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Short note

Molecular cloning and relative tissue expression of keratocan and mimecan in embryonic quail cornea

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Abstract

We have cloned and sequenced the cDNAs for quail cornea keratan sulfate proteoglycan core proteins, keratocan and mimecan. The deduced quail keratocan protein contains a single conservative amino acid difference from the chick sequence, whereas quail mimecan protein contains a 58 amino acid-long avian-unique sequence that shares no homology with mammalian mimecan. Ribonuclease protection assay of Day 16 embryonic quail tissues reveals that keratocan and lumican are expressed at highest levels in cornea, whereas mimecan mRNA is expressed at a much lower level. Keratocan is expressed only in quail cornea, whereas mimecan is expressed in many different tissues as four transcripts of different sizes. Both lumican and mimecan are expressed at lowest levels in brain, liver and sternum. © 2000 Elsevier Science B.V./International Society of Matrix Biology. All rights reserved.

Keywords: Cornea; Extracellular matrix; Keratocan; Mimecan; Proteoglycans; Quail

1. Introduction

The remarkable transparency of the cornea is a function of the very small, uniform diameter and regular spacing of stromal collagen fibrils and minimal hydration of the extracellular matrix. These properties are regulated in part by the keratan sulfate proteoglycans (KSPGs) of the corneal stroma. Three major corneal KSPGs have been identified in vertebrates: lumican (Blochberger et al., 1992), keratocan (Corpuz et al., 1996; Dunlevy et al., 1998) and mimecan (Funderburgh et al., 1997; Dunlevy et al., 2000). The gene that encodes mimecan (Funderburgh et al., 1997) was originally identified as that for osteoglycin (Madisen et al., 1990). Core protein analyses have shown that KSPGs belong to the small leucine-rich proteoglycan (SLRP) family (Iozzo, 1997). In adult mammals, keratocan mRNA is expressed almost exclusively in cornea and sclera (Corpuz et al., 1996), whereas lumican and mimecan mRNAs are expressed in many different tissues (Corpuz et al., 1996; Funder-

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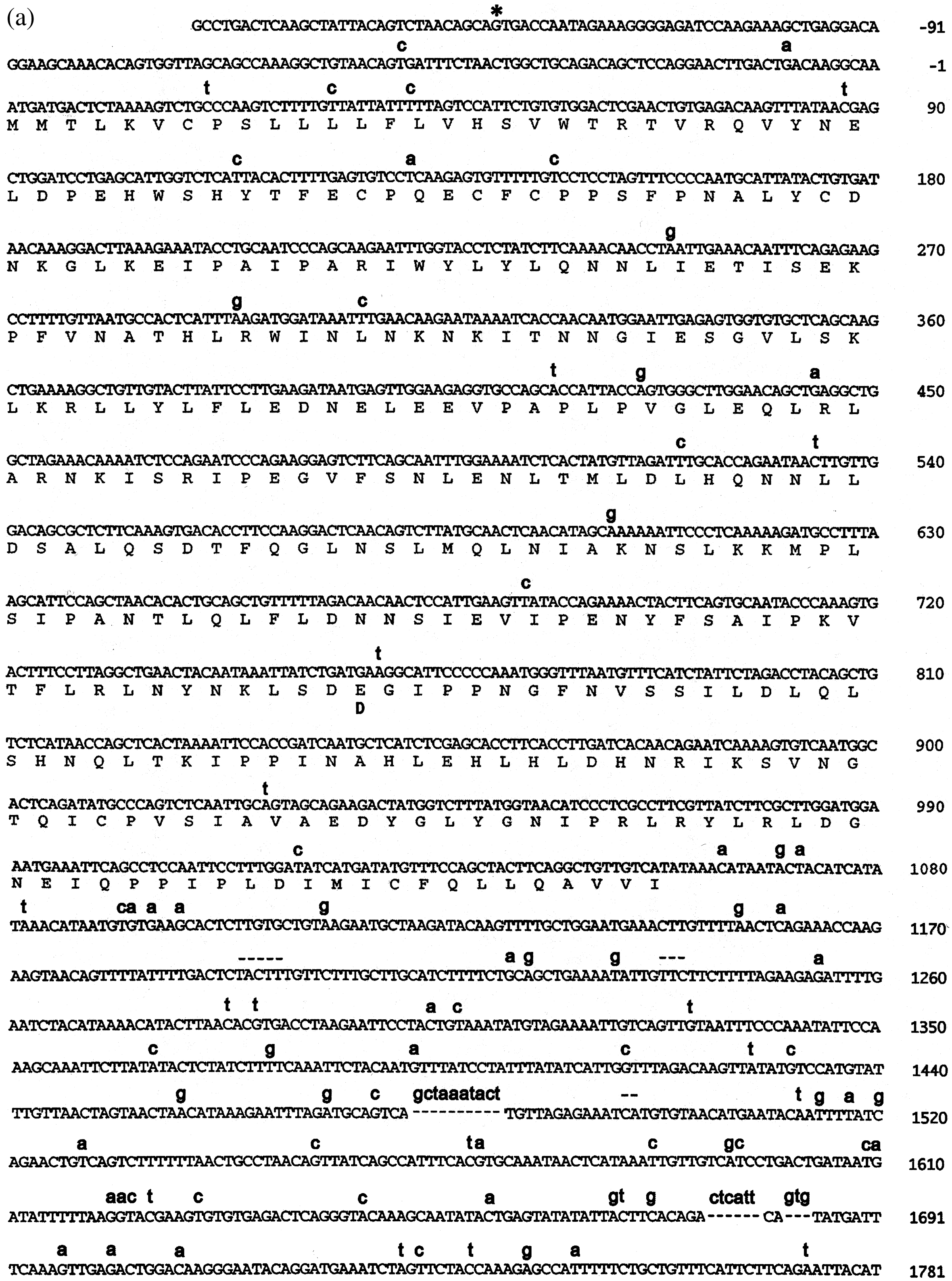

Fig. 1. Nucleotide and deduced amino acid sequences of quail keratocan (a) and mimecan (b), and comparison with chick cDNA. GenBank accession numbers are: keratocan, AF128223 (quail), AF022890 (chick); and mimecan, AF128224 (quail), AF126963 (chick). The nucleotides are numbered beginning at the translation start site. Bold, lowercase letters above the quail nucleotide sequence and bold, uppercase letters below the amino acid sequence provide the corresponding data from the chick sequence and mark sites of variations in nucleotides and amino acids, respectively, from the quail sequence. A hyphen denotes absence of a nucleotide or an amino acid. The 5′-end of the chick sequence is indicated by an asterisk (*). The 3′-ends of both chick keratocan and mimecan cDNAs, which include poly(A) tails, are at quail positions 2306 and 1745, respectively.

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  c         at      c             g  a   c    t            c  c    t
TTTTAATTTTGACACCATTTTTTCCTTCAGCTTTAGGTTGGTCATTCAGTATTTGGTTCATATTTTGATTCTACAGAGACAAAACCAAGA  1871

        g  a  ac                               g  g  c        a   t g   t  g   a         a
AAAGTAATCATTATTTATTTCACAGAGTAGGTGTTTTACAGAAATTACAAAGATAAGTTAATGTATCAATGAAAAACATTAATTTAAGAG  1961

t     gaattta g  g               c a       ca     a  t         g
ATTAA-------A-AGATAAAACCCTCCCTCCTACCACTAACGCTAACTTGTTCACTGAACTAAACAAATATTTTCATAGGAAGTGTATC  2043

     a    t   caac              a              tg      c g     c   ac       a           c
AGTTGGAGCAAATA----AAACTCAGGGCAGTGTTTAGCTATCTGCCAACTCATATAAATACATATACTTCTGGACTCTAGAGATGTCTA  2129

  -a            a    t     t        t              t tg   c     t             -------- --
GTTTTTTTAGATTTCAGTAGCAAACAAACAATATATAAGCAGGCATCTTTACACAAAATCTGGTGGATTTTGATTTTTGTTGTTGTTGAT  2219

         t     g   c   t                 t          t  aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
GTTGTTTGGATGTTT-TTTATTTGTGTTTTGTTTAGTAAAACAATACTGGGAGTTCAATACAAAATTCAAATATTTGAGCCAACAATTCA  2308
CTCTATTCACTGTTAATTTTTGTTATTTCTAATACCCAACAAAGCAGACTATCAATCAGCAATATTATTTTACTTTTCATGTATTTGTAG  2398
ATAGAAGTAATTAGTTGAATCCATTCCTAACATTTTAGAAGAGAATTTAGAATTTAGAACATTCTAAATTTTAAAACTGGCCTCAGCAAC  2488
AATCAATTTAAACTGTATATGTGGCATAGTCTTCTTCTTTGGAGGTTAAACATTTTCTTAAAAGCAGATCTTTAGAAGCATTGAGAATTT  2578
CATTTTCTAACCTTCAGTCTCATACTGTTTGAAAATTGGGACTTATGCTCTCTGGCACTTACAGATATAACCCAGTATAGAAGCCCAGTG  2668
TAGAATTAACTTATTCAGAAATCCTCTTTTGAAAACCTTTATTATCACAGTTCATTGAATATGATTGCTGGAAAATTTTAGCAAAATGTT  2758
TCCTGTACCTTTATATTCATTCTGTATGAAAAAAATACACATCTAAAGTCTCTACAGTATGTATGCATGTTCACGCATGTTCTTTCATAA  2848
TAACATAGTAATTTATTTTTTGAATAAGAAATGATTTTGGGGACAGTACTACTAATTAAATTATAATGATCATCAAAAAAAAAAAAAAAA  2938
AAAAAAAAA                                                                                   2947
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(b)

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                                                                                GTCAAGAGAAT  -523
CATTTGTTCCATCCTTTATTGCATCCTTGGGCAGCATTTCATAATCCTGCTGGATCAGACCTCTAGCCTGCACAATTAATACTCAATTCA  -433
GAGAAAAAGCCATAGCAATTAGAAAGGGTAAATCACCACACAGCAGAAAACTGCCACTTTCACCAAGCAGGATGAACCAGACTGGATAAC  -343
ATTTGTAAAGGTTAAAGCTAAGTACAGCCCCCTGCTCCAGGATATTACCTCTTACCCTAGGGACAGAATGCTGGCAAAGCATTTTTTACC  -253

                                       *  tc  c                   ttcattgttggattg
CCATGGAAAGCACTTTTAAGACTGTCAATCTTGGAAACCTTATTCATTGCAGCAACCTGGATGTAA---------------TTCATAACT  -178

 t                g   tg       a             tt     c     cca      g       a t    at
TCACTCTTTTATCAGTCTACACTGGTTTAACGTATGCATAAGAACAACTGGTTTTAG---CACAGCCACAGAAGAGCCTGGACCCTGCTA  -91

                                                                                 c
AAACGTCACAAAGAAAGTATTCTCCACAGTCAACACACGAGCAGACCTCCATCAGCTCAGTGCAAGCCAAGGACATTGCTCTGTGAGAAA  -1

           g  a   g                                           a
ATGAAGACTTTACAGGCTACTTTTTTCCTGGTTGCATTTGTACCTTTGGTGAAGCCAGCACCTCCTATACAGCAAGATTCACCCAAGTTT  90
M  K  T  L  Q  A  T  F  F  L  V  A  F  V  P  L  V  K  P  A  P  P  I  Q  Q  D  S  P  K  F
                  A
        tgtt   g     t   c                  a                           a
TATGAGTAC---GATACAGATATTGTCACGGGAAGCCTGATCCAGCAGGATTATGAAATGCTGCCCAAGGATGCAATAAAGGATGGAACA  177
Y  E  Y  -  D  T  D  I  V  T  G  S  L  I  Q  Q  D  Y  E  M  L  P  K  D  A  I  K  D  G  T
         V     A     F  A                                               T
                   c                           a
AATGTTTCTCTTGACACTGGCCTGAGACTGCAAGCAGATGACAGCGAGCTGAGTGCCAGACCCACAAAGGATACAAATTTGCCTACTTGT  267
N  V  S  L  D  T  G  L  R  L  Q  A  D  D  S  E  L  S  A  R  P  T  K  D  T  N  L  P  T  C
                  A
                                                                                      c
CTGCTCTGCGTGTGTTTAAGCGGATCAGTGTACTGTGAGGAAATAGATATTGAAGCTGTACCCCCACTGCCAAAGGAAACAGCATATCTT  357
L  L  C  V  C  L  S  G  S  V  Y  C  E  E  I  D  I  E  A  V  P  P  L  P  K  E  T  A  Y  L

        a                                            c                 t
TATGCACGGTTTAATAAAATCAAGAGGATAGCTGTCTCAGATTTTGCTGACATTACTACTTTGAGAAGAATCGATTTTAGTGGGAATATG  447
Y  A  R  F  N  K  I  K  R  I  A  V  S  D  F  A  D  I  T  T  L  R  R  I  D  F  S  G  N  M

                                                                       g     a     t
ATAGAAGAAATAGAAGATGGAGCCTTTTCAAAGCTTTTGTTATTGGAAGAACTTTCTCTTGCTGAAAACCGACTTGTGAAACTGCCAGTT  537
I  E  E  I  E  D  G  A  F  S  K  L  L  L  L  E  E  L  S  L  A  E  N  R  L  V  K  L  P  V

                          c        g                 a              c
CTACCTCCCAAACTAACAACATTTAATGCGAACCAAAACAGAATCAAGAGCAGGGGAATCAAAAACAATGCTTTCAAGAAACTGACAAAT  627
L  P  P  K  L  T  T  F  N  A  N  Q  N  R  I  K  S  R  G  I  K  N  N  A  F  K  K  L  T  N

                          t                 t
TTGGCCTACCTCTACTTAGGACATAACGCACTGGAATCTGTTCCCCTTAACCTACCAGAAAGTCTGCGCATTCTGCACCTTCAGCACAAC  717
L  A  Y  L  Y  L  G  H  N  A  L  E  S  V  P  L  N  L  P  E  S  L  R  I  L  H  L  Q  H  N

  c  c          a                     t        c              a
AATATTACTACAATCACTGATGACACTTTCTGCAAGTCCAATAACACACGGTACATCCGCACGCGTATGGATGAGATAAGGATGGAAGGA  807
N  I  T  T  I  T  D  D  T  F  C  K  S  N  N  T  R  Y  I  R  T  R  M  D  E  I  R  M  E  G
               N
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Fig. 1. (*Continued overleaf*).

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                            t                              g
AATCCAATCCTCCTGGCAAAGCACGTCAATGCTTTCTCCTGCTTGAAAACACTGCCTGTAGGAACATACTACTAGCTACTACTTACATAA   897
N  P  I  L  L  A  K  H  V  N  A  F  S  C  L  K  T  L  P  V  G  T  Y  Y
                                             R
  ca c                     a    g    c                     t      g      c
TTACATGTGTCAAAACTCAAGCATGGTAACAAAAGATCATTCTTATTACTGTTTACATGTTTATATCTTCTCCCCACTAAAGCAATTTTC   987
  c                     g            g                     -- c -      g c   t
CCTGTATACCCAGAACCTTTACTACGTTGAAATGGATTCTCCTCTGAAATAATTATTTCACAGCAGCCATCCTATTTAGGAGTTACAAT   1077
                         g  a cc  - g  g  g  cg    a      t     t  t     t   t tg
GCCACCCAAACTTTCAGAATCTTTTATT-ATTCAAGATATA-AAATGTACACATTCATATACTACCAACATTGAATTCAACAATTACTCA   1165
  t-- tg ag    t      c  c       tt               -     -------     g  t
TTACAAAATCCTGTAAAACCCTGCAAGTGTTACTTCACACACTTTACCAGTTTCCAGAATAATCAATGTATTTAACTGTGATAAACCAAA   1255
                   g a      a    t t    -    cg      c           cttcaaaactgcat
ACATTTCTTGAGTAAGCTAAGTTTTTACTCCAGTCAAAATGGTTTTTGGCCTTAAATGAAATTTTTTCAGTTAAAA --------------   1331
ggaaaaaaatagcatatgaaagaaagttatgtttagagacagtatttctccaagtttaacttcactgaagtttacttaaaaacttggttt
------------------------------------------------------------------------------------------   1331
gtggaaaa         a   tt   g     tg          a                       g  c       ---  a
---------TGTGAAGAATTTTT-ATCAAGCTTAGCATACCAAGCAACAAAGTGAATGGAACATTTCAAGTATGTATCAAATCATCTTTTT   1412
 t                  c        t          c          a                                 a
ACATTTTTCTTTCAAAATAATCTAAATGCCATTATATATTAAAATTGAATGTATGCATATAAACACATTCAACTGTATATGTTACATAAC   1502
      t    a          g   t              tat         cc              a
TATTTTT-ACACTTTATATTTTTAATTCAAATATATAATATA---TATAGACATTTTGTTCAGCTGTGCTCATAGGAGTCATTCTTGCCA   1588
   -- cc            ca   c     c                    g     att     ac aagac      ag
CTTCCATTTTAATACAACAGTCAGTATGCAGTAGGGTATGACTCAAGCACTTATTTTT--CTTTA--T----TATGACAT--ACTGAGCC   1668
   c                                               g  a c g caaaaaaaaaaaaaaaaaaa

TTTTTCCACTGCTTTCTCTTGACAGGAAACATGACAATAAAGTTAACAACAGATTCAGTTAATGCTCTTACCTGTAAATGAATACATGTT   1758
CAACTAGAAGTCAGATCGAAGAAACAAATACCTCTGTACCCAATACTTACACAATATACAGAAACATAACATATCTTTAGAATTCTAACT   1848
TAATACTATTCTGTTAACCTCAAACAAGTTAAACAAGAATTATTTCACTCAACTCCTTTAAACCAGATGTTGAAAAGAGAAAGTGTGTGC   1938
AGACCTCAAAGCACATTCATAATTTATTACAGAAAACAGTATTTCCATTTACTTCACTACACACTTCTCCTGCCTTGTCAGCTTATCTCC   2028
TCAAATACCAACCACAAGCTTAAACTCTTCTAACAGACAAATGCCCAAAAAAGTGCTAATTACCGTGACTAAGAACATCGTACTAACCTT   2118
GTAGCTAGTGTAGATTTCTAATTTGTAGAAATAGTTAAGCACAAATACACGTATCACTGCCATCAAAAAGAATACTGATAAAATATTTTG   2208
TATTATGTCTATCTTATTTAGCTACACCCCCATTTGTTAGTGCTTGTGCTCTAGAGAATTATTACTACGTTTAACATACTGATGCTCTGC   2298
CACTATAAGTGCTGATATCTAGGTGACTTTGTCAAGCACCAACTAAGGGTTTGTCTTTCTTGATAGAAGATGCAATTATGAAATTCAAAA   2388
CTAAATATTTTCACCCTGAACCTGTTAGTCAAGAAATAGCATAGCAGTCCCTATCTTTTCCATTTAAATGCTTACTGTATGATAATGTAA   2478
ACATTTGTTTACCTTGTATGGATTCTCAGCTCAATAAATAATTACATACATGATA                                    2533
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Fig. 1. (*Continued*).

burgh et al., 1997). Mimecan is transcribed into several mRNAs of different sizes that give rise to the same amino acid sequence (Funderburgh et al., 1997; Tasheva et al., 1997).

KSPGs increase in chick cornea during embryonic development (Hart, 1976; Funderburgh et al., 1986; Takahashi et al., 1999). Lumican, keratocan and mimecan mRNA levels are highest during early development, but decline as the cornea becomes transparent (Dunlevy et al., 2000). In mouse embryos, keratocan mRNA is detected in neural crest cells migrating toward the cornea and sclera at E13.5 (embryonic Day 13.5), then becomes restricted to corneal stromal cells by E18.5 (Liu et al., 1998). However, very little is known about when neural crest cells begin to make the other corneal KSPG core proteins, or whether there are regional differences across the cornea in core protein synthesis, polymerization, accumulation, distribution or turnover. Quail/chick chimeric corneas provide an excellent experimental system in which to test the functional roles of individual proteoglycans in specific embryonic regions (e.g. by transfecting a quail neural crest graft with a quail-specific antisense RNA before transplantation into a chick embryo host). To obtain quail-specific molecular probes for such studies, we have cloned and now report the cDNA and deduced amino acid sequences for quail keratocan and mimecan, their comparison with the corresponding chick sequences, and their expression in quail cornea and other tissues. (Lumican and another corneal proteoglycan, decorin, are presented in Corpuz et al., 2000) Our results confirm the presence of an avian-specific mimecan splice variant that gives rise to a unique amino acid sequence (Dunlevy et al., 2000), and demonstrate that in birds, as in mice, keratocan appears to be expressed only in the cornea.

2. Materials and methods

All the experimental procedures used here have been described previously (Corpuz et al., 2000). The quail (*Coturnix coturnix japonica*) cornea/sclera cDNA library was screened with chick keratocan and mimecan cDNA probes (Dunlevy et al., 1998, 2000). Three clones for each of the two KSPGs were sequenced. The quail primers used for the generation of DNA templates are: keratocan, sense: 5′-GCTAACACACTGCAGCTGT-3′ (position 640), antisense: 5′-TCTTACAGCACAAGAGTGC-3′ (position 1116);

and mimecan, sense: 5′-GATACAGATATTGT-CACGGG-3′ (position 100), antisense: 5′-ATTC-CAGTGCGTTATGTCC-3′ (position 664). The polymerase chain reactions (PCRs) generated 477-bp and 565-bp PCR products for keratocan and mimecan, respectively, which were used to synthesize antisense riboprobes for ribonuclease protection assay (RPA) studies.

In conducting RPA experiments, we attempted to use both quail β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal controls. However, we found their mRNA expression to be quite variable from tissue to tissue (Fig. 2). We therefore used 28S and 18S ribosomal RNAs as loading controls, for ethidium bromide staining reveals their uniform expression across all quail tissues. Preparation of β-actin and GAPDH antisense riboprobes has been described (Corpuz et al., 2000).

3. Results and discussion

The cDNA and deduced amino acid sequences for quail keratocan and mimecan, including comparisons with published chick sequences (Dunlevy et al., 1998, 2000), are shown in Fig. 1. The quail keratocan clone (Fig. 1a) is 3108 bp long and encodes a protein containing 353 amino acids. At the nucleotide level, the quail coding region is 98% identical to the chick sequence (Dunlevy et al., 1998), resulting in a single conservative amino acid difference at residue 253. In contrast, quail and chick 3′UTRs are only 86% identical. The three quail keratocan clones sequenced are identical in the 5′UTR and translation regions, but are variable in the 3′UTR (data for two other clones not shown). Variable 3′UTRs were also found in chick (Dunlevy et al., 1998).

The quail mimecan clone (Fig. 1b) is 3066 bp long and the derived protein contains 293 amino acids. Quail mimecan shares 96% nucleotide identity with the chick sequence in the coding region, but only 76% in the 3′UTR (Dunlevy et al., 2000). This results in 97% identity at the protein level: one amino acid difference (residue 7) in the putative signal peptide region, six differences in the amino-terminal region of the mature protein, and two amino acid differences toward the carboxy-terminal end. Most significantly, the 58 amino acids (residues 28–85) immediately following the signal peptide in quail are 94% identical to the corresponding 59-amino acid region in chick [previously reported as containing 60 amino acids in Dunlevy et al. (2000)], whereas this region shows no homology to corresponding bovine, human and mouse mimecan regions. The nucleotide sequence in this avian-unique cDNA (quail nucleotides 82–255; chick nucleotides 313–489) is 46% (quail) and 47% (chick) identical to bovine intron 4 nucleotides 13 907–14 096 (Accession No. AF105150), suggesting that birds have novel splice sites in their mimecan gene that replace the 3′ end of mammalian exon 3 and all of exon 4 with a new avian exon 4 from intron sequences located upstream of exon 5 (Dunlevy et al., 2000). This results in an avian-specific amino acid sequence that is distinct from the mammalian sequence.

RPA analysis demonstrates that keratocan and lumican mRNAs are expressed most highly in Day 16

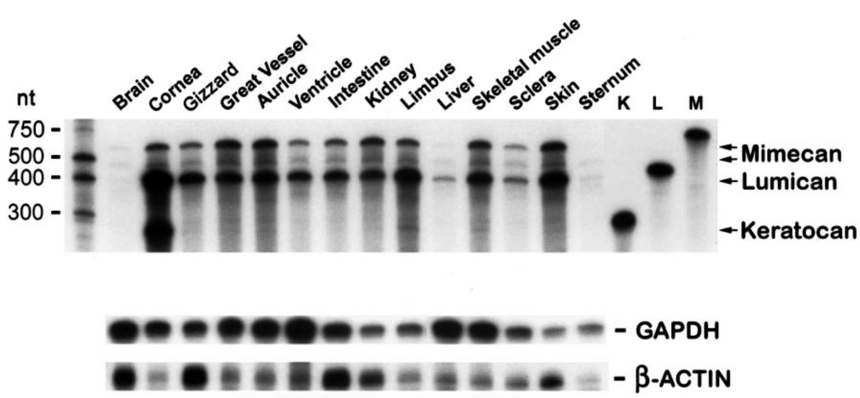

Fig. 2. Relative expression of quail keratocan, lumican and mimecan genes in various tissues as determined by ribonuclease protection assay. Total RNA (2.5 μg) from each Day 16 quail embryo tissue was hybridized with [α-^{32}P]UTP-labeled keratocan, lumican and mimecan antisense riboprobes, and the electrophoresed protected fragments were exposed to Hyperfilm with intensifying screen for 18 h. Arrows show the keratocan, lumican and mimecan transcripts. RNA size markers (RNA Century Marker-Plus, Ambion) are shown on the left. Unreacted keratocan (K, 280 nt), lumican (L, 401 nt) and mimecan (M, 565 nt) antisense riboprobes, 1500 c.p.m. each, are shown on the right. Ribosomal RNAs (28S and 18S) serve as loading controls. GAPDH and β-actin mRNA expression profiles are included to show their variable expression in different tissues and, therefore, their inappropriateness as loading controls in this study.

embryonic quail cornea, whereas mimecan mRNA is significantly less expressed (Fig. 2). Quail keratocan expression appears to be limited exclusively to the cornea. No detectable message is observed in the cartilaginous quail sclera, in agreement with data from embryonic Day 18.5 and adult mouse sclera (Liu et al., 1998). However, keratocan mRNA is found in the non-cartilaginous fibrous bovine sclera (Corpuz et al., 1996). Conversely, lumican and mimecan mRNAs are expressed in multiple quail tissues, with lumican generally more highly expressed than mimecan. Both transcripts are low or undetectable in quail brain, liver and sternum. Our quail data agree with previous studies on chick cornea, which showed a much lower level of mimecan mRNA compared to lumican and keratocan mRNAs (Dunlevy et al., 2000), but not with results in bovine cornea, which showed higher levels of both mimecan and lumican mRNAs compared to keratocan mRNA (Funderburgh et al., 1997). This study is the first to assess the avian expression of keratocan and mimecan transcripts in multiple tissues. It shows that in most quail tissues four mimecan transcripts are detectable by RPA: one major and one minor transcript, as well as two minimally detectable transcripts that migrate with and just below the lumican transcript. Northern blotting and RPA studies have also identified multiple mimecan transcripts in bovine cornea and various other tissues (Funderburgh et al., 1997; Tasheva et al., 1997).

Acknowledgements

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