Regulatory Activities of the 5′- and 3′-Untranslated Regions and Promoter of the Human Aggrecan Gene*

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Expression of aggrecan by chondrocytes is subject to modulation by several factors, including cytokines, growth factors, and mechanical stimuli. Interleukin-1 and tumor necrosis factor-α both suppress aggrecan gene expression and synthesis, whereas insulin-like growth factor I and transforming growth factor-β stimulate aggrecan gene expression and synthesis. Mechanical loading of cartilage has been shown to modulate (increase and decrease) aggrecan gene expression and synthesis depending on the type and extent of the load imposed (1–7). Changes in aggrecan synthesis have also been identified as important events in cartilage pathology (8–10). Although aggrecan is expressed predominantly in cartilage, it is also known to be expressed in other tissues such as neural tissues (11–14) and neonatal lungs (15). In the embryonic central nervous system, aggrecan is involved in regulating neural crest migration (14, 16). Exposure of the lungs of neonatal rats to hyperoxia up-regulates aggrecan expression (15). Mutations that preclude the proper synthesis and deposition of aggrecan into the extracellular matrix are often lethal. A point mutation identified in exon 12 of the aggrecan core protein gene of the nanomelic chick introduces a premature translation termination codon that precludes synthesis of the third globular domain (G3) (17), and a 7-bp deletional mutation in exon 5 of the cartilage matrix-deficiency (cmd) mouse aggrecan gene leads to a premature translation termination codon in exon 6 (18). Despite these observations, the molecular mechanisms governing the regulation of aggrecan gene expression and synthesis remain obscure.

The core protein of aggrecan consists of an amino-terminal globular domain (G1) that binds hyaluronan, an interglobular domain, a second globular domain (G2) whose function is yet unknown, an extended glycosaminoglycan attachment region, and G3, which is located at the carboxyl-terminal end of the molecule (19–21). Comparison of the aggrecan core protein structure and deduced amino acid sequences from various species (22–25) with the recently determined exon and intron organizations of the chick (26), human (27), mouse (25), and rat (28) aggrecan have revealed a highly modular structure of the gene. Various domains of the protein are coded by groups of exons or single exons, most of which must be expressed as modules of two or more exons because of restrictive exon splicing requirements (27). Alternative splicing of the epidermal growth factor-like (23, 29, 30) and complement-regulatory protein-like motifs (23) of the G3 domain in some species have, however, been reported.

The coding region of the aggrecan gene is flanked by exons 1 and 19 (27), from which the 5′- and 3′-untranslated regions (UTRs) are transcribed (23, 27). The UTRs of other genes, for example ferritin, have been shown to function as regulatory elements of the genes (31–35). We hypothesize that the UTRs of the aggrecan gene are involved similarly in regulating expression of the gene, potentially in concert with the promoter. Thus, in this study we have cloned and investigated the regulatory activities of the promoter and the 5′- and 3′-UTRs of the human aggrecan gene.

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2 The abbreviations used are: G1, G2, G3, globular domains 1, 2, and 3, respectively; bp, base pair(s); UTR, untranslated region; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; CMV, cytomegalovirus; LUC, luciferase; β-GAL, β-galactosidase; kb, kilobase(s); PCR, polymerase chain reaction; TES, N,N,N,N-tetrakis(hydroxymethyl)methyl-2-aminoethanesulfonic acid; BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; SSRE, shear stress response element; NF-xB, nuclear factor-xB.
cDNA sequence (23). The amplified product was digested with HinI primers 5′ gene was amplified from the genomic cosmid cosHA-G3 (27), using the human aggrecan 5′ stream of the luciferase reporter gene in pLUCneo to generate the and BamHI designed from nucleotides for investigation of the activities of the human aggrecan Constructs— For investigation of the activities of the human aggrecan 5′- and 3′-UTRs independent of the human aggrecan promoter, luciferase constructs containing the 5′- and 3′-UTRs of the human aggrecan mRNA (Fig. 1A) were prepared in the cytomegalovirus (CMV) promoter-driven pcDNA plasmid pLUCneo. The previously cloned 5′- and 3′-UTRs upstream of the luciferase reporter gene in pLUCneo to generate the human aggrecan 5′-UTR construct pCMV5UTR. Exon 19 (containing the 3′-UTR and two polyadenylation signals) of the human aggrecan gene was amplified from the genomic cosmid cosHA-G3 (27), using the primers 5′-TTT CGC TGC AGC CAC CAC CTA CAA ACC ACG CAG A-3′ and 5′-TGC ACT GGG CCG TCG AAA GGC AGC ATG G-3′, and cloned into the XhoI sites of pcDNA3 to generate the control luciferase reporter plasmid pLUCneo. The previously cloned 5′-UTR of the human aggrecan mRNA (27) was amplified using the primers 5′-ATC ACT AAG CTT GGC CCG ACC ACC TAC CTC-3′ and 5′-TCA CGA GGA TCC AGA GTA AAG TGG TCA TAG TTC AC-3′. The underlined sequences indicate restriction sites for HindIII and BamHI, respectively. To preserve the relative sequence (38) of the human aggrecan mRNA, TRP-2 was designed from nucleotides +78 to +54 of the published human aggrecan cDNA sequence (23). The amplified product was digested with HindIII and BamHI and cloned in the HindIII/BamHI site immediately upstream of the luciferase reporter gene in pLUCneo to generate the human aggrecan 5′-UTR construct pCMV5UTR. Exon 19 (containing the 3′-UTR and two polyadenylation signals) of the human aggrecan gene was amplified from the genomic cosmid cosHA-G3 (27), using the primers 5′-TTT CGC TGC AGC CAC CAC CTA CAA ACC ACG CAG A-3′ and 5′-TGC ACT GGG CCG TCG AAA GGC AGC ATG G-3′, and cloned into the XhoI site of pcDNA3 immediately downstream of the reporter gene in pLUCneo to generate the 3′-UTR construct pCMV3UTR. The construct pCMV5/3UTR was generated by cloning, respectively, the 5′- and 3′-UTRs in the HindIII/BamHI and XhoI sites of pLUCneo. The suffix -US denotes the upstream position of the UTRs relative to the CMV promoter. All inserts were cloned, by design, in the same orientation. The β-galactosidase (β-GAL) gene cassette of the pSV-β-galactosidase expression vector was subcloned into the HindIII/XhoI site of pcDNA3 for use as transfection efficiency control.

Preparation of the Human Aggrecan Promoter Constructs—For investigation of the activities of the human aggrecan 5′- and 3′-UTRs independent of the human aggrecan promoter, luciferase constructs containing the 5′- and 3′-UTRs of the human aggrecan mRNA (Fig. 1A) were prepared in the cytomegalovirus (CMV) promoter-driven pcDNA plasmid. The luciferase gene cassette of the pCMV construction contained the HindIII/BamHI and XhoI sites of pcDNA3 to generate the control luciferase reporter plasmid pLUCneo. The previously cloned 5′-UTR of the human aggrecan mRNA (27) was amplified using the primers 5′-ATC ACT AAG CTT GGC CCG ACC ACC TAC CTC-3′ and 5′-TCA CGA GGA TCC AGA GTA AAG TGG TCA TAG TTC AC-3′, and cloned into the XhoI site of pcDNA3 immediately downstream of the reporter gene in pLUCneo to generate the 3′-UTR construct pCMV3UTR. The construct pCMV5/3UTR was generated by cloning, respectively, the 5′- and 3′-UTRs in the HindIII/BamHI and XhoI sites of pLUCneo. The suffix -US denotes the upstream position of the UTRs relative to the CMV promoter. All inserts were cloned, by design, in the same orientation. The β-galactosidase (β-GAL) gene cassette of the pSV-β-galactosidase expression vector was subcloned into the HindIII/XhoI site of pcDNA3 for use as transfection efficiency control.

Preparation of the Human Aggrecan Promoter Constructs—A 1.05-kb fragment (containing 0.7 kb of the proximal promoter and 0.35 kb of exon 1) of the upstream region of the human aggrecan gene was amplified by PCR using the human genomic PromoterFinder kit and the Elongase Enzyme Mix according to the supplier’s instructions. The PCR product was cloned in the pcRII TA cloning vector and sequenced in both directions. The promoter/exon 1 fragment was then digested with MluI (located in the PromoterFinder adaptor) and BsrBI (located at +25 of the human aggrecan exon 1 or 5′-UTR). The digested human aggrecan promoter (~701 to +25) was ligated to the 5′-UTR at the BsrBI site and subcloned, in place of the CMV promoter, in pCMV5/3UTR to generate the chimeric luciferase constructs pAGC1(−701)/5UTR and pAGC1(−701)/5UTR (Fig. 1B). Similarly, constructs pAGC1(−701) and pAGC1(−701)/3UTR were prepared by cloning the aggrecan promoter fragment into the MluI/BamHI site immediately upstream of the luciferase gene in pLUCneo and pCMV3UTR. The luciferase gene symbol AGC1 here denotes the human aggrecan promoter.
Cis-acting Regulators of the Human Aggrecan Gene

Measurement of Luciferase mRNA Half-life—To assess the effects of the UTRs on stability of the luciferase mRNA, primary bovine articular chondrocytes were transfected with pLUCneo and the pCMV/5'UTR and pCMV/3'UTR constructs. 45 h later (t = 0), the cells were treated with 65 μM DRB, an RNA synthesis inhibitor specific for RNA polymerase II (37, 38). Total RNA was subsequently isolated from the cells at 30-min intervals, starting at t = 0, and the luciferase mRNA levels were quantified by PCR (39).

RESULTS

Effects of the Human Aggrecan 5'- and 3'-UTRs on Activities of the CMV Promoter—The human aggrecan mRNA contains a G/C-rich 382-bp leader sequence and a relatively short 3'-UTR. The current sequence of exon 1 differs from that obtained previously by cDNA cloning (27) at two nucleotide positions. At position +1, the current sequence reads as a C (Fig. 4), whereas it was determined as a G previously. Also, at position +7 the genomic sequence is a C compared with an A at the same position in the cDNA sequence. To verify the accuracy of these sequences, two separate clones containing the promoter and exon 1 were sequenced. The genomic sequence at position +7 is consistent with those reported for the rat and mouse aggrecan gene (25, 28).

The sequence was analyzed for promoter motifs and transcribed by transiently transfecting chondrocytes with 10 μg of pLUCneo, pCMV/5'UTR, or pCMV/3'UTR per 60-mm dish for 45 h and then treated with the RNA synthesis inhibitor DRB at a concentration of 65 μM. At the indicated times after initiation of DRB treatment, total RNA was isolated from the transfected cells and analyzed for luciferase mRNA levels using quantitative PCR (39). Each set of data was fitted by linear regression, and the half-lives of the respective luciferase transcripts were calculated from the slopes and intercepts of the regression lines.
scriptional factor binding sites using the TSSG\(^2\) and MatInspector (41) programs at the Baylor College of Medicine Search Launcher site. The promoter region is highly G/C-rich and lacks canonical TATA and CAAT motifs (Fig. 4). However, a search of the promoter database predicted a TATA box at \(-31\), which coincides with the beginning of the TATGTATG sequence (reverse font). This sequence is conserved in the rat and mouse aggrecan promoters (25, 28) but not in the chick aggrecan promoter (42). The transcriptional start site of the human aggrecan gene was predicted by TSSG to be at \(-6\), 5 bp downstream of the transcriptional start site (Fig. 4) determined previously by PCR-based cDNA cloning (27). Several putative binding sites for ubiquitous transcription factors such as SP-1, AP-2, and AP-4 are located in both the promoter and exon 1. Recognition sequences for growth factor- and cytokine-inducible factors are also present. The promoter lacks a TATA box, but a TATGTATG sequence at \(-31\) (reverse font) might function as a TATA element.

Effects of the Human Aggrecan 5\(^{\prime}\)- and 3\(^{\prime}\)-UTRs on Activities of the Human Aggrecan Promoter—To investigate the roles of the human aggrecan promoter and the UTRs in expression of the aggrecan gene, chimeric luciferase constructs containing the human aggrecan promoter 5\(^{\prime}\)-UTR (exon 1) and/or 3\(^{\prime}\)-UTR (exon 19) (Fig. 1B) were prepared and used in transient transfection assays. The level of expression induced by the promoter was 89-fold higher than that of the promoter-less luciferase vector (data not shown), indicating that this was a functional promoter. In the following experiments the activity of pAGC1(\(-270\)) containing the proximal promoter region (\(-270\) to \(-125\)) of the human aggrecan gene, served as baseline activity. The presence of the 5\(^{\prime}\)-UTR immediately downstream of the aggrecan promoter, construct pAGC1(\(-270\))/5UTR, significantly stimulated the expression of luciferase activity 7.7-fold relative to the activity of pAGC1(\(-270\)) (Fig. 5A). The expression of luciferase activity was suppressed by 68% (\(p, 0.001, n = 6\)) when cells were transfected with pAGC1(\(-270\))/3UTR, the construct containing the human aggrecan exon 19 (3\(^{\prime}\)-UTR) immediately downstream of the luciferase reporter gene. The activity of pAGC1(\(-701\)/3UTR, with the UTRs flanking the luciferase gene, was 45% lower than that of pAGC1(\(-270\)) and 93% lower than the activity of pAGC1(\(-270\))/5UTR (Fig. 5A).

In NIH 3T3 fibroblasts transfected with the aggrecan promoter/UTR constructs, a pattern of luciferase expression similar to that in chondrocytes was observed (Fig. 5B). Relative to the activity of the pAGC1(\(-701\)) construct, expression of luciferase activity in fibroblasts transfected with pAGC1(\(-701\))/5UTR

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was 39.6-fold higher \((p < 0.001, n = 12)\). However, there was no significant difference between the activities of pAGC1\((-701)/3\)UTR and pAGC1\((-701)/5\_3\)UTR compared with that of pAGC1\((-701)\).

To determine regions of the promoter which are essential for promoter activity, unidirectional deletion of the aggrecan promoter in pAGC1\((-701)/5\)UTR was performed using endogenous restriction enzyme sites. Deletion of segment \(-701 \text{ to } -452\) moderately stimulated luciferase activity by 47\% \((p < 0.001)\) compared with the activity of pAGC1\((-701)/5\)UTR (Fig. 6A). Further deletion of the promoter down to \(-179\) resulted in luciferase activity that was approximately the same as that of pAGC1\((-701)/5\)UTR. Removal of all but 52 bp of the promoter reduced luciferase expression by only 20\% \((p < 0.001)\). These results indicated that most of the human aggrecan proximal 701 bp promoter activity resides downstream of nucleotide \(-53\). Deletion of the 5’-UTR (exon 1) from the construct containing the 52-bp promoter fragment completely inhibited expression of luciferase activity (Fig. 6B), demonstrating the requirement of sequences within exon 1 (i.e. downstream of \(+25\)) for promoter activity.

**DISCUSSION**

The structural gene for aggrecan is large and complex, giving rise to an mRNA approximately 8 kb in size for the human gene. With elucidation of the gene structure for aggrecan in several different species (25–28), isolation and characterization of regulatory regions of the gene can now be performed. We have cloned the human aggrecan promoter and performed functional studies to determine regulatory activities of the promoter and the 5'- and 3'-UTRs of the gene. The human aggrecan promoter contains several putative SP-1/AP-2 binding sites (-CCCGCC-), including a cluster of SP-1/AP-2 binding sites around the transcriptional start site (Fig. 4). SP-1 plays a key role in regulating transcription initiation of TATA-less promoters (44, 45). The transcriptional start site of the human aggrecan promoter is spanned by three overlapping SP-1/AP-2 binding sites. In the rat gene, there is only one copy of the SP-1/AP-2 site in a position equivalent to that of the overlapping SP-1/AP-2 sites. Nonetheless, in both genes, transcription is initiated 23–24 bp downstream of a conserved TATG direct repeat (Fig. 4, reverse font), suggesting that this sequence is important in transcriptional initiation of the aggrecan gene. Search of the eukaryotic promoter data base using the human aggrecan promoter sequence predicted a TATA box at \(-31\), the position of the TATG repeat. Thus, this sequence may be the equivalent of a TATA box in TATA-less promoters such as the aggrecan promoter. Transcription initiation of the bovine aggrecan gene, however, occurs at four different sites upstream of this TATG repeat (25), suggesting other mechanisms of transcription initiation.

**FIG. 5.** Modulation of the activities of the human aggrecan promoter by the 5’- and 3’-UTRs. Chimeric luciferase constructs containing the proximal 701-bp human aggrecan promoter and the 5’- and/or 3’-UTR were transfected into chondrocytes (panel A, \(n = 6\)) or NIH 3T3 cells (panel B, \(n = 12\)) for 48 h and assayed for expression of luciferase activities. The result of each group is presented as mean ± S.D.

**FIG. 6.** Deletional analysis of the human aggrecan promoter. Panel A, the 701-bp proximal human aggrecan promoter in the construct pAGC1\((-701)/5\)UTR was unidirectionally deleted (5’ to 3’) using endogenous restriction enzymes and blunt end ligated to generate constructs with promoter fragments ending at the indicated positions. Monolayer bovine articular chondrocytes were then transiently transfected with the deletion constructs and assayed for luciferase activities. Panel B, the 5’-UTR (exon 1) was deleted from the construct containing 52 bp of the proximal promoter (panel A), and its activity was compared with the full-length (701-bp) promoter with or without the 5’-UTR. Note the marked suppression of luciferase activity when the 5’-UTR is deleted from the full-length or 52-bp promoter constructs.
Expression of aggrecan can be modulated by mechanical forces, cytokines, and other serum factors. The two NF-κB sites in the promoter may confer responsiveness to cytokines. This is suggested by the overlapping NF-κB and STAT binding sites at −375 to −362 (Fig. 4) and the fact that NF-κB is often activated by cytokines such as tumor necrosis factor-α (46). The four SSREs (one in the promoter and three in exon 1) are potential sites for regulating aggrecan gene expression by shear stresses. The suppressive effects of interleukin-1 on aggrecan gene expression can be blocked or reversed by treatment with platelet-derived growth factor (47). This stimulatory effect of platelet-derived growth factor on aggrecan expression may be mediated through the SIF response elements in the promoter (Fig. 4).

The 5′- and 3′-UTRs of the human aggrecan gene strongly modulated the activities of the human aggrecan promoter (Fig. 5) as well as those of the CMV promoter (Fig. 2). In chondrocytes and NIH 3T3 cells, the 5′-UTR stimulated the activities of the human aggrecan promoter greater than 7-fold (Fig. 5). In contrast, the 3′-UTR inhibited the activities of the human aggrecan promoter in both chondrocytes and NIH 3T3 cells. The stimulatory activity of the 5′-UTR was inhibited in the presence of the 3′-UTR. The mechanism by which the 3′-UTR suppresses aggrecan promoter activity is unclear, but it might be mediated through negative transcriptional elements including the Gfi-1 response elements found in both the promoter (Fig. 4) and the 3′-UTR (not shown). With respect to the CMV promoter, the 5′-UTR suppressed the expression of luciferase activity in chondrocytes or NIH 3T3 cells, but the 3′-UTR modulated the activity of CMV promoter in a cell type-specific manner. The 3′-UTR was inhibitory in chondrocytes (Fig. 2A) and stimulatory in NIH 3T3 cells (Fig. 2B). This cell type-specific effect of the 3′-UTR may be caused by the presence or absence of specific transcription factors in the transfected cells which bind the 3′-UTR and regulate transcription.

The effects of the 5′- and 3′-UTRs were mediated at the transcriptional level since 1) placement of the UTRs upstream of the CMV promoter did not alter their effects on expression of luciferase activities significantly (Fig. 2), and 2) their presence or absence from the luciferase transcript did not affect luciferase mRNA stability. The involvement of the 3′-UTR in modulating gene expression at the level of transcription is an unusual finding that has been demonstrated only for a few other genes. Typically, the 3′-UTR has been reported to control gene expression by modulating stability and translation of mRNAs (31–33). However, a role for the 3′-UTR in suppressing both basal and growth hormone-induced transcription has been reported in the rat serine protease inhibitor gene (35). Other examples of the transcriptional involvement of the 3′-UTR in regulating gene expression include the interaction between the tumor necrosis factor promoter and 3′-UTR in mediating the response of the gene to bacterial endotoxin (48) and the identification of an estrogen response element in the c-fos 3′-UTR which is capable of conferring estrogen responsiveness to a heterologous promoter such as the thymidine kinase promoter (34).

In the absence of inhibitory modulators, high level expression of the human aggrecan gene is critically dependent on sequences present in exon 1 (5′-UTR) (Fig. 6). This is indicated by the fact that sequential deletions of the promoter from −701 to −52 have little effect on promoter activity when the 5′-UTR is present. However, when the 5′-UTR is removed, leaving a 5′-flanking sequence of −701 to +25 or −52 to +25 (Fig. 6B), luciferase activity is reduced 85–100%. It is possible that sequences within this region bind to general transcription factors required during the assembly of the transcription initiation complex. This region contains several putative SP-1 binding sites, characteristic of TATA- and CAAT-less promoters, and may be involved in recruiting the TFIID protein to the transcription initiation complex.

Aggrecan is a member of a family of hyaluronan-binding proteoglycans that have been designated as hyalectans (49) or lecitans (50). Other members of this group include versican, neurocan, and brevican. The hyalectans share striking similarities, both at the protein and genomic levels (49). They differ primarily in the glycosaminoglycan attachment regions and also in the presence of the G2 domain in aggrecan. Promoter sequences for aggrecan (Fig. 3) (25, 28, 42), versican (51), neurocan (52), and brevican (53) have now been described. The versican (51) and neurocan (52) promoters both contain TATA boxes, whereas the aggrecan (Fig. 3) and brevican (53) promoters do not. Like the aggrecan promoter, the versican promoter contains multiple AP-2 sites (51). However, the 150-bp promoter region immediately upstream of the transcriptional start site of the versican gene lacks AP-2 or SP-1 sites, in contrast to that of the aggrecan promoter region (Fig. 3). This, along with the presence of a TATA box in the versican gene, suggests different mechanisms for transcriptional initiation of the aggrecan and versican genes.

Perlecan, a modular heparan-sulfate proteoglycan that is expressed predominantly in vascular tissues (49, 54), is also expressed in developing and adult cartilage (55, 56). The perlecan promoter, like the aggrecan promoter (Fig. 3), lacks canonical TATA and CAAT boxes and contains multiple SP-1/ AP-2 binding sites in the promoter region and exon 1 (57). Most of the perlecan SP-1/AP-2 binding sites are in the reverse orientation compared with those of the aggrecan promoter. Notwithstanding, the aggrecan (Fig. 6A) and perlecan (57) proximal promoters exhibit very similar activities. Unidirectional deletion of the promoters to −52 (for aggrecan) and −132 (for perlecan) retained 80% and 40–50% of the wild-type promoter activities, respectively. This is suggestive of the sufficiency of the clusters of SP-1 and AP-2 sites near the transcriptional start sites, in conjunction with sequences in exon 1, in mediating basal activities of the aggrecan and perlecan promoters. The similarities in the organizations and activities of the aggrecan and perlecan promoters may provide clues to the mechanisms governing expression of the aggrecan and perlecan genes, especially in relation to their expression in cartilage (55, 56).

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