Species-specific alternative splicing of the epidermal growth factor-like domain 1 of cartilage aggrecan

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Aggrecan transcripts contain two alternatively spliced exons that code for two epidermal growth factor-like domains (EGF1 and EGF2). Whereas the EGF2 sequence is expressed at a uniform level among different species, the EGF1 sequence has been detected only in human aggrecan transcripts. In this study we have used the nested primer reverse transcription–PCR (RT–PCR) method to compare the expression of the EGF1 exon in human, bovine and dog aggrecan transcripts. Our results indicate that this exon is expressed in a species-specific manner. In addition to its significant expression level in human transcripts, the EGF1 sequence can be detected in a small portion of bovine aggrecan transcripts as shown with nested primer RT–PCR. In contrast, the same module is not detectable in dog aggrecan transcripts, although an EGF1 sequence is present in the dog aggrecan gene. The expression level of the EGF1 exon in the aggrecan transcripts correlates with the strength of the polypyrimidine tract upstream of the exon. The EGF1 sequence also shows much less conservation between the species than the EGF2 sequence. The species-specific expression and high sequence variation of the EGF1 exon imply that this sequence is likely to code for an aggrecan domain having no cartilage-specific function.

INTRODUCTION

Aggrecan is the most abundant non-collagenous component of the extracellular matrix of cartilage. It is a multi-domain molecule characterized by a large core protein (220–250 kDa) and an extremely high degree of glycosylation [1]. Aggrecan belongs to the family of aggregating proteoglycans, a group that also includes versican [2], neurocan [3] and brevican [4]. As a common structural feature, these proteoglycans possess an N-terminal globular domain that gives the molecules their ability to attach to hyaluronan. Furthermore all the family members have a highly similar C-terminal globular domain whose function is still obscure. The middle segment of the core protein bears sulphated glycosaminoglycan chains but the number of these chains differs significantly between the members of the family, aggrecan having the most (more than 100). The high degree of substitution of aggrecan with negatively charged glycosaminoglycan chains enables this molecule to play a key role in both the regulation of water content and the compressibility of cartilage.

The C-terminal globular domain of the aggregating proteoglycans is composed of three types of structural subunit: epidermal growth factor (EGF)-like, C-type lectin-like and complement regulatory protein-like modules. Versican and neurocan have been shown to have two consecutive EGF-like domains [2,3], whereas only one is present in brevican [4]. An alternatively spliced EGF-like domain is expressed in a small portion (approx. 5%) of the aggrecan transcripts in different species [5]. This domain (EGF2) is highly similar to the second EGF-like domain of versican and neurocan. Furthermore human aggrecan mRNA contains an additional alternatively spliced EGF-like sequence (EGF1) [6] that is present in approximately one-quarter of the transcripts [5] and its expression level does not change with age [7]. Aggrecan cDNA sequences from other species seem to lack this domain, although a recent study has shown the presence of a similar sequence in the rat aggrecan gene [8].

In this study we present evidence for the species-specific expression of the EGF1 sequence in human, bovine and dog aggrecan transcripts. Analysis of the splicing and branch sites indicates that sequence variations in the polypyrimidine tract upstream of the EGF1 exon are most probably responsible for this species-specific expression.

EXPERIMENTAL

Isolation of RNA

In most cases, chondrocytes from human, bovine and dog articular cartilages were used as sources of RNA. Femoral condyles of 30–40-week-old human foetuses that had died as a consequence of malfunction of the heart or central nervous system were obtained from the Southern Division of the Co-operative Human Tissue Network (University of Alabama, Birmingham, AL, U.S.A.). Cartilage samples were collected from the tarsal joints of 1–2-year-old steers and from femoral condyles, tibial plateaus, costae and tracheas of 4-week-old and 1.5-year-old dogs. Chondrocytes were released from cartilage by pronase–collagenase digestion of the tissue [9]. Cartilage samples were treated with 0.9% pronase (Calbiochem, La Jolla, CA, U.S.A.) in PBS for 90 min, then digested overnight with 0.4% collagenase (Worthington, Freehold, NJ, U.S.A.) in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) foetal bovine serum (Hyclone, Logan, UT, U.S.A.). Isolation of total RNA was performed with the guanidinium thiocyanate/phenol/chloroform method [10], modified in that the phenol/chloroform/isopentanol extraction

Abbreviations used: CS, chondroitin sulphate; EGF, epidermal growth factor; EGF1 and EGF2, the first and second EGF-like domain of aggrecan; RT–PCR, reverse transcription–PCR.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers L29488 (human), L29486 (bovine) and U43729 (dog).

Table 1 Primers used for amplification reactions

Letters in parentheses indicate sense (S) or anti-sense (A) primers.

Primers	Sequence (5′ → 3′)	Species specificity	Domain specificity
P1HD (S)	CGAAACAGCCACCTCCCCAACAG	Human, dog	CS
P1B (S)	TCAGGAGAAGAGACCCAAACAGC	Bovine	CS
P2HB (S)	AGCCCTGTAGAGCTGGGACCTGC	Human, bovine	EGF1
P2D (S)	GCCAAGGAGCCATGCGGGACCTG	Dog	EGF1
P3 (A)	GCTCGCCAGTGTAGCCAGGGGGG	All	EGF1
P4 (A)	CCCTCTGTAGCTGGGAAGGCATAAGCAT	All	EGF2
P5 (A)	ACTCCTGCTCCTCGGGGGTGACG	All	LEC

was repeated three times. RNA was also isolated directly from dog hyaline cartilaginous tissues by extracting the cartilage pieces by the method described above.

Reverse transcription–PCR (RT–PCR)

Primers for PCR (Table 1) were chosen from published sequences of human [6,11], bovine [12] and dog [5] aggrecans and synthesized by Research Genetics (Huntsville, AL, U.S.A.). Reverse transcription of RNA species and subsequent amplification of cDNA were performed with a GeneAmp RNA PCR kit in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus Instruments, Norwalk, CT, U.S.A.). Total RNA (1 μg) was reverse-transcribed in 20 μl of 10 mM Tris/HCl buffer, pH 8.3, that contained 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 0.75 μM of the appropriate anti-sense primer, 1 unit of RNAse inhibitor and 2.5 units of Moloney murine leukaemia virus reverse transcriptase. The reaction was conducted at 42 °C for 20 min and then the reverse transcriptase was inactivated by heating the mixture to 99 °C for 5 min. The cDNA produced was amplified in a 100 μl reaction volume of 10 mM Tris/HCl buffer, pH 8.3, containing 2 mM $MgCl_2$, 200 μM of each dNTP, 0.15 μM of both primers and 2.5 units of AmpliTaq DNA polymerase. Annealing and chain extension were conducted at 60 °C for 1.5 min in 35 cycles. After amplification, a 7-min final extension period was allowed at 60 °C. Amplification products were analysed on 1% (w/v) agarose gel in 0.9 M Tris/borate buffer containing 0.02 M EDTA. PCR products were revealed with ethidium bromide staining.

Nested primer RT–PCR was performed to amplify sequences expressed at low levels. RT–PCR was performed first with primers outside the sequence to be amplified. In a subsequent amplification, one of the primers was replaced with an inner primer (either sense or anti-sense) specific for that sequence. Usually 1 μl of the first amplification product was used in the second amplification step. Reaction conditions were the same as described above.

PCR on genomic DNA

To locate exons that code for EGF1 domains in aggrecan genes and to determine the intron sequences that might play a role in the splicing of exons, PCR was performed on genomic DNAs (Promega) with exon-specific primers. DNA (0.5 μg) was amplified in the same buffer as described above. The reactions were conducted at 60–68 °C, depending on the primer pairs and templates.

To amplify DNA regions of more than 4 kb, long-range PCR was used. The regular PCR buffer was replaced with 20 mM Tris/HCl buffer, pH 8.8, containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 and 0.1 mg/ml nuclease-free BSA. The amount of *Taq* polymerase was increased to 5 units and an equal number of units of *Taq* Extender PCR additive (Stratagene, La Jolla, CA, U.S.A.) was added. All other components had the same concentrations as described above. Extension times were increased to 7 min.

DNA sequencing

PCR products were purified with a Magic PCR Preps DNA purification kit (Promega). Purified DNA samples (40–100 fmol) were sequenced from both strands with the Sanger dideoxy chain termination method [13] with a fmol® DNA sequencing kit (Promega) and [α-^{35}S]dATP (more than 600 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.). The primers were identical with those used for amplifications. The reactions were performed in a thermocycler, and primer annealing and extensions were at 60 °C for 1.5 min. Bands were separated on an 8% (w/v) polyacrylamide/50% (w/v) urea gel and detected by autoradiography.

RESULTS

The presence of the EGF1 sequence in the aggrecan gene

To detect the EGF1 sequence in the bovine and dog aggrecan genes, amplification reactions were performed on genomic DNAs. Because sequence information for these species was lacking, a human EGF1 domain-specific primer (P3) was used. The PCR resulted in products similar in sizes to the human product (Figure 1) and with 5′ end sequences identical with the chondroitin sulphate (CS) domain sequences of aggrecans. The 3′ end of the PCR products was homologous with the human EGF1 sequence, indicating that the same sequence was present in both bovine and dog aggrecan genes.

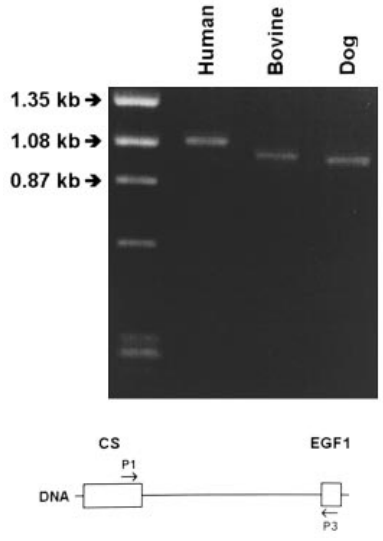

Figure 1 Localization of the EGF1 sequence in the aggrecan gene of different species

An ethidium bromide-stained 1% (w/v) agarose gel is shown. The sense primers were P1HD for human and dog, and P1B for bovine DNAs.

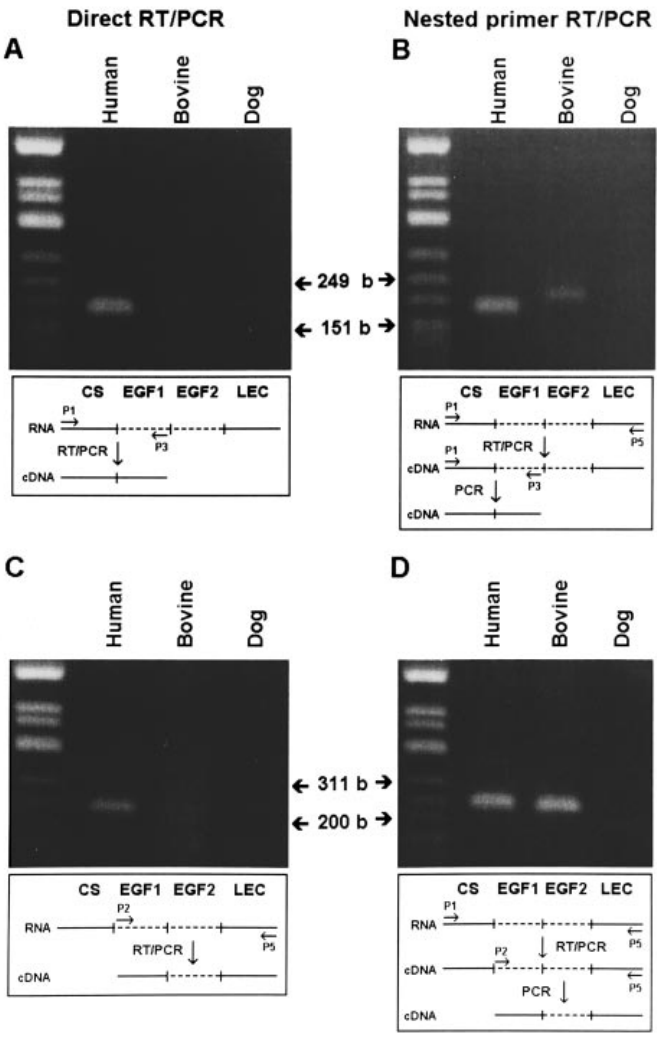

Figure 2 Direct and nested primer RT–PCRs to detect aggrecan transcripts with EGF1 sequence in different species

Ethidium bromide-stained 1% (w/v) agarose gels are shown. Primers used for reverse transcriptions and subsequent amplifications are represented by arrows on the diagrams beneath the gels. The CS domain-specific sense primers were P1HD for human and dog, and P1B for bovine RNA species. The EGF1 domain-specific primers were P2HB for human and bovine, and P2D for dog RNA species. Broken lines indicate alternatively spliced sequences. Abbreviation: LEC, lectin.

Differential expression of the EGF1 domain in human, bovine and dog aggrecan transcripts

As earlier results demonstrated [5], the human EGF1 sequence was expressed in approximately one-quarter of the aggrecan transcripts. To determine the expression of this domain in bovine and dog aggrecan transcripts, RT–PCRs were performed on RNA species isolated from chondrocytes of articular cartilages. These reactions failed to produce any detectable band on agarose gel (Figure 2A). The inability of the human EGF1 sequence-specific primer to bind both bovine and dog RNA templates could be dismissed as a cause of the negative result, because this primer, paired with the appropriate CS domain-specific primers, readily produced specific PCR products from genomic DNAs (Figure 1). Thus the failure to obtain a PCR product was caused by either the lack of or a low level of expression of the EGF1 sequence in the aggrecan transcripts. In a subsequent experiment, nested primer RT–PCR was used to investigate whether or not the EGF1 sequence was present at all in the transcripts. A single product of the expected size was detected when bovine RNA was used, but the reaction with dog RNA was still unsuccessful (Figure 2B). The sequence of the bovine RT–PCR product proved that it was amplified from aggrecan transcripts. Additional RT–PCR experiments performed on RNA samples isolated from either chondrocytes or tissues of articular, costal and tracheal cartilages of dogs of different ages (4-week-olds and 1.5-year-olds) also failed to detect the EGF1 sequence in dog aggrecan mRNA species (results not shown). These results suggested that the EGF1 exon was spliced into a very small portion of the bovine aggrecan transcripts, whereas its expression level, if any, in dog aggrecan transcripts was below the detection limit of our nested primer RT–PCR technique.

To corroborate these findings, EGF1 exon-specific primers were designed on the basis of the 3′ end sequence of the genomic PCR products. Direct and nested primer RT–PCRs were performed with these primers together with lectin-like domain-specific primers. The reactions gave the same results as in the previous experiments (Figures 2C and 2D), implying differential expression of the EGF1 module between the three species.

Sequence of EGF1 domains

The products of the direct and nested primer RT–PCR and genomic DNA PCRs were isolated, purified and sequenced (Figure 3). The human EGF1 domain showed the same sequence as previously described [6]. The bovine PCR products also contained an EGF-like sequence that was 84.2% identical with the human sequence. However, the bovine sequence had a single nucleotide deletion close to its 3′ end (Figure 4). This deletion caused a frame shift and the formation of a stop codon at the end of the domain (Figure 3).

Because RT–PCR products could not be detected in dog RNA, only the PCR products amplified from genomic DNA were sequenced. First the 3′ sequence of the PCR product shown in Figure 1 was determined. Then long-range PCR was performed on dog genomic DNA with an EGF1 sequence-specific sense primer (P2D) and an EGF2 sequence-specific anti-sense primer (results not shown). The 5′ sequence of this PCR product provided the missing part of the dog aggrecan EGF1 sequence. The nucleotide sequence of the dog EGF1 module showed 76.3% and 74.6% identity with the human and bovine EGF1 domains respectively. The dog sequence contained a six-nucleotide deletion between the second and third cysteine positions (Figure 3). Moreover, if this sequence were translated, the first cysteine residue of the domain would be mutated into phenylalanine and the fourth cysteine residue into glycine.

Differences in splice site sequences

The differences in the splicing efficiencies of the EGF1 exons between the three species (human, bovine and dog) could result from either different expression levels of certain splicing factors or from specific changes in intron sequences that flank the EGF1 exon. Because RNA species were isolated from normal articular chondrocytes, the expression levels of the splicing factors were assumed to be similar in these cells. Therefore we focused on specific intron sequences known to take part in the splicing of the primary transcript.

Exon skipping might be caused by sequence changes in the 5′ splice site of the intron downstream of the EGF1 exon, as suggested by the exon definition theory of splicing [14]. A 26-

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Nucleotide sequence
                    ↓
Human    GCCCCCGCCAGGTCCTGTGCAGAGGAGCCCTGTGGAGCTGGGACCTGCAAGGAGACAGAG -  60
Bovine      T TA    T        C  AA        A C            C       G    -  60
Dog            A T       T   CA       A  C------        TC  C   TGA  -  54

                                                          ↓
Human    GGACACGTCATATGCCTGTGCCCCCCTGGCTACACTGGCGAGCACTGTAACATAGAC    - 117
Bovine                 T          -   C        A       AG  G         - 116
Dog            A     G T             C        C       C  TG         - 111

Protein sequence

Human    AlaProAlaArgSerCysAlaGluGluProCysGlyAlaGlyThrCysLysGluThrGlu -  20
Bovine      SerThrSer            Lys      Ser           Gln           -  20
Dog            Thr      Phe    Lys        ------         GlnGlnMetLys -  18

Human    GlyHisValIleCysLeuCysProProGlyTyrThrGlyGluHisCysAsnIleAsp      39
Bovine                              AlaThrLeuAlaSerThrGluThrSTOP       -  37
Dog            Ile   Gly                        Gln         Val        -  37
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Figure 3 Nucleotide and deduced amino acid sequences of EGF1 domains present in aggrecans of different species

The complete sequences are shown for the human module. In other EGF1 modules, only differences are indicated. Arrows mark the first and last nucleotides of the EGF1 sequences.

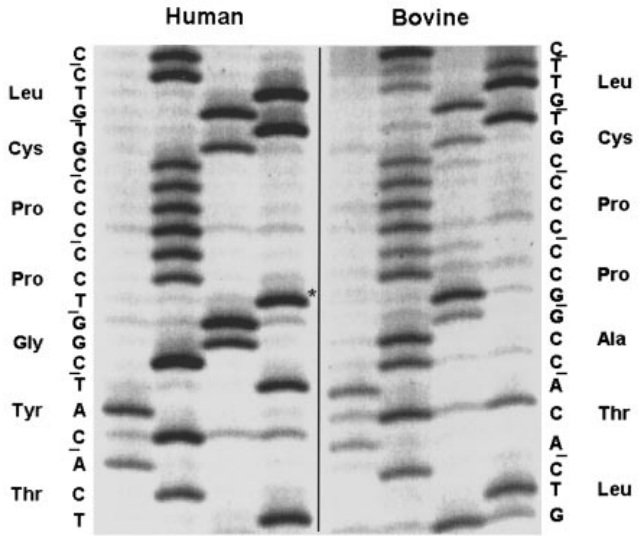


Figure 4 Single nucleotide deletion in the bovine EGF1 sequence

Autoradiograms of sections of the human and bovine EGF1 sequences are shown. The nucleotide (T) marked by an asterisk in the human sequence is missing from the bovine sequence. Nucleotide and protein sequences are indicated at the sides of the gels. Codons are separated by horizontal lines.

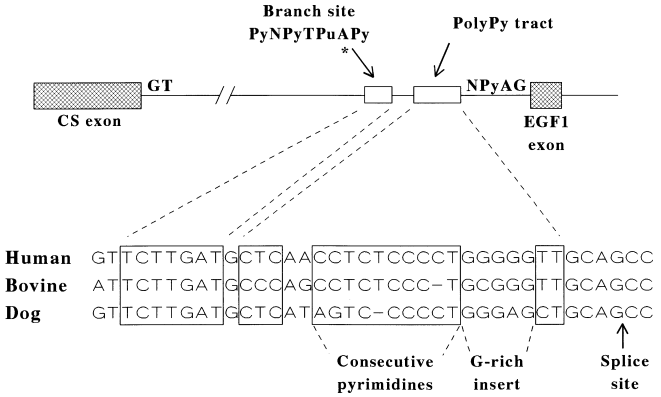


Figure 5 Consensus splicing sequences in the intron between the CS and EGF1 exons

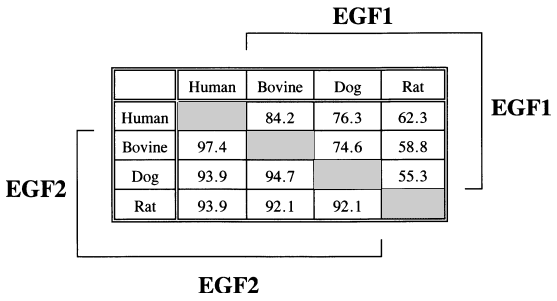
Cross-hatched boxes indicate exons, and the line between them represents the intron. Empty boxes illustrate consensus sequences, which are also boxed on the sequences. The asterisk marks the putative branching nucleotide (A).

nucleotide-long sequence for this splice site was obtained from the dog genomic 'long-range' PCR product (see above) and showed 84 % identity with the recently published human sequence [15]. The immediate splice sites of both the dog and human introns downstream of the EGF1 exon had the GTAAG sequence that fitted the consensus GTRAG sequence [16]. Thus this site could be ruled out as a cause for different splicing levels of the EGF1 exons.

As to the intron upstream of the EGF1 exon, three consensus sequences should be of particular interest: the immediate 3′ splice site [16], the polypyrimidine tract [17] and the branch sequence [18,19]. These specific sites were determined by sequencing the genomic PCR products amplified with CS and EGF1 domain-specific primers (Figure 1). The sequence of these products revealed that all three species contained the GCAG sequence at the immediate 3′ splice site (Figure 5). This sequence was identical with the consensus NYAG [16]. However, it should be noted that the first nucleotide of the EGF1 exon in all species was a cytosine and not the more frequent guanine. A potential branch point with a sequence of TCCTGAT was identified approx. 28 or 29 nucleotides upstream of the splice site (Figure 5). This sequence also was invariant between the species and fits well with the consensus YNYTRAY [18,19]. A relatively weak polypyrimidine tract was found between the branch sequence and the 3′ splice site. Its most remarkable feature was that it was interrupted with a guanine-rich pentanucleotide (Figure 5). This pentamer reduced the tract of consecutive pyrimidines to 10 nucleotides in the human sequence. A deletion in the corresponding bovine intron shortened the tract to nine nucleotides, whereas a single deletion and two pyrimidine–purine trans-

versions resulted in a seven-nucleotide continuous tract in the dog intron (Figure 5).

It should be emphasized that the consensus sequences described above are putative, i.e. they are identified by their location and similarity to previously described sequences and not by functional assays. However, the 34-nucleotide region upstream of the EGF1 exon shows 73–88 % identity between the three species. This degree of conservation is very high for an intron sequence unless it sustains specific information. In comparison, the remaining portion of the sequenced introns exhibits 52–67 % identity between the three species, whereas the same value is 74–85 % for the coding sequence of the EGF1 module (see above). Thus it is reasonable to conclude that this 34-nucleotide region immediately upstream of the EGF1 exon carries information for splicing.



	Human	Bovine	Dog	Rat
Human		84.2	76.3	62.3
Bovine	97.4		74.6	58.8
Dog	93.9	94.7		55.3
Rat	93.9	92.1	92.1	

Figure 6 Sequence conservation of aggrecan EGF-like domains between the species

Numbers indicate percentage identities between nucleotide sequences of the EGF1 (above the shaded diagonal) and the EGF2 (under the shaded diagonal) domains, respectively. The EGF2 sequences were obtained from [5], whereas the rat EGF1 sequence was taken from [8].

DISCUSSION

In contrast with the uniform expression level of the EGF2 sequence between the species [5], the EGF1 domain is expressed in a species-specific manner. Whereas this sequence is present in human aggrecan mRNA at a substantial level, only a small portion of bovine aggrecan transcripts contain it. Furthermore this sequence is undetectable in dog aggrecan transcripts, even with nested primer RT–PCR. A recent study on the structure of the rat aggrecan gene [8] has also found an EGF1 sequence at approximately the same position as the human EGF1 sequence in the aggrecan gene. However, transcripts that possess this sequence could not be detected in rat chondrocyte mRNA [8]. This observation also supports the species-specific expression of the aggrecan EGF1 domain.

The different expression levels of the EGF1 sequence in the three species can most probably be explained by changes of intron consensus sequences that are important in splicing. The 5′ splice site of the intron upstream of the EGF1 exon has not been investigated because this site is always utilized regardless of whether or not the transcript contains the EGF1 sequence. Of the other consensus intron sequences, only the polypyrimidine tract shows variations. This tract contains a guanine-rich pentanucleotide that is inserted six nucleotides upstream of the 3′ splice site. In addition there are two other purines that interrupt the tract further upstream. The pyrimidine content of the tract is 13–15 of the 21 or 22 nucleotides, but owing to insertions, mutations and deletions, the length of the uninterrupted pyrimidine runs in the human, bovine and dog sequences is 10, 9 and 7 nucleotides respectively. The recently described rat aggrecan gene [8] does not contain a prominent 3′ splice site, polypyrimidine tract or branch site upstream of the EGF1 sequence. The absence of these consensus sequences correlates well with the lack of the EGF1 exon in the rat aggrecan transcripts.

The polypyrimidine tract seems to be one of the most important sequence motifs necessary for efficient splicing of metazoan pre-mRNA. It is required for the assembly of the splicing complex, the cleavage at the 5′ splice site and the lariat formation [17,20]. It is known to bind several splicing factors such as U2 auxiliary factor [21], heteronuclear ribonucleoproteins [22], intron binding protein [23,24] and polypyrimidine tract binding-protein [25], and also has been implicated in branch point selection [26–28] and alternative splicing [29–31]. Deletions, truncations or mutations of this sequence can reduce or abolish its ability to bind splicing factors and prevent spliceosome formation and intron removal [17,20,32–34]. It also has been shown that purine stretches inserted into the polypyrimidine tract can severely impair splicing *in vitro* [27]. A recent study has indicated that a seven-nucleotide polypurine string close to the 3′ splice site of one of the introns of a deficient adenosine deaminase gene also prevents the removal of the intron *in vivo* [35]. The purine-rich pentanucleotide in the aggrecan intron upstream of the EGF1 exon is also located at the 3′ acceptor site and thus may cause a decreased level of splicing. Other elements that might influence the splicing efficiency are the length and base composition of the polypyrimidine tract. The length of the tract before the EGF1 exon is close to the minimum and might cause a lower binding affinity for splicing factors [28]. It also has been demonstrated that uracils and cytosines might have different roles in the tract, uracils being preferred to cytosines [33]. The tract upstream of the EGF1 exon contains mainly cytosines, which may further curtail its ability to bind splicing factors.

The sequences of the 3′ acceptor sites upstream of the EGF1 exons indicate that there is a certain correlation between the length of the consecutive pyrimidines and the expression levels of the EGF1 exon between the species: the longer the tract, the higher the expression level of the exon. A 10-nucleotide consecutive pyrimidine tract results in a substantial level of expression of the EGF1 exon in human aggrecan transcripts [5]. Decreasing the length of the tract by one nucleotide severely diminishes the expression of the exon in bovine aggrecan, and further decreasing it by two nucleotides lowers the splicing efficiency of the exon in dog aggrecan transcripts below the detection limit of the nested primer RT–PCR. The evolutionarily (*in vivo*) occurring splice site differences are in accord with previous findings *in vitro* that gradual weakening in the polypyrimidine tract by deletions and mutations causes progressively less efficient splicing [34].

Contrary to the highly conserved EGF2 domain of aggrecan [5], the EGF1 module shows much larger sequence variations (Figure 6). Its higher mutational rate is related to the fact that the rat [8] and probably the dog EGF1 sequences are present as part of an intron (as pseudoexons). Intron sequences generally mutate at much higher rates than exons because they do not bear any coding information. The loss of coding information in the dog and rat [8] EGF1 pseudoexons is indicated by long deletions and the mutation of cysteine residues that otherwise would be very important for the folding of the domain. Even the bovine EGF1 sequence (which is expressed at a minute level) contains a single nucleotide deletion, generating a translational frame shift and a premature stop codon at the end of the exon. Therefore the translation of the bovine aggrecan transcript possessing the EGF1 sequence results in a core protein truncated after the

EGF1 domain. The lack of the other subdomains of the C-terminal globular domain could lead to serious consequences because this part of the molecule has been suggested to be essential in the intracellular trafficking of aggrecan [36,37]. However, the mutated polypyrimidine tract upstream of the EGF1 exon prevents significant expression of this exon in the bovine aggrecan transcripts and down-regulates the production of a 'defective' core protein, eliminating any potentially negative effects.

Although a different role for the EGF1 domain between the species cannot be ruled out, the high mutational rate and the species-specific expression of the EGF1 sequence indicate that this module might not have any cartilage-specific function in the aggrecan molecule.

We thank Dr. Erzsébet Walcz and Dr. Márta Valyon for their valuable help and Dr. Björn Olsen and Dr. Vincent C. Hascall for critical reading of the manuscript. This work was supported by grants from the National Arthritis Foundation and by National Institutes of Health Grant R01 AR40310.

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Received 30 May 1996; accepted 12 July 1996