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Identification of an osteogenic protein-1 responsive element in the aggrecan promoter

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Abstract

Previous studies have demonstrated that osteogenic protein-1 (OP-1), a member of the bone morphogenetic protein (BMP) subfamily of the TGF-β superfamily, stimulates aggrecan synthesis. To investigate transcriptional regulation of the aggrecan gene by OP-1, we constructed a clone containing a 1 kb region of the 5′-upstream sequence of the mouse aggrecan gene fused to the promoter-less luciferase reporter gene in pGL2-Basic vector. A series of promoter deletion constructs were also generated. Studies of the promoter activity of these DNA constructs in transient-transfected cells revealed that deletion of a 33 bp region rendered the promoter unresponsive to OP-1, BMP-6, and CDMP-1 without affecting BMP-2 and TGF-β1 responsiveness. Thus, the expression of the mouse aggrecan gene in response to BMPs appears to be the result of a unique combination of different *cis*-acting elements.

Keywords: Aggrecan promoter; Bone morphogenetic protein; Osteogenic protein-1; BMP-7; Transcription regulation

Aggrecan is a large chondroitin sulfate proteoglycan and a major cartilage extracellular matrix component. Aggrecan participates in matrix organization and in regulating growth factor activity. Increased aggrecan synthesis usually is associated with matrix assembly and, in turn, cartilage formation [1,2].

Osteogenic protein-1 (OP-1) is a member of the bone morphogenetic protein (BMP) family that belongs to the transforming growth factor-β superfamily [3–7]. Several BMPs have been shown to induce formation of new bone and cartilage [8–11]. Previous in vivo and in vitro studies showed that OP-1 stimulates aggrecan expression in several different cell types, such as goat cartilage [12], calf periosteum-derived cells, bovine articular cartilage slices, isolated chondrocytes grown in alginate beads, articular chondrocytes from fetal, adolescent, and adult humans, as well as mouse MC615 chondrocytes [13–18]. Bovine chondrocytes transfected with an adenovirus vector containing the human BMP-7 gene also showed an increased expression of aggrecan [14]. In the present study, we identified a sequence within the mouse aggrecan promoter that is involved in its up-regulation by OP-1. An inhibitory element of the basal transcription activity was also detected in the aggrecan promoter.

Materials and methods

Cell culture. C2C12 cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM containing 10% FBS and penicillin/streptomycin in a humidified 5% CO_2 atmosphere at 37 °C as described [19].

Construction of promoter–reporter clones. A parent plasmid containing a 1.02-kb of the mouse aggrecan promoter composed of nucleotides ranging from −523 upstream to +499 downstream from the transcription start site was fused to the promoter-less luciferase reporter gene in the pGL2-Basic vector. The transcription start site is +1. The 1.02-kb promoter sequence was generated by PCR using the mouse genomic DNA as the template, the forward primer 5′GAG CTC TTG AGA TGT CCT TGG TCT ATG3′ and the reverse primer 5′AAC TCG AGT TGG ACT CGC TCC TCC AA3′. Five aggrecan

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promoter fragments were subsequently generated by unique restriction enzyme digestion of the parent 1.02-kb clone and subcloned into pGL2-Basic vector preceding the reporter luciferase gene. The six clones contained the following promoter sequence: −523/+499, −404/+499, −48/+499, −523(Δ−303/−48)/+499, −523(Δ−303/−48)/+221, and −48/+221. Three additional clones (−365/+221, −335/+221, and −300/+221) were generated by PCR, using the forward primers 5′CTA TGA AGA GCT CTT TCA AA3′, 5′GAG TAG GGA GCT CGT CCT GT3′, and 5′TGG GTA TGA GCT CCA AAT CG3′, respectively; with a common reverse primer 5′GAT GCT CGA GCC GGC GAG GA3′. All plasmids were checked for purity on agarose gels and their identities were confirmed by restriction enzyme mapping and double-stranded DNA sequencing.

Transient transfection. The promoter activities of these constructs were measured in transiently transfected, multipotent C2C12 cells using FuGene6 following the manufacturer's direction. The transfected C2C12 cells were treated with two concentrations of OP-1 (50 and 200 ng/ml) for 24 h. The promoter activity was measured by the luciferase activity as previously described [20]. The activity was normalized to the β-galactosidase activity and presented as the relative activity to the vehicle control (as 1).

Results

Construction of different aggrecan promoter constructs

To identify the OP-1 responsive elements in the aggrecan promoter, a series of mouse aggrecan promoters were constructed (Fig. 1A). These DNA constructs were used in transient transfection experiments to define regions of the promoter essential for OP-1 response.

An OP-1 responsive element is located within a 101 bp region

Fig. 2A shows that the promoter activity for the pGL-2-Basic vector was very low and was not affected by OP-1. Both clones containing the −523/+499 and the −404/+499 sequence responded to OP-1 with a 2-fold stimulation beyond the control. The promoter activity of the clone containing the −48/+499 sequence was reduced dramatically but was still about 3-fold higher than the parent pGL-2-basic vector. This promoter sequence was not OP-1 responsive. These observations suggest that an OP-1 responsive element lies within the region containing −404 and −48. However, the clone containing the −523(Δ−303/−48)/+499 sequence was responsive to OP-1, though to a lesser degree (∼50%) than the −523/+499 clone. These promoter constructs retained significant levels of basal promoter activity compared to the pGL2-Basic. Considered together, these observations suggest that an OP-1 positive response element lies between −404 and −303.

A general transcription inhibitory element is located within a 278 bp region

Transfection experiments with 3′ deletion constructs revealed the presence of an inhibitory element(s). For example, clones −48/+221 and −523(Δ−303/−48)/+221 displayed a 2-fold higher basal activity and were

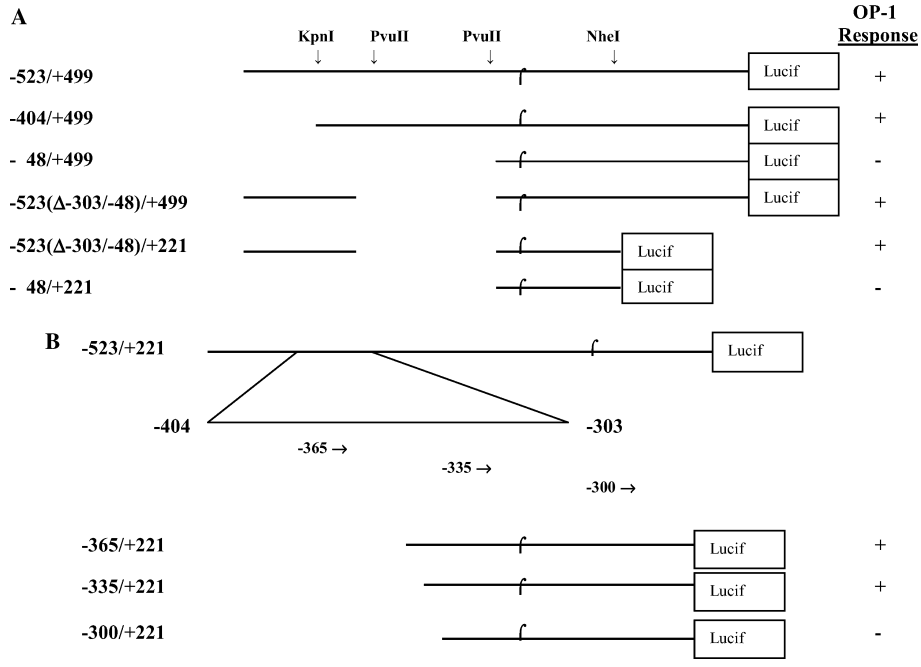

Fig. 1. Schematics of DNA constructs containing different mouse aggrecan promoter regions fused to the promoter-less luciferase (Lucif) reporter gene. The transcription start site is +1 and is indicated as (ʃ). (A) The 1.02-kb DNA fragment was subcloned into the pGL2-Basic vector containing the promoter-less luciferase reporter gene (Luc). The unique restriction sites used to produce the deletion clones shown are indicated by arrows. (B) Subclones generated by PCR. Arrows indicate positions of the 5′-end PCR primers used to generate the indicated subclones. A summary of the promoter activity in response to OP-1 is shown to the right of the diagram.

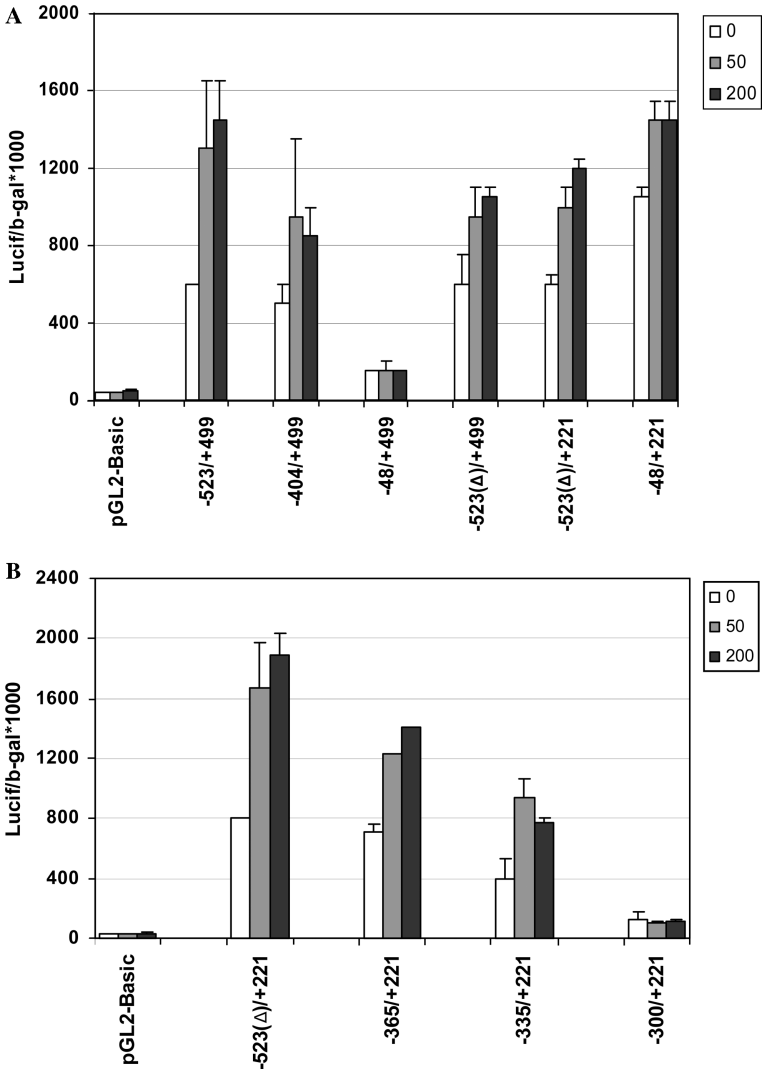

Fig. 2. Effects of OP-1 on the aggrecan promoter activity in transiently transfected cells. Cells were transfected with the DNA constructs containing different deletions of the aggrecan promoter shown in Fig. 1. Cultures were treated with solvent (open bars), OP-1 at 50 ng/ml (gray bars), or OP-1 at 200 ng/ml (black bars) for 24 h followed by measurement of the reporter gene activity. Values were corrected for the β-galactosidase activity. Values represent means ± SEM of 5–8 independent determinations. The −523(*Δ*)/+499 and −523(*Δ*)/+221 represent −523(*Δ*−303/−48)/+499 and −523(*Δ*−303/−48)/+221 clones, respectively.

more responsive to OP-1 than clones −48/+499 and −523(*Δ*−303/−48)/+499, respectively. The observations suggest that an inhibitory element is located within the region containing +221 and +499.

An OP-1 responsive element is located between −335 and −303 bp

Subsequent studies used clones with a combination of the 3′ deletion and 5′ as well as internal deletions to further define the OP-1 responsive elements in the mouse aggrecan promoter. OP-1 stimulated, in a dose-dependent manner, the −523(*Δ*−303/−48)/+221 aggrecan promoter activity by about 2-fold above the control (Fig. 2B). The aggrecan promoter, consisting of −365/+221, remained responsive to OP-1 stimulation. The promoter consisting of −335/+221 remained responsive, though at a lower level. However, the promoter consisting of −300/+221 was not responsive to OP-1 stimulation, although the promoter activity was still higher than the basal activity. These findings strongly suggest that the element(s) responsible for the up-regulation of the aggrecan promoter by OP-1 is located in the 33-bp region between −335 and −303.

The OP-1 responsive element is also required for BMP-6 and CDMP-1 responsiveness

To determine whether the 33-bp region of the mouse aggrecan promoter is responsive to other members of the BMP family, the activity of different promoter constructs was measured in cells treated with BMP-2, BMP-6,

CDMP-1, and TGF-β1. Fig. 3 shows that the background promoter activity of the pGL-2-Basic vector was extremely low and was not stimulated by any of the proteins tested. Clones containing the sequences −523(Δ− 303/−48)/+221, −365/+221, and −335/+221 responded to OP-1, BMP-6, BMP-2, CDMP-1, and TGF-β1, though to varying extent (from 2- to 4-fold, compared to its own vehicle control). The clone containing the sequence −300/+221 was no longer responsive to OP-1, BMP-6, and CDMP-1. By contrast, the promoter construct containing the sequence −300/+221 remained responsive to BMP-2 and TGF-β1, though to a lesser degree.

Discussion

Previously, we showed that OP-1 stimulated aggrecan transcription in cartilage cells [18]. The current study reveals the presence of a *cis* regulatory element located in a 33 bp region in the proximal aggrecan promoter region. Deletion of the region abolished the up-regulation of aggrecan mRNA expression by OP-1. The base sequence of this 33 bp region is GTCCTGTGCGATGG CATCTTTCTGGGTATCAG C and appears to be unique, containing no recognition sites for any known transcription/regulatory factors.

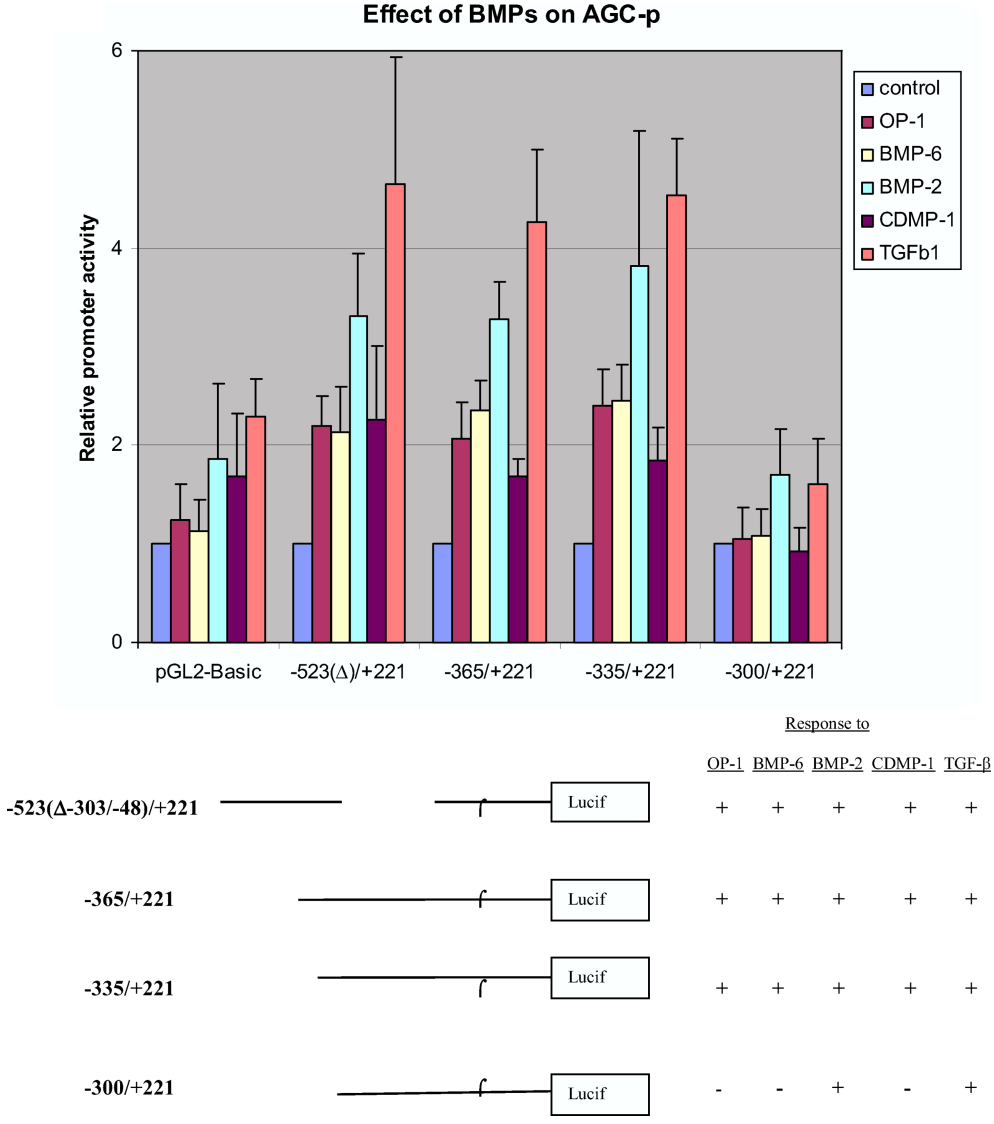

Fig. 3. Effects of other BMPs and TGF-β1 on the aggrecan promoter activity in transiently transfected cells. Cells were transfected with DNA constructs containing different deletions of the mouse aggrecan promoter shown in Fig. 1. Cultures were treated with solvent, 200 ng/ml OP-1, BMP-2, BMP-6, and CDMP-1, or 2 ng/ml TGF-β1 for 24 h followed by determination of the reporter gene activity. Values were corrected for total protein level and normalized to solvent control as 1. Values represent means ± SEM of three independent determinations. The raw values for pGL-2-Basic, −523(Δ−303/−48)/+221, −365/+221, −335/+221, and −300/+221 clones were 41, 575, 1413, 595, and 211, respectively. A summary of the responsiveness to OP-1, BMP-2, BMP-6, CDMP-1, and TGF-β is shown at the bottom of the graph and on the right of the schematics of the clones.

The sequence TTAAAAATAAAAAGGGTGAATC ATCATTCCATC in the mouse collagen X promoter was reported to be involved in OP-1 stimulation of its activity [21]. The sequence contains a myocyte-specific enhancer binding factor (MEF)-2-like sequence (TTAAAAATAAAAA) and an AP-1-like sequence (TGAATCATCA). Both sequences are necessary for stimulation by OP-1. In a separate study, a 317-bp region in the chicken collagen X promoter was reported [22]. The region appears to be more responsive to BMP-4 than BMP-2 or -7. The sequence contains numerous putative regulatory sites, but neither the MEF-like sequence nor the AP-1-like sequence is found within this region. The newly identified 33-bp OP-1 responsive sequence in the aggrecan promoter is not found within the BMP-responsive elements in either collagen X promoters. Additionally, the current OP-1 responsive element also differs from the OP-1 negative response element found in the promoter of the IGFBP-5 gene, which was down-regulated by OP-1 [20].

The current data are the first to reveal that the 33-bp region in the mouse aggrecan promoter contains responsive element(s) not only for OP-1, but also for BMP-6 and CDMP-1. Based on the extent of amino acid sequence homology, OP-1 and BMP-6 belong to the same subgroup of the BMP family and share 87% amino acid homology. BMP-2 and CDMP-1 belong to different subgroups that are rather distal from the OP-1/BMP-6 subgroup. Thus, it is not surprising that deletion of the OP-1 responsive element in the mouse aggrecan promoter resulted in a loss of BMP-6 responsiveness. However, it is unexpected that the element is also responsive to CDMP-1. Deletion of the OP-1 responsive element in the mouse aggrecan promoter resulted in partial loss of BMP-2 responsiveness. It is conceivable that additional sequence(s) located outside the 1.02 kb promoter region examined is required for BMP-2 responsiveness. Thus, the current study reveals, for the first time, unique *cis*-acting elements in the mouse aggrecan proximal promoter for the different BMPs.

The data further reveal an inhibitory element(s) within the +221 to +499 region. Although the nature of the element is not clear at present, previous studies on the chick aggrecan promoter identified three *cis* elements that repressed chick aggrecan gene expression. They are CTCCTCC, CTTCA(G/C), and TCCCC [23]. No homologous sequence can be identified within the +221 to +499 region of the mouse promoter. However, two sequences (CTTCAt and CTTCgC) that differ in one base can be found in the mouse promoter at +275 and +453, respectively. Whether these sequences are responsible for the inhibitory activity is not clear at present.

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