Differential Splicing and Alternative Polyadenylation Generate Multiple Mimecan mRNA Transcripts*

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We previously showed the 25-kDa corneal keratan sulfate proteoglycan to be a translation product of the gene producing osteoglycin and proposed the name mimecan for this gene and its product. We also demonstrated three mimecan RNA transcripts using Northern blot analysis. In this report, we investigate the mechanisms accounting for these transcripts. Ribonuclease protection analysis and reverse transcription-polymerase chain reaction of bovine corneal mRNA detected a mimecan transcript that lacked 278 base pairs of the 5′-untranslated region between residues 62 and 340. This splice variant represents the predominant form of mimecan mRNA in bovine cornea and sclera. It was also detectable in other bovine tissues as a minor transcript. Two additional cDNA clones that were isolated contained 398 bases of nucleotide sequence at the 3′-end of mimecan cDNA, not present in the published sequence. Ribonuclease protection analyses with the 3′-probe, which included the new sequence, allow detection of three RNA transcripts while 5′-probes recognized only two. These results indicate that the three canonical polyadenylation sites in the 3′-untranslated region of mimecan mRNA are alternatively selected. Possible roles for this previously undetected degree of diversity of mimecan RNA isoforms transcribed in the same tissue are discussed.

Keratan sulfate proteoglycans (KSPGs)† are members of a group of small, leucine-rich proteoglycans (SLRPs). These proteins share a common core structure which consists of a central domain with varying numbers of leucine-repeat repeats flanked by cysteine-rich clusters (1). Seven members of SLRPs have been described so far. These include the keratan sulfate-containing proteoglycans keratan (2), lumican (3, 4), and fibromodulin (5) and the dermatan sulfate-containing proteoglycans decorin (6), biglycan (7), and epiphycin (8). A seventh member of the family, osteoglycin, previously called osteoinductive factor (9), was isolated initially as a 12-kDa protein representing the C-terminal 33% of the coded protein. Our recent studies demonstrated that the full-length translation product of the gene producing osteoglycin is a corneal KSPG (previously called KSPG 25), also present in many other tissues, but lacking a keratan sulfate chain. We named this gene and its protein product mimecan (10) and use this nomenclature in the present paper.

Whereas information on protein structure of KSPGs has increased over the past decade, only modest information is available regarding their transcriptional regulation. For example, mimecan appears to be encoded by a single gene because a simple pattern of bands is observed in genomic Southern blots probed with PCR-amplified cDNA probes (11). However, two and three mRNA species that are selectively expressed in different cell types have been detected by Northern blots of bovine, mouse, and human tissues (9–11). Multiple forms of mature mRNA transcripts from a single gene can be produced by several different types of reactions, such as alternative transcription start sites, alternative splicing of pre-mRNAs, and alternative polyadenylation site selection (12). Interestingly, alternative splicing has been detected for some proteoglycans, e.g. decorin and agrin (13–15). However, no published information is available regarding the origin of mimecan mRNA species.

In this report, we investigate the molecular mechanisms by which the three mimecan mRNA transcripts detected in cornea are generated. First, we detected an RNA transcript that lacked 278 bp in the 5′-UTR of mimecan cDNA. In addition, we showed by RPA that splicing of this putative exon occurs in a tissue-specific fashion. Second, we isolated sequence at the 3′-end of cDNA that is present in only a portion of cDNA clones and demonstrated that three alternative polyadenylation sites generated the three mimecan mRNA transcripts. Our results suggest a previously undetected degree of diversity of mimecan RNA isoforms transcribed in the same tissue.

**EXPERIMENTAL PROCEDURES**

*Isolation and Analysis of Mimecan cDNAs—*Seven cDNA clones were isolated by screening a Uni-ZAP XR cDNA library made from cultured bovine keratocytes as described previously (10). All clones were completely sequenced by the dideoxy chain termination method (16) using the fmolDNA sequencing system (Promega Corp.). Sequencing primers were synthetic oligonucleotides deduced from the published sequence (9, 10). All primers used in this study are shown in Table I.

**RT-PCR Analysis of Mimecan mRNA—*Total RNA from cultured keratocytes and different bovine tissues was isolated by the guanidinium thiocyanate-LiCl method as described (10). RNA (1 μg) was reverse transcribed in a 20-μl reaction containing 10 μM of the anchor primer oligonucleotide (dT)18 or gene-specific primers, Superscript reverse transcriptase (Life Technologies, Inc.), and 0.5 μm dNTPs for 50 min at 42 °C in buffer supplied by the manufacturer. Reactions were terminated by addition of 1 μl of RNase H (1 unit) followed by incubation at 37 °C for 20 min and heat inactivation (95 °C, 5 min). The single strand cDNA products (2 μl) were used as templates in a 50-μl PCR amplification reaction that contained 10 μM of TRIS, pH 9, 50 mM KCl, 0.1% Triton X-100, 1.6 mM MgCl2, Taq DNA polymerase (5 units), 0.2 mM dNTPs, and 100 ng of each primer pair (Fig. 1) and that was carried out for 30 cycles (95 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min).

**Resulting PCR products** were analyzed by electrophoresis on 3% agarose gels with DNA visualized by ethidium bromide staining. The PCR products were eluted from the gel, subcloned into pGEM-T vectors

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‡ The abbreviations used are: KSPG, keratan sulfate proteoglycan; SLRP, small leucine-rich proteoglycan; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR, RPA, ribonuclease protection analysis; bp, base pair(s); UTR, untranslated region; pBS, pBluescript; PIPES, 1,4-piperazinediethanesulfonic acid; kb, kilobase(s).
Structural Variation in Mimecan mRNA Transcripts

RNAse Protection Experiments—The following restriction fragments from mimecan cDNA were subcloned into pGEM-1 vector and used to synthesize antisense RNA probes: EcoRI to Sccl positions 1–584, EcoRI to HinclII (positions 1–746), and 662-bp HindIII fragment (positions 298–960). Antisense riboprobes were synthesized from these subclones after linearization with EcoRI. The clone containing the EcoRI-ScaI DNA fragment was linearized also with KpnI (position 106, Bst1107I (position 274), and SspI (position 309). Two DNA clones in pBluescript SK vector were also used for RPA: pBS-60, containing 2166 bp of mimecan cDNA (10), and pBS-62, containing an additional 398 bp at the 3'-end of mimecan cDNA (Fig. 1). Prior to synthesis of antisense riboprobes, these pBS clones were linearized with NdeI (position 1696).

Linearized plasmid (1.7 μg) was transcribed with 20 units of T7 or SP6 RNA polymerase (Promega) for 1 h at 37 °C. Transcription reactions contained transcription buffer (Promega), 10 mM dithiothreitol, 500 μM each ATP, GTP, and CTP, 15 μM UTP, and 50 μCi of [32P]UTP (3000 Ci/mmol; NEN Life Science Products) and 1 μl of RNasin (Promega). To remove the DNA template, RNase-free DNase (3 units/μg of DNA) was added to the transcription reaction with incubation continued for 30 min at 37 °C. Riboprobes were purified three times by phenol-chloroform extractions followed by ethanol precipitation. Amounts of labeled RNA probes were estimated from the [32P] incorporated for 30 min at 37 °C. Riboprobes were purified three times by phenol-chloroform extractions followed by ethanol precipitation. Amounts of labeled RNA probes were estimated from the [32P] incorporated for 30 min at 37 °C. Riboprobes were purified three times by phenol-chloroform extractions followed by ethanol precipitation. Amounts of labeled RNA probes were estimated from the [32P] incorporation. Concentrations of nonradioactive RNAs were determined spectrophotometrically and integrity of all RNA transcripts was examined by agarose gel electrophoresis. Total RNA (1 μg) and 1 × 10^6 cpm of each probe were resuspended in 30 μl of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA), and hybridization was carried out at 60 °C overnight (14–16 h). RNase T1 (200 units) or RNase A/T1 mixture (Ambion Inc., Austin, TX) was added to each sample, and digestion was carried out for 30 min at 37 °C. Twenty μl of 10% SDS and 10 μl of 10 mg/ml proteinase K were added to each sample, and incubation was continued for 30 min at 37 °C. Samples were extracted with phenol-chloroform, and the protected fragments were resolved on 6% denaturing polyacrylamide gels.

Northern Blot Analysis of Bovine mRNA—Total RNA (10 μg) from cultured keratocytes and various bovine tissues was subjected to electrophoresis through a 1.5% agarose gel containing 2.2 M formaldehyde and blotted onto nylon membrane using a Turboblotter apparatus (Schleicher & Schuell). The membranes were probed with a 32P-labeled bovine mRNA clone insert probe at 42 °C in 50% formamide as described previously (2). Filters were washed and exposed to Hyperfilm-MP (Amersham Life Science, Inc.) at +70 °C for 8–24 h.

Genomic DNA Hybridization—Bovine genomic DNA was purchased from CLONTECH. Ten μg of genomic DNA was digested with an appropriate restriction endonuclease, fractionated in a 0.8% agarose gel, and transferred onto nylon membrane (GeneScreen Plus, NEN Life Science Products) as described previously (17, 18). The membranes were prehybridized for 1 h at 65 °C in a solution containing 0.2% polyvinyl pyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 0.05 M TRIS-HCl, pH 7.5, 1 mM NaCl, 0.1% sodium pyrophosphate, and 1% SDS. Two DNA probes were generated by PCR from pBS62 cDNA clone, a 914-bp probe (between primers P5-P8) and a 1314-bp probe (between primers P5-P9), 32P-labeled using Prime-a-Gene labeling system (Promega) and used at 2 × 10^6 cpm/ml of the above solution. Hybridization was carried out overnight at 65 °C. Following hybridization, the filter membranes were washed and exposed to X-Omat AR film (Kodak Scientific Imaging System) at -70 °C for 1–3 days.

RESULTS AND DISCUSSION

Identification of Differentially Spliced Mimecan mRNA—During the course of our previous study, we subcloned overlapping restriction fragments spanning the entire mimecan cDNA sequence. These clones were used for synthesis of RNA probes for RPA. Of several antisense probes utilized, most of them produced more than one protected fragment with RNA from cultured keratocytes. Restriction mapping of mimecan cDNA and the inferred RNA probes are summarized in Fig. 1A.

Initially, a ScaI-EcoRI fragment containing 584 nucleotides of the 5'-sequence of mimecan cDNA was used as a probe in RPA experiments. RNA from cultured keratocytes was hybridized with an antisense RNA probe corresponding to the above fragment. A fully protected band of 584 bp was revealed (Fig. 2A, lanes 1 and 2) as expected. Surprisingly, a more intense additional band of about 250 bp also was observed. No protected band was detected when RNA was omitted from the reaction mixture in control experiments (Fig. 2A, lane 3). These data indicate the existence of an alternative splice site in the 5'-UTR of mimecan mRNA. To demonstrate whether the extra protected band was specified by the 5'- or the 3'-end of the probe, we performed RPA with three shorter RNA probes. These were made by digestion of the same construct with KpnI, Bst1107I, or SspI restriction sites within the cloned EcoRI-ScaI DNA fragment. As shown in Fig. 2B, when ScaI-KpnI fragment lacking 106 bp at the 5'-end of the first probe was used in RPA, the same 250-bp major protected band was detected, in addition to the fully protected band of 480 bp. Similar data were obtained with ScaI-Bst1107I and ScaI-SspI RNA probes (Fig. 2, panels C and D). The major band of 250 bp was seen in addition to the less intense fully protected 300- and 260-bp bands. Considering the fact that the 5'-end of the described RNA probes used in these experiments is located at position 584 (ScaI site) and that the size of the partially protected band was about 250 bp, we concluded that an alternative splice site exists between positions 309 and 309 of mimecan mRNA.

To confirm our data, we used a second construct containing a HinclII-EcoRI DNA fragment between positions 746 and 1 of the published mimecan cDNA sequence(10) and thus overlapping the ScaI-EcoRI clone. We reasoned that if an alternative splicing site existed at the 5'-end, the additional 398-bp sequence should be protected at the 3'-end of mimecan cDNA. The two polyadenylation signals located in this region are indicated in bold letters.

FIG. 1. A, schematic representation of the mimecan cDNA map, which is based on clone pBS60 (10), plus additional 398 nucleotides at the 3'-end. Restriction endonuclease sites are shown as follows: E, EcoRI; K, KpnI; Bst, Bst1107I; H, HindIII; Ss, SspI; Sc, ScaI; Hc, HincII; and N, NdeI. The open rectangle represents the protein coding region. The positions and lengths of antisense RNA probes (SE, SK, SB, SS, HE, HH, PH5, and pBS-60) are shown by the thin horizontal arrows and are not to scale. The vertical arrowheads indicate the positions of the three polyadenylation signals. B, the additional 398-bp sequence found at the 3'-end of mimecan cDNA. The two polyadenylation signals located in this region are indicated in bold letters.
The nucleotide sequences of larger PCR subclones were identical with that of the published mimecan cDNA (10). Thus, data presented here show that a 278-bp exon located between positions 62 and 340 is spliced out to generate an abundant shorter mRNA message in bovine cornea and cultured keratocytes. This 278-bp sequence appears to represent the second exon of the mimecan gene, whereas the 62 bp at the 5’-end of the cDNA corresponds to the first exon. However, only the isolation and characterization of mimecan genomic clones will provide the definitive answer to whether additional exons exist upstream of the first 62 bp. For example, two leader exons separated by over 12 kb that are alternatively spliced to exon 2 have been described in the human decorin gene, another member of the SLRP gene family (19).

The number of reports on pre-mRNAs that are subjected to regulated splicing has been increasing steadily (reviewed in Ref. 20). In some cases, alternatively spliced mRNAs lead to the synthesis of different proteins from the same pre-mRNA. Well documented examples of protein isoforms generated by alternative RNA splicing include neural cell adhesion molecule (21, 22) and human paired-box-protein PAX8 (23). In other cases, differential splicing can generate mRNAs that contain or lack an open reading frame and therefore function as an on-off switch (24). It is presently unclear what function the alternatively spliced exon may confer on mimecan, especially if it is located within the 5’-UTR. Exon absence, however, must affect the mRNA secondary structure. RNA secondary structure has been implicated in the regulation of initiation of translation (25). At present, it seems likely that the single, full length translation product of the mimecan gene is subjected in situ to proteolytic cleavage to form the 25-kDa form predominant in bovine cornea or the 12-kDa form (osteoglycin) found in other tissues. Elucidating the possible roles of this splicing event in regulating mimecan translation will be important.

### Tissue Distribution of Differentially Spliced Mimecan mRNA Transcripts—

As discussed above, tissue- and stage-specific pre-mRNA splicing events are important for post-transcriptional gene control. Therefore, ascertaining whether and how the two mimecan splice variants are present in other bovine tissues becomes an important question. To address this matter, we have isolated total RNA from several bovine tissues. A 662-bp HindIII DNA fragment between positions 298 and 960 was subcloned into pGEM-1 vector and used for synthesis of an antisense RNA probe. We chose this probe because, first, it would allow us to confirm the two splice variants detected so far, and, second, it covers a portion of protein-coding region of mimecan cDNA that would enable us to detect a possible third splice variant, if such existed within this region in other tissues. The results of an RPA experiment with HindIII probe are shown in Fig. 4.

Consistent with data obtained from the initial five probes, an intense, partially protected band of about 620 bp was the major product detected in RNA from bovine cornea and cultured keratocytes. The 620-bp splice variant also was found to be more abundant than the full-length protected probe in bovine sclera, but less abundant in RNA from cartilage and vagus nerve. In most of the RNA samples tested, the full size mRNA transcript (662 bp) was the major protected band. As discussed earlier, the physiological significance of these findings remains to be determined.

### Identification of Alternative Polyadenylation Sites in the Generation of Mimecan mRNA Transcripts—

The differentially spliced exon described above can account for only two mRNA transcripts. However, Northern blot analysis of total RNA from 18 bovine tissues showed the presence of three mimecan transcripts of 2.4, 2.5, and 2.6 kb (Fig. 5A). These mRNAs were
highly expressed in cornea, cultured keratocytes, and sclera. They were detectable at a significant level in cartilage, aorta, ligamentum nuchae, intestine, kidney, and vagus nerve but also were present at low levels in other tissues. How the third message was generated still remained unclear. To approach this question, we utilized our mimecan cDNA clones and have analyzed seven additional pBluescript clones containing the largest inserts. Restriction enzyme mapping and sequence analysis revealed that two clones, pBS-62 and pBS-14, contained 398 additional nucleotides at their 3'-end (see Fig. 1B for complete sequence) compared with the published mimecan cDNA sequence from clone pBS-60 (10). All seven clones showed sequence identity over the protein coding region.

To confirm that the 398-bp sequence was present at the 3'-end of mimecan mRNA and was not the result of cloning procedures and to test for differential splicing at the 3'-end of mimecan mRNA, we designed PCR primers spanning this region (Table I). Primers were used to perform RT-PCR. The experiments were done with RNA from cultured keratocytes, a cell type in which mimecan mRNA is highly abundant. The PCR strategy and the results of these experiments are shown in Fig. 6. Reverse transcription was performed with either oligo-dT or gene-specific primer P9 located at the 3'-end of the region in question. A single band of expected size was obtained using each of six primer pairs (P7-P8, P6-P8, P5-P8, P7-P9, P6-P9, and P5-P9). A second band indicative of an alternative splice site was not detected. The PCR results confirmed that the 398-bp sequence at the 3'-UTR found in cDNA clones pBS-62 and pBS-14 exists in mimecan mRNA. To further test whether the 3'-untranslated region of mimecan mRNA is a product of the same gene, we performed Southern blot analysis with bovine genomic DNA. The DNA was digested with four different restriction enzymes and was hybridized with a 914-bp PCR-amplified DNA probe (generated by primers P5-P8) and
with 1314-bp PCR-amplified DNA probe (primers P5-P9). The results of this experiment are shown in Fig. 5B. The two overlapping probes span the entire 3′-UTR of mimecan mRNA. Because identical bands on Southern blots were detected with both probes, we concluded that all of these regions belong to the same gene, encoding the mimecan protein.

Inspection of the published mimecan cDNA sequence reveals that of the total size of 2565 bp, one-half is located at the 3′-UTR. This observation prompted us to search for regulatory elements recognized in mammalian downstream mRNAs. Indeed, the hexanucleotide AAUAAA, involved in the cleavage and nuclear polyadenylation of pre-mRNA, was found at three locations: positions 1880, 2200, and 2540. Interestingly, besides the canonical AAUAAA, the sequences AAUUA and AUAUU, shown also to be functional polyadenylation signals (26), were located at positions 1960 and 2000, very close to the AAUAAA hexamer, shown also to be functional polyadenylation signals found in this mRNA. The two bands of 210 and 220 bp were detected in addition to the fully protected band of ∼530 bp and a fully protected band of 880 bp. Because it is known that RNA has a lower mobility than DNA of the same length on denaturing polyacrylamide gel (18), the corrected size should be ∼5% smaller. Moreover, considering the fact that the polyadenylation signal is located 10–30 nucleotides upstream of the actual pre-mRNA cleavage, i.e. RNA cleavage in most genes occurs between the AAUAAA hexamer and so-called G/T cluster (26), the sizes of the bands protected by both 3′-end probes are consistent with the predicted sizes of the RNA transcripts that would result from the polyadenylation signals found in this mRNA. The two bands of ∼210 and 220 bp corresponding to the 3′-end of the shortest RNA transcript are most likely due to staggered RNase cuts. However, it is worth noting that two additional imperfect polyadenylation hexamers are located closely downstream from the AAUAAA site and could also give rise to this heterogeneity. Complete elucidation of the 3′-end site selection in this mRNA will require further study with deletion mutants of the gene. Taken together, the present results give definitive proof that alternative polyadenylation sites are utilized in the generation of mimecan mRNA isoforms. All three transcripts were detectable in bovine cornea and keratocytes.

From the rapidly accumulating data in the literature, faithful 3′-ends appear to be important for gene regulation. Many genes have multiple polyadenylation sites, and in some cases, these are used differentially during development (reviewed in Ref. 28). For example, negative translational regulation of Cae-
norhabditis elegans gene glp-1 (germ line proliferation defective) has been shown to be mediated through sequences in the 3′-UTR (29). In Drosophila, posteriorly localized nanos activity represses translation of the hunchback RNA through sequences in the hunchback 3′-UTR (30). Whether such regulatory elements are located in the 3′-UTR of mimecan mRNA and how the 3′-end choice affects gene function will be important to determine.

Conclusions—In this paper, we have characterized the mechanisms that generate multiple mimecan mRNA transcripts. The demonstrations of a differentially spliced exon and of alternative polyadenylation signal selection suggest the presence of complex mechanisms involved in the regulation of mimecan gene expression. The first consequences of the variety of alternative splicing patterns of mimecan mRNAs are clear: different transcripts are expressed in different tissues. However, what the selective advantage of transcription of different mRNA isoforms might be, how the expression pattern changes during development, and the physiological consequences of this regulated expression of the mimecan gene remain to be determined in future studies.

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Fig. 7. RNase protection analysis using 3′-probes. A, pBS-60 linearized with NdeI as described in the text was used to generate the RNA probe. Lanes: P, undigested probe; M, 100-bp DNA molecular size marker; lane 1, digested probe; and lanes 2 and 3, total RNA from bovine cornea and cultured keratocytes, respectively. B, pBS-62, containing the additional 390 bp at its 3′-end, linearized with NdeI was used to generate the RNA probe. Lanes: P, undigested probe; M, marker; lane 1, poly(A)+ RNA from cultured keratocytes hybridized to the probe; and lane 2, total RNA from cultured keratocytes hybridized to the probe. Arrows indicate the protected bands corresponding to the three RNA transcripts. The band above 100 bp seen in lanes 1 and 2 was also detected with digested probe and, therefore, is not representative of mRNA.