter.
RESULTS AND DISCUSSION

Cloning and Sequence Analysis of the Human Perlecan 5'-Flanking Region—By screening a chromosome 1-specific phage library with a human cDNA probe encompassing the 5' region of the perlecan gene, we isolated about 4.5 kb of the sequence 5' to exon 1. A computer-assisted analysis, using the Signal and Eukprom programs from the GCG and PCGENE packages, revealed that the 5'-flanking region (Fig. 1) lacked canonical TATA or CAAT boxes but contained several features of a promoter including: 

1. A relatively high GC content, primarily evident in the proximal region, with 80% GpC, typical of CpG islands.
2. The presence of numerous cognate cis-acting elements.

![FIG. 1. Regulatory elements of the 5'-flanking region of the human perlecan HSPG2 gene. Putative regulatory motifs (boldface and underlined) are labeled above the coding strand. Palindromic inverted repeats are double-underlined and boldface. The numbers on the right refer to the nucleotide position relative to the major transcription start site (open diamond) at the 5' end of exon 1 (shaded sequence). The candidate cis-acting elements (57) include: ubiquitous zinc finger transcription factor (Sp1); a primary target of signal transduction induced by epidermal growth factor, phorbol ester and serum (PEA3); developmentally regulated dimer that binds to a palindromic sequence most abundant in neural crest cells (AP2); GT-IC binding motif of the SV40 promoter, related to AP3 (TEF-2); glucocorticoid receptor, member of the steroid hormone receptors superfamily (GR); ubiquitous factor, binds to cAMP-responsive element and forms homo- or heterodimers with members of the AP1 or Jun families (ATF/CREB); related to members of the Ets family and most abundant in B lymphocytes and macrophages (Pu.1); induces the regulation of the SV40 promoter, related to AP3 (TEF-2); induced by IL-6, cooperates with NF-IL6 (H-APF-1); ubiquitous factor, inducible by IL-6 and IL-1, member of C/EBP family (NF-IL6); a component of signaling network of B cells and resting T cells, binds to PEAS motif, acts in combination with AP1 (Ets-1); expressed in erythroid cells, megakaryocytes and mast cells, activates globin gene, plays autoregulatory roles in its own expression (GATA-1); ubiquitous, induced by TGF-β, interacts with DNA polymerase to enhance DNA replication (NF1); ubiquitous member of rel-related proteins, released after cytokine stimulation of an inactive complex formed with IxBa and IxBb (NF-κB).

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elements and palindromic inverted repeats. These features are typical of housekeeping and growth factor-encoding genes that characteristically lack TATA or CAAT boxes and contain multiple transcription initiation sites, as in the human HSPG2 gene (27). The majority of the cognate cis-acting elements were clustered in the first 1.5 kb as related to the major transcription initiation site (Fig. 1). The proximal region contained four GC boxes and 15 consensus hexanucleotide binding sites for the zinc finger transcription factor Sp1, 5 of which were located in the first exon. Another striking feature of the perlecan promoter was the presence of numerous AP-2 motifs (38), eight residing in the first 1.5 kb and two in the most distal area. In addition, numerous AP-2 binding sites were scattered throughout the noncoding strand (not shown). These potential binding sites for the AP-2 transcription factor possess the following two important features: (a) their binding and transcriptional activity are inhibited by SV40 T antigen, and (b) they confer phorbol ester 12-O-tetradecanoylphorbol-13-acetate and cAMP inducibility (39). Of note, SV40 large T antigen inhibits transcription of perlecan in renal tubular epithelial cells (40), and perlecan expression is markedly up-regulated by 12-O-tetradecanoylphorbol-13-acetate in colon cancer (41) or K562 leukemia (6) cells. The proximal promoter region also contained three palindromic inverted repeats in close proximity of each other (between –438 and –204) that, by forming secondary structure, could influence the regulation of perlecan gene expression. The perlecan promoter contained several motifs that bind transcription factors involved in hematopoiesis (42) including two PEA3 motifs, primary targets for signal transduction that bind to Ets members of oncogene, one Ets-1 and one PU.1 box that confers transcriptional activation to B lymphocytes (43), and nine GATA-1 motifs that are involved in erythrocyte differentiation (42). In addition, multiple GATA-1 sequences were present in the noncoding strand (not shown). The adjacent binding sites for GATA and Ets transcription factors at –1830 and –1802, respectively, are similar to those described in the human α2 integrin promoter (44) and suggest the possibility of either cooperative interaction or competitive inhibition. The distal promoter region contained a binding site for NF-kB, a factor that has been involved in interleukin-1 stimulation of transcription in a variety of genes (39). Two fully conserved CTF-NF1 elements at position –2524 and –1947 in a gene that lacks both TATA and CAAT box sequences are very close to those identified in the human α2 integrin (44) and in a variety of genes including the chicken β globin, mouse SPARC and a silencer site in the mouse α2(I) collagen gene (45). In these genes, the CTF-NF1 sequences serve as negative regulatory elements which suppress gene transcription in a cell type-specific fashion (45).

The 5'-Flanking Region of the Human Perlecan Gene Exhibits Promoter Activity in Cells with a Diverse Histogenetic Origin—To prove that the sequence upstream to exon 1 can exhibit transcriptional activity, we transfected a full-length promoter-CAT construct harboring a –2.5-kb sequence and 86 bp of exon 1, into various human cell lines of epithelial or mesenchymal origin and also into two murine cell lines (Fig. 2). When the values were normalized on β-galactosidase activity, an internal control for changes in transfection efficiency, functional promoter activity was detected in all the cells tested with maximal activity in the WiDr colon carcinoma cells (Fig. 2B). This is consistent with the fact that these tumor cells express high levels of this proteoglycan (20, 23). The activity of the perlecan promoter in such diverse cell types is in harmony with the widespread tissue distribution of perlecan (4). The activity of perlecan promoter in HL-60 promyelocytic leukemia cells (Fig. 2B) corroborates our previous study which has shown that perlecan is expressed in lymphoreticular cells (6) and further emphasizes the importance of the numerous motifs that could bind cognate transcription factors involved in hematopoiesis (see above). Relatively high perlecan promoter activity was also observed in murine cells, e.g. NIH-3T3 fibroblasts and M2 melanoma cells (Fig. 2B, filled bars), indicating that the human perlecan promoter is active in another species. Taken together, these findings validate the widespread distribution of perlecan demonstrated before by immunohistochemical (4, 7, 46) and in situ hybridization (4, 7) analyses and further suggest that the levels of perlecan gene expression may be regulated by tissue-specific factors.

Deletion Analysis of the Perlecan Gene Promoter—To investigate in more detail the functional properties of the perlecan gene promoter, we performed reporter gene analysis using various perlecan promoter-CAT fusion plasmids harboring either 5' stepwise or internal deletions (Fig. 3A). In these experiments we used HeLa cells because of their ease of transfection
and because we found that they express perlecan, a fact that was confirmed by the activity of the full-length promoter–CAT construct in transient cell transfection assays (Fig. 2). After normalization on β-galactosidase activity, several perlecan promoter constructs exhibited considerable CAT activity. The relative CAT activity of nine constructs based on data from several (n = 6–14) independent experiments is summarized in Fig. 3B. The largest construct, harboring the 2.5 kb of 5′-flanking region, contained maximal promoter activity set arbitrarily to 100%. Shortening the upstream sequence from −2500 to −1800 bp did not produce significant changes in CAT activity. However, three internal deletion constructs (−2500/−283, −2500/−560, and −2500/−1800) showed a marked reduction of CAT activity. Indeed, when these values were corrected for nonspecific baseline activity expressed by the two promoterless control plasmids (pBSCAT and pUC19), the internal deletions showed essentially complete loss of activity. Consistent with these data, the −461-bp construct maintained 80–90% promoter activity as compared with that detected in the two longest constructs, respectively. Additional reduction was observed with other constructs (Fig. 3B). Sequence analysis of this proximal region revealed, in addition to the multiple AP-2 sites and three palindromic inverted repeats, a regulatory element located at position −450 that consisted of a 5′-GT-GAGCGTG-3′ sequence with homology to the consensus sequence of the DE1 element 5′-TGACGGTG-3′ of the mouse α-crystallin gene (47). This cis-acting motif resembles the cAMP-responsive element sequence (ATF/CREB), 5′-GT-GAGCGTA/C(A/C)-3′, and behaves as a functional CREB site (47). Therefore, it is possible that this region in the perlecan promoter may be connected with a cAMP transduction. Present in the most proximal promoter region (−132-bp construct) were four GC boxes and several binding sites for Sp1, a well-characterized transcription factor that binds GC boxes and stimulates transcription in promoters that contain these sites (39), including the murine syndecan 1 (48) and thrombospondin 1 (49) genes. These results indicate that the 5′-flanking region of the perlecan gene can act as a functional promoter and contains a complex array of cis-acting elements necessary for driving the expression of the perlecan gene in both epithelial and mesenchymal cells.

Induction of Perlecan Gene Expression in Human Skin Fibroblasts by TGF-β but Not by TNF-α—In the next series of experiments, we wished to investigate the modulation of perlecan gene expression by TGF-β and TNF-α. We utilized normal human diploid skin fibroblasts because, in contrast to HeLa cells, these cells respond to various cytokines and their proteoglycan makeup has been well investigated (7, 29, 50). To this end, post-confluent skin fibroblasts were preincubated for 5 h with 1% fetal calf serum and then incubated for 24 h in the absence or presence of TGF-β (5 ng/ml) or TNF-α (5 ng/ml), either alone or in combination. Total RNA was then extracted and analyzed for perlecan or glyceraldehyde-3-phosphate dehydrogenase gene expression (Fig. 4). While TGF-β up-regulated the steady state levels of perlecan mRNA (Fig. 4, A and B, lane 2), TNF-α had no significant effects (Fig. 4, A and B, lane 3). Interestingly, TNF-α did not block the up-regulation of perlecan gene expression induced by TGF-β (Fig. 4, A and B, lane 4).

To verify that the increased perlecan mRNA levels correlated with an increased proteoglycan synthesis, we performed immunoblotting experiments using a monoclonal antibody directed against domain III of perlecan (4). A ∼2-fold increase in perlecan protein core biosynthesis in the presence of TGF-β, but no effect in the presence of TNF-α, was observed (Fig. 5). Because most of the synthesized perlecan (>90%) is released as soluble proteoglycan (3), these results indicate that the transcriptional effects of TGF-β are also seen at the protein level, albeit at a degree lower than that observed at the mRNA level. Our results are in agreement with the stimulation of perlecan biosynthesis and secretion by TGF-β observed in other cellular systems including colon carcinoma (24), uterine epithelial (25), and 3T3 fibroblastic (26) cells.

Identification of a TGF-β-responsive Region in the Perlecan Promoter—The results presented above suggested that the promoter of the perlecan gene harbored sequences that could respond to TGF-β. To characterize these putative TGF-β-respon-
sive element(s), we initially tested the largest construct of 2.5 kb for sensitivity to the cytokine. In this series of experiments, the cells were transfected with the promoter CAT-containing plasmid and subjected to subsequent culture in the presence or absence of the cytokine. The results (Fig. 6) showed that the entire promoter of human perlecan was transcriptionally activated by about 2.6-fold upon treatment with TGF-β. To delineate more accurately the TGF-β-responsive region, we made additional promoter-CAT constructs and subjected them to the same transient cell transfection assays in which the cells were cultured in the presence or absence of the cytokine. The results showed that the −461-bp construct was markedly stimulated by TGF-β at a level even higher than the largest (−2500-bp) construct, whereas all additional 5’ deletions, including −285-, −189-, −158-, and −132-bp constructs, lacked TGF-β responsiveness (Fig. 6). Taken together, these results indicate that the proximal 461 bp of the human perlecan promoter contains nearly all the regulatory elements to provide full functional promoter activity and further show that a region of about 177 bp, located between −461 and −285 bp, harbors the TGF-β responsive sequence(s).

DNase Footprinting and Electrophoresis Mobility Shift Assay of the Proximal Promoter Region—The clear geographic demarcation of TGF-β action on the perlecan promoter indicated that the major TGF-β activating element was located between −461 and −285 bp. DNase I footprinting was used to discover cis-acting elements in this relatively short sequence. The results showed at least five distinct footprints, designated a-e (Fig. 7). Interestingly, similar results were obtained with nuclear proteins isolated from either diploid fibroblasts (Fig. 7A, lane 3) or HeLa cells (Fig. 7A, lane 4). However, only footprinting a was located in the region which was responsive to TGF-β (Fig. 7B,
Footprinting was performed by incubating the purified labeled template with 100 milliunits of DNase I either in the absence (lane 1) or the presence of 10 μg of nuclear extracts from fibroblasts (lane 3) or HeLa cells (lane 4). Lane 2 is the probe alone whereas lane 5 contains radioactive DNA size markers. The footprints a-e are labeled by the vertical double arrows. The binding sites for NF-1 and AP-2 transcription factors are boldface and underlined. The region responsive to TGF-β is shaded.

**TABLE I**

| Gene                          | Position | Sequence                      | Reference   |
|-------------------------------|----------|-------------------------------|-------------|
| Human perlecan                | -314     | 5′-TGCCCGGGCCGGCCGGCC...-3′   | Present study|
| Rat α1 (I) collagen           | -1643    | 5′-TGCCCGAG...GCCAG-3′        | 37          |
| Mouse α2 (I) collagen         | -308     | 5′-TGGCTTT...GCCAG-3′         | 52          |
| Human elastin                 | -138     | 5′-TCCGAG...GCCCT-3′          | 53          |
| Human type I plasminogen activator inhibitor | -560 | 5′-TGCTGGCAT...GCCCT-3′       | 55          |
| Human growth hormone          | -285     | 5′-TGCCGGCC...GCCAG-3′        | 54          |
| Human α2 integrin             | -235     | 5′-TGCTAGGGCCGGCA...-3′       | 44          |
| NF-1 consensus                | 5′-TGGCTGAA...GCCAG-3′   | 58          |
| AP-2 consensus                | 5′-TGGCGGGCG...GCCCAT-3′ | 38          |
| TβRE consensus                | 5′-TGGGCCG.NN...GCC...-3′ |             |

**shaded sequence**. Footprints b and e harbored Sp1 binding sites, footprint d overlapped with an AP-2 consensus sequence, whereas footprint c was adjacent to an Sp1 binding motif.

A closer inspection of the 17-bp sequence footprinted by nuclear proteins from either fibroblasts or HeLa cells, e.g. footprint a in Fig. 7, showed that this sequence harbored a 14-bp motif that was very similar to a TGF-β-responsive element (TβRE) previously observed in the promoter of rat α1 (I) collagen (37, 51), mouse α2 (I) collagen (52), human elastin (53), human growth hormone (54), human α2 integrin (44), and human plasminogen activator inhibitor (55) genes (Table I). All of these sequences, with the exception of the human α2 integrin, have been shown to be responsive to TGF-β by transient cell transfection, DNase footprinting, or electrophoretic mobility shift assay analyses. Structurally, all of these TβREs are characterized by internal NF-1 and AP-2 motifs, which may mediate the action of TGF-β.

To examine the binding of the perlecan TβRE to nuclear protein, a double-stranded oligomer encompassing the perlecan TβRE was end-labeled and incubated with equal amounts of nuclear proteins extracted from either untreated or TGF-β-stimulated (5 ng/ml for 24 h) fibroblast nuclei. Electrophoretic mobility shift assay analysis showed that the TGF-β-stimulated nuclear extracts contained >3-fold more protein binding activity than unstimulated samples and that the binding was specific because it could be displaced by 10-fold molar excess of unlabelled TβRE competitor (Fig. 8A). The results further showed that a comparable response was also obtained with...
nuclear extracts from cells exposed to TGF-β for either 12 or 18 h (Fig. 8B).

An NF-1 Binding Site Mediates the TGF-β-induced Stimulation of Perlecan Transcriptional Activity—To investigate the specificity of perlecan TβRE, competition studies were performed. A 32P-labeled perlecan TβRE oligomer (−3 × 10^6 cpm/pmol of DNA) was incubated with or without TGF-β (5 ng/ml) for 10-fold excess, respectively. Incubation mixtures were separated on 6% polyacrylamide gels in low ionic-strength buffer (51). The migration of the DNA/protein complex is indicated by an asterisk on the left margin.

The results showed that perchloric acid (−3 × 10^6 cpm/pmol DNA) with binding buffer in the presence or absence of 10-fold molar excess of perlecan TβRE cold competitor. After a 15-min incubation, the mixture was incubated with 10 μg of nuclear proteins as indicated on the top. B, in these experiments, 20 μg of nuclear proteins from cells treated with TGF-β (10 ng/ml) for 12 or 18 h were tested for their ability to bind the 32P-labeled perlecan TβRE oligomer as indicated on the top. The concentrations of the TβRE cold competitor was 10-fold (lane 5) and 20-fold (lane 6) molar excess, respectively. Incubation mixtures were separated on 6% polyacrylamide gels in low ionic-strength buffer (51). The migration of the DNA/protein complex is indicated by an asterisk on the left margin. Electrophoretic conditions are the same as those detailed in the legend to Fig. 8.
with competing AP-2 oligonucleotide. Collectively, these stud-

ies support the conclusion that the perlecan TβRE binds to transcription proteins of the NF-1 family, and that the collagen α1(I) TβRE may contain additional transcription factors that differ from those binding to the perlecan TβRE.

Finally, to investigate more directly the sequence specificity of the perlecan TβRE, we generated double-stranded oligomers harboring various mutations in the 5′, 3′, or central region of the perlecan TβRE (Fig. 10B). Three-bp substitutions in either the 5′- or 3′-GCG region reduced almost completely the ability of the perlecan TβRE to be an effective competitor of the specific DNA-protein complex (Fig. 10A, lanes 5 and 6, respectively). In contrast, 3-bp or 4-bp substitutions in the central domain of the core binding oligomer did not appreciably influence the complex formation (Fig. 10A, lanes 3 and 4, respectively). These results are similar to those obtained with the murine α2(I) collagen promoter where an NF-1-like sequence is responsible for a 5–10-fold transcriptional induction by TGF-β (52).

NF-1 was initially identified as a cellular factor that stimulates in vitro replication of adenovirus DNA (56), but it was subsequently shown that this family of transcription factors plays a role in both DNA replication and RNA transcription (57). Indeed, the CAAT-binding/NF-1 family comprises at least six distinct nuclear proteins that are composed of heterologous subunits with binding affinity for imperfect palindromes of TGCC sequences separated by 6 or 7 bases, although they can also bind to a single TGCC sequence (57, 58). Flanking sequences, length and composition of the spacer regions can greatly influence their binding affinity (57). Taking into account the homology and the mutational analysis, thus, a consensus for TβRE (5′-TGCCC.N8...GCC-3′) could be generated (Table I).

Conclusions—We have cloned and sequenced 2.5-kb of 5′-flanking region of the human perlecan HSPG2 gene and determined for the first time functional promoter activity using transient cell transfection assays. The 5′-flanking region possesses strong basal and inducible promoter activity and is thus assumed to represent the promoter as it operates in vivo. The ubiquitous distribution of perlecan correlates well with functional promoter activity detected in cells with disparate histogenetic origin and also of different species. This study has also identified a TβRE in the proximal promoter region with affinity for members of the NF-1 family of transcription factors. Our data indicate that this sequence is primarily responsible for the transcriptional activation of the perlecan gene by TGF-β. The results show a complex array of regulatory factors for this proteoglycan and further indicate that the level of constitutive expression in both epithelial and mesenchymal cells is likely guided by the proximal promoter region. The structural and functional characterization of the perlecan gene promoter will now allow dissection of the complex regulation of this molecule during normal development, tissue repair, and tumor growth.

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