INTERACTING POSITIVE AND NEGATIVE ELEMENTS IN THE PROXIMAL PROMOTER AND FIRST EXON OF THE AGGRECAN GENE

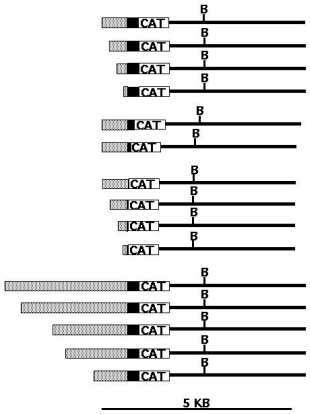
+Doege, K., *Stephens, D.T., Garrison, K. *+ Shriners Hospital for Children, Research Dept., 3101 S.W. Sam Jackson Park Rd., Portland, OR 97201: (503) 221-3438, FAX (503) 221-3451, KJD@SHCC.ORG

Introduction: Aggrecan is a definitive and essential component of cartilage. This proteoglycan is a major functional determinant of the cartilage matrix, expressed from the earliest time that a chondrocyte is identifiable. Knowledge of the regulation of the aggrecan gene would allow the modulation of many aspects of cartilage structure and function, but of equal importance, it would serve as a model or paradigm for those mechanisms at work in the process of chondrogenesis in general. The structure of the rat aggrecan gene has been previously published, along with the identification of the transcriptional start site, and preliminary characterization of the proximal promoter[1]. This 920-bp promoter contained 280 bp of untranslated first exon, as well as 640 bp of upstream flanking DNA. This promoter, called B18, was active only in the forward orientation, could be enhanced by exongenous enhancers, and was active in both chondrocytes and fibroblasts (1). Additional DNA elements in the gene therefore must interact with this promoter to confer tissue specific regulation; but the proximal promoter is very likely to provide essential elements for regulated expression, as well as for basal treanscrioption. Deletions of both the 5' and 3' portions of the B18 proximal promoter were performed to identify important funcitonal elements in cell transfection assays using reporter vectors. Sequences within the funcitonal regions were probed for nuclear factor binding activity by electorphoretic mobility shift assay.

Methods. Constructs. A 1.9 kb EcoRI-BamHI fragment encompassing exon 1 of the rat aggrecan gene was sequenced by the random fragment method. The EcoRI-PstI fragment containing 640 bp of 5' DNA and 280 bp of the first exon was HindIII linkered and cloned into the pCAT-Basic reporter vecotr (Promega); this construct is designated B18. A larger promoter fragment contained 3.2 kb of 5' DNA and the same first exon endpoint as B18; this construct is P0. A PCR strategy was used to constuct a series of deletions of the 5' and 3' flanking DNA of B18. The 5' DNA was deleted in approximate 200-bp increments (9, 10, 11); these were prepared in the presence (4) or absence (5) of the first exon DNA. Two intermediate-size deletions of 100 bp each of the 3' first exon DNA were also prepared in combination with the full-length 5' B18 DNA (16-14 and 16-15). A 3' deletion which removed the entire first exon through the start of transcription was also prepared (1-7). Deletions of 500 bp increments of the P0 promoter from the 5' end were prepared by ExoIII/mung bean nuclease digestion. Transfection assays. Primary cell cultures of 15-day chick embryo sternal chondrocytes and skin fibroblasts in F-12/10% FBS were co-transfected with a mixture of a test CAT plasmid and a control luciferase plasmid in a 20:1 ratio, using Superfect reagent (Clontech). Following overnight exposure to DNA, media was changed, and cells incubated an additional 36 hours before harvesting. Sonicated lysates were prepared, and luciferase and CAT activities were measured. CAT activities were normalized by the internal luciferase controls, and expressed relative to the B18 proximal promoter. EMSA assays. Nuclear extracts were prepared from cultured cells using NP-40 lysis; probes were end-labeled annealed oligonucleotide pairs, or PCR products. Competitors were present in a 200-fold molar excess.

Results. A series of 5' and 3' deletions of the aggrecan proximal promoter region was created and analyzed; the data shown in Figure 1 are the average of several separate experiments. Deletion of the 5'-flanking DNA results in a gradual loss of about 50% of the promoter activity (9-4 to 11-4 series). Deletion of most of the first exon DNA from the 3' end (1-5) results in almost complete loss of promoter activity; much of this loss is seen in a short deletion of the extreme 3' DNA (construct 1-15). A surprising result is seen when the two deletion series are combined. Deletion of the most 5' DNA restores the activity lost upon first exon deletion (9-5 construct; compare to 1-5). This result suggests that a negative element exists in the most 5' DNA of the B18 fragment, but the first exon sequence over-rides this negative influence with a positive factor. Upon further deletion of 5' DNA (construct 10-5), promoter activity is again dramatically lowered, suggesting that an additional positive element is located in the 9-10 interval. Deletion of the interval from 10-11 (construct 11-5) has little additional effect. When the 3' DNA is deleted past the transcription start site, activity drops to background (not shown).

The P0 construct consistently shows slightly lower activity than the B18 proximal promoter, suggesting that there are no additional essential elements in the 2.5 kb DNA upstream of B18. Nevertheless a deletion series was tested in case an altered context might reveal a masked activity in the upstream DNA. Consecutive 500-bp deletions of P0 from the 5' end showed no major changes in activity, except for the 6-1 fragment which showed enhanced activity in chondrocytes, but not in fibroblasts. This result is under further investigation.



	RELATIVE CAT ACTIVITY	
	FIB	CHON
B18-CAT	1.0	1.0
9-4	0.8	1.1
10-4	0.8	0.8
11-4	0.5	0.4
1-15	0.20	0.25
1-14	0.13	0.30
1-5	0.02	0.07
9-5	0.5	1.7
10-5	0.02	0.03
11-5	0.05	0.15
P0-CAT	0.44	0.62
2-2	0.6	0.66
6-1	0.45	1.7
8-1	0.39	1.0
10-1	0.38	1.1

FIGURE 1. Transient transfection analysis of aggrecan promoter deletions. Shaded bar is upstream sequence, black bar is first exon sequence. Results are averages of at least three experiments.

Analysis of the sequence of the B18 promoter suggests numerous potential interacting sites for transcription factors; gel shift experiments were undertaken to determine which if any predicted binding activities may correlate with the functional regions identified in the deletion series transfections. Preliminary results have localized two chondrocyte-specific binding activities: one within the 5' region (interval 1-9), deleted in the 9-4 and 9-5 constructs; and one within the 3' end of exon 1, in the region deleted in 1-15. One of these activities may be novel.

Discussion. The analysis of these deletion series has revealed several functionally important loci in the aggrecan proximal promoter which appear to interact in a complex fashion. Sequence(s) within the untranslated first exon appear to be important for basal expression by overcoming a negative regulatory activity in the 5' flanking region of the promoter. An additional positive element has been indicated, also in the 5' flanking DNA of B18. A construct (P0) larger than B18 by 2.5 kb of 5' flanking sequence does not show altered expression from the proximal B18 construct, but a sequence embedded in the larger promoter may help confer regulated expression, at least in isolation. These functional loci are under analysis to pinpoint the specific DNA sequences within the intervals which are active, and to identify transcription factors which recognize these sequences. These functional regions have been identified out of context of the fully-regulated gene, but are likely to be important parts of the operating mechanism, and will be of great interest to examine in combination with other regulatory components of aggrecan expression as they are identified.

References. 1. Doege, K., Garrison, K., Coulter, S., and Yamada, Y.: J. Biol. Chem. **269:**, 29232-29240, 1994

Acknowledgements. Funded by Shriners of North America

☐ One or more of the authors have received something of value from a commercial or other party related directly or indirectly to the subject of my presentation.

☒ The authors have not received anything of value from a commercial or other party related directly or indirectly to the subject of my presentation.