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Characterization of the gene for the membrane and secretory form of the IgM heavy-chain constant region gene (Cμ) of the cow (*Bos taurus*)

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SUMMARY

Our present understanding of the evolution of immunoglobulins is derived from a few vertebrate species. In order to obtain additional information on the development of the humoral immune system, we cloned and determined the nucleotide sequence of the bovine cDNA and genomic IgM heavy-chain constant region gene (Cμ). The gene contains four constant region domain-encoding exons (CH1 to CH4) and two exons encoding the transmembrane domain (TM1, TM2), expressed in the membrane-bound receptor form of the IgM. The sequence of a cDNA clone encoding the 3′ portion of the membrane form of the μ-chain revealed that the TM1 exon is spliced to the CH4 exon, as occurs in other mammals. Comparison of deduced amino acid sequence data from different vertebrates revealed a high similarity to sheep Cμ (88%) and a lower degree of similarity to pig (62%), rat (62%), rabbit (58%) human (56%), hamster (55%), mouse (54%), chicken (28%) and horned shark (22%) Cμ.

INTRODUCTION

Immunoglobulins are divided into five major classes, IgM, IgG, IgA, IgD and IgE, a classification which is based on antigenic differences in the heavy-chain constant regions as defined by serology. IgM is present in the sera of all vertebrates[1,2] and is the first antibody produced in response to antigen. The IgM molecule is usually a pentamer but a tetrameric configuration is found in Actinopterygii such as bowfin,[3] channel catfish,[4] rainbow trout[5] and Atlantic cod[6] and hexameric forms in amphibians.[7] Due to its multiple binding sites, IgM has a high binding avidity for micro-organisms such as viruses, which are made of identical capsid subunits, and bacterial flagella which are made up of repetitive structures.

In cattle and other ruminants, IgM has been shown to be important in combating septicaemia when administered passively to calves[8] and as an important bactericidal antibody against mastitis.[9] Both colostrum and mature milk from cows contain appreciable amounts of IgM[10,11] and have been used as starting material from which to purify and biochemically to characterize IgM.[12]

The gene or the corresponding cDNA of the IgM heavy-chain constant region (Cμ) has been characterized in many species including human,[13,14] rabbit,[15,16] mouse,[17] rat[18] (incomplete cDNA sequence), hamster[19] (cDNA), sheep[20] (cDNA), pig[21] (cDNA), horned shark,[22] chicken,[23] channel catfish[24] and rainbow trout.[25] A partial nucleotide sequence of the secreted form of bovine Cμ cDNA has previously also been determined.[26]

Diagnostic tests for IgM responses require appropriate reagents as well as purified IgM. The availability of sequence data on IgM permits the making of specific peptides which could be an alternative method for immunization for obtaining pure and sufficient amounts of anti-IgM-reagents. Furthermore, the phylogeny is based on comparison of amino acid or nucleotide sequences from different species[27–29] and it may thus be of importance to characterize also the bovine Cμ gene.

MATERIALS AND METHODS

The recombinant phage EMBL4 containing the bovine Cμ gene (phage clone 15), has previously been identified and restriction mapped and was a kind gift from Dr Katherine Knight (Loyola University, Chicago, IL).[30] Digestion of this clone with restriction enzymes *Xba*I and *Xho*I (Boehringer Mannheim Biochemicals Mannheim, Germany) gave rise to the main part of the Cμ gene in a 2·6-kb fragment. This fragment was subcloned into pBLUESCRIPT KS (±) (STRATAGENE, La Jolla, CA), transferred into the *Escherichia coli* strain JM109 (heat-shock) and sequenced by both the Taq Dye Deoxy Terminator cycle sequencing (ABI model 377 sequencing station) and the nucleotide chain termination method using the fmol® DNA sequencing System (Promega, Madison, WI). The two exons encoding the transmembrane domain (TM1, TM2) were amplified from both a cDNA library and the phage clone 15 by polymerase chain reaction (PCR), using a sense primer from the CH4 domain

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(one of four constant regions, CH1 to CH4) of bovine Cμ and an antisense primer from the TM2 of the sheep Cμm.[20] The PCR product was cloned into the pGEM-T vector (Promega), transferred into *E. coli* and sequenced. The primers for sequencing were vector primers, T7, SP6, T3 and additional internal primers (Table 1, Fig. 1). All of these internal primers, with the exception of the TM2, are bovine Cμ-specific primers and are designed based on our sequence from the genomic Cμ.

Screening of cDNA phage library

Screening of a bovine spleen cDNA phage library purchased from Uni-Zap XR (STRATAGENE, La Jolla, CA), was performed according to instructions from the manufacturer using a 2·6-kb *Xho*I–*Xba*I bovine Cμ DNA fragment as a probe (phage clone 15).[30] The primers for sequencing of the excised phagemid (Cμ-containing) were vector primers, T7, T3 and additional internal primers (Table 1., Fig. 1).

Southern blot

Southern blot analysis was performed after single- and double-digestion of different bovine genomic DNA samples (Swedish red and white strain) with the restriction enzymes *Eco*RI, *Hin*dIII and *Taq*I. The digested samples were separated in 0·8% agarose gels and transferred to Hybond membranes (Amersham, Slough, UK). Hybridizations and washing were carried out as previously described[31] although the methods for probe labelling and detection were modified.

Probe

Labelling of the probe was carried out by PCR. In the PCR reaction, we used 1 mg template [pBLUESCRIPT KS (±) with an insert of a 2·6-kb *Xho*I–*Xba*I bovine Cμ DNA fragment), 1 ml 1·0 mM dATP, 1 ml 1·0 mM dCTP, 1 ml 1·0 mM dGTP, 0·95 ml 1·0 mM dTTP, 0·5 ml 0·125 mM Digoxigenin-11-dUTP (Boehringer Mannheim), 4 ml 25 mM MgCl, 5 ml PCR buffer, 5 U Taq DNA polymerase (Promega) and 5 ml 10 pM T7 and T3 primers. The final volume was adjusted to 50 ml with dH_2O. After an initial denaturation at 95° for 3 min, 30 cycles were performed consisting of 95° for 1 min, 55° for 1 min and 72° for 1 min followed by a final extension at 72° for 7 min.

Detection

Chemiluminescent detection was carried out with a DIG Luminescent Detection Kit according to instructions from the manufacturer (Boehringer Mannheim). The hybridized bands were detected by multiple film exposures (Lumifilm, Boehringer Mannheim) in 2 hr.

Table 1. The bovine internal Cμ primers which are used in amplifying and sequencing. The approximate positions of these are shown in Fig. 1

Primer*	Oligonucleotide 5′ → 3′
CH1S	AATTCAGTCAGCTTCTCCTGGAA
CH1As	GCGATCACCCTCACGGTGCCGACGGTCTTT
CH2S	TGCAGTCCTCACCCATAACCTT
CH2–CH3As	GGTCTGGGGCGGTGTCTGCA
CH3As	AAGGATGGGGGGATGGTGAA
CH3S	CACCTCAACGACACCTTCAG
CH4S	CCTGACGGTGGCCGAGGAGGACTGGAGCAAA
IS	CACCCCCGCCTCCCCACTCGCTTC
IIS	CCAGGCTCACACACTCGGAT
IIIS	CACTCTCCCTGGGTCTCAGATGTCTAT
IVS	ACCAGAGACAGGACGCCCCACCA
TM1S	CAGCACCACGGTCACCCTGTTCAA
TM2As	TATTACTGCGGGCTCCATGCTTG

*As, antisense; S, sense.

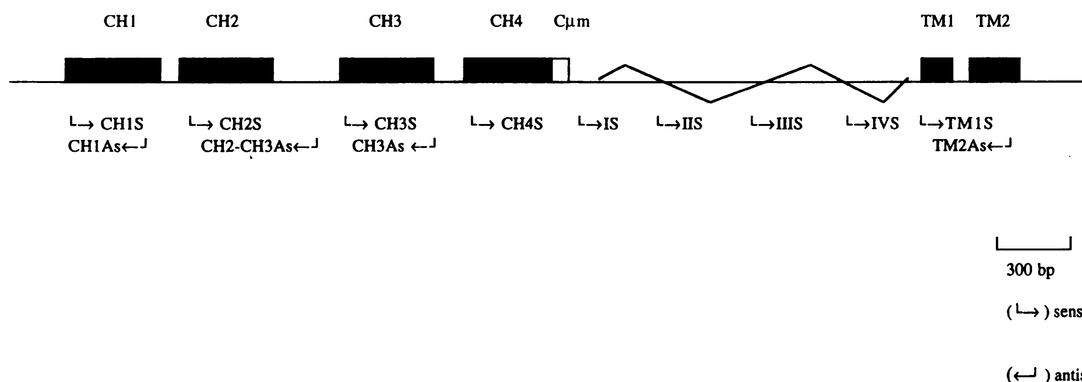

Figure 1. The approximate position of the bovine internal primers.

```
        10                  30                  50                  70                  90                  110
gtcctggtcaacagtgggctggcctcagtgatctggttgtgctgaggactgggggcctgagtgtgtatagtcttattgatgaccccagacccccagagcaggccccaggtggctgagctg  120
tgggcagtggagggtgggctggtagggctgagtgtgccctccactccactgtcccagagagaaggtagagctgcccacacccccaaccagcaggatgcctcacacccccctcttctcctg  240
tgtcctctctcgggtccccaGAAGGTGAATCACACCCGAAAGTCTTCCCCCTGGTGTCCTGCGTGAGCTCGCCATCCGATGAGAGCACGGTGGCCCTGGGCTGCCTGGCCCGGGACTTCG  360
                     E  G  E  S  H  P  K  V  F  P  L  V  S  C  V  S  S  P  S  D  E  S  T  V  A  L  G  C  L  A  R  D  F  V
TGCCCAATTCAGTCAGCTTCTCCTGGAAGTTCAACAACAGCACAGTCAGCAGCGAGAGATTCTGGACCTTCCCCGAAGTCCTGAGGGACGGCTTGTGGTCGGCCTCCTCTCAGGTGGTCC  480
  P  N  S  V  S  F  S  W  K  F  N  N  S  T  V  S  S  E  R  F  W  T  F  P  E  V  L  R  D  G  L  W  S  A  S  S  Q  V  V  L
TGCCCTCCTCAAGCGCCTTTCAAGGCCCGGATGACTACCTGGTGTGCGAAGTCCAGCACCCCAAGGGAGGAAAGACCGTCGGCACCGTGAGGGTGATCGCTACAAgtgagtcgggcccgt  600
  P  S  S  S  A  F  Q  G  P  D  D  Y  L  V  C  E  V  Q  H  P  K  G  G  K  T  V  G  T  V  R  V  I  A  T
cccgtggttgggtgcaggggagggtccaggccccgctgacctcttgtccttctctgcagAGGCGGAAGTGCTGTCCCCAGTCGTGAGTGTCTTTGTCCCGCCTCGCAACAGCCTCTCTGG  720
                                                              A  E  V  L  S  P  V  V  S  V  F  V  P  P  R  N  S  L  S  G
TGACGGCAATAGCAAGTCCAGCCTCATCTGCCAGGCCACGGACTTCAGCCCCAAACAGATCTCGTTGTCCTGGTTTCGTGATGGAAAGCGGATAGTGTCTGGAATTTCTGAAGGCCAGGT  840
 D  G  N  S  K  S  S  L  I  C  Q  A  T  D  F  S  P  K  Q  I  S  L  S  W  F  R  D  G  K  R  I  V  S  G  I  S  E  G  Q  V
GGAGACTGTGCAGTCCTCACCCATAACCTTCAGGGCCTACAGCATGCTGACCATCACGGAGAGAGACTGGCTCAGCCAGAACGCGTACACCTGCCAGGTAGAACACAACAAGGAAACCTT  960
 E  T  V  Q  S  S  P  I  T  F  R  A  Y  S  M  L  T  I  T  E  R  D  W  L  S  Q  N  A  Y  T  C  Q  V  E  H  N  K  E  T  F
CCAGAAGAACGTGTCCTCCTCATGTGATGTTGgtgagtgcagccctggggggcgggcgctcaccctcaggtctgcagacaccgccccagacctgccagctgctccctgagccttggcttc  1080
 Q  K  N  V  S  S  S  C  D  V  A
ccagagcggcaagggcaggaggggctgtgcagggcggctgggggccggcacccctccaacagggccccaggttcacaggggactcagccaagtgggccctggtctttgggcggacctctc  1200
ccttcacctgatttcactccaagcaactctctcccacctccagCCCACCATCTCCGATCGGGGTCTTCACCATCCCCCCATCCTTCGCCGACATCTTCCTCACGAAGTCAGCCAAGCTG  1320
                                             P  P  S  P  I  G  V  F  T  I  P  P  S  F  A  D  I  F  L  T  K  S  A  K  L
TCCTGTCTGGTCACAAACCTGGCCTCCTATGATGGCCTGAACATCAGCTGGTCCCGTCAGAACGCCAAGGCCCTGGAGACCCACACGTATTTTGAGCGACACCTCAACGACACCTTCAGC  1440
S  C  L  V  T  N  L  A  S  Y  D  G  L  N  I  S  W  S  R  Q  N  A  K  A  L  E  T  H  T  Y  F  E  R  H  L  N  D  T  F  S
GCCCGGGGTGAGGCCTCGGTCTGCTCGGAGGACTGGGAGTCCGGAGAGGAGTTCACGTGCACAGTGGCCCACTCGGACCTGCCCTTCCCAGAAAAGAACGCCGTCTCCAAGCCCAAAGgt  1560
A  R  G  E  A  S  V  C  S  E  D  W  E  S  G  E  E  F  T  C  T  V  A  H  S  D  L  P  F  P  E  K  N  A  V  S  K  P  K  D
aggccctgccctgcccctgccctccaccccagaccctcccccggcttctgccttctgaggacagcaggctagggccagcagaggtcccacactcgccgatctcaccactgtgacccccctc  1680
ccccacccagACGTCGCCATGAAACCGCCGTCCGTGTACCTGCTGCCTCCAACGCGGGAACAGCTGAGCCTGCGCGAGTCGGCCTCCGTCACCTGCCTGGTGAAGGCGTTCGCGCCCGCG  1800
            V  A  M  K  P  P  S  V  Y  L  L  P  P  T  R  E  Q  L  S  L  R  E  S  A  S  V  T  C  L  V  K  A  F  A  P  A
GACGTGTTCGTGCAGTGGCTGCAGAGGGGGGAGCCCGTGACCAAGAGCAAGTACGTGACCAGCGCCCCGGCGCCCGAGCCCCAGGACCCCAGCGTGGTGTACTTTGTGCACAGCATCCTG  1920
D  V  F  V  Q  W  L  Q  R  G  E  P  V  T  K  S  K  Y  V  T  S  A  R  A  P  E  P  Q  D  P  S  V  V  Y  F  V  H  S  I  L
ACGGTGGCCGAGGAGGACTGGAGCAAAGGGGAGACCTACACCTGCGTCGTGCACGAAGCCCTGCCCCACATGGTCACCGAGCGGACCGTGGACAAGTCCACCGGTAAACCCACCCTGTAC  2040
T  V  A  E  E  D  W  S  K  G  E  T  Y  T  C  V  V  H  E  A  L  P  H  M  V  T  E  R  T  V  D  K  S  T  G  K  P  T  L  Y
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N  V  S  L  V  L  S  D  T  A  S  T  C  Y  *
tgctgcgttctctaaaaattagaaataaaaagatccattcaaagtgtggttgtgagtgagcaaagctctccctgctaggcccgtggctctctgcctcaccttgcaagaaccacctccatc  2280
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aggcagacatctgcccccagggcttgtgcctcactggctagggagccgacctcaaaccaccagagacaggacgccccaccaccgctgtcagccccaagtggccctgagtcctccagaggg  3600
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                                   E  G  E  V  S  A  E  E  E  G  F  E  N  L  N  T  M  A  S  T  F  I  V  L  F  L  L  S  L  F
TCTACAGCACCACGGTCACCCTGTTCAAGgtagccgcatcgtcccgaggagggggtgaggccacagagccccggggccgcagatgcccacgcacgcactcacgctgtctctgtcgcctgc  4200
  Y  S  T  T  V  T  L  F  K
agGTGAAGTGAtggccagccaagaacatggggcacggagacggaacacgaggggctgccttggggccgggtccctggcctatgtggcttgtccgcttgtactgaaattttccctgcgtc  4320
  V  K  *
ctctccagcttcaagctgtaagaaactggcttttctcggagcagctgagtgccatggccaagcatggagcccgcagtaatagcctccacctggccctgctttgcaatgtcgcatttgtgg  4440
ccttgaaataaa  4452
```

Figure 2. Nucleotide and inferred amino acid sequence of the bovine IgM heavy-chain constant region gene. The exons encoding the four secretory domains (CH1, CH2, CH3 and CH4) and the two C-terminal transmembrane domains (TM1 and TM2) are indicated. The inferred amino acid sequence (single letter code) is indicated under the first base of each codon, and stop codons are indicated by an asterisk. The splice sites, inferred by comparison with the cDNA sequence are indicated by small letters. The secretory signal is indicated by underlining letters, following the CH4. The polyadenylation signal sequences for the secretory and transmembrane mRNAs are double underlined.

```
───────────────── CH4 ─────────────────┬──────── TM1 ──────────
GAGACCTACACCTGCGTCGTGCACGAAGCCCTGCCCCACATGGTCACCGAGCGGACCGTGGACAAGTCCACCGAGGGGGAGGTGAGCGCCGAGGAAGAAG 100
E  T  Y  T  C  V  V  H  E  A  L  P  H  M  V  T  E  R  T  V  D  K  S  T  E  G  E  V  S  A  E  E  E  G

───────────────── TM1 ──────────────────────────────────┬ TM2 ┐
GCTTTGAAAACCTCAACACCATGGCCTCCACCTTCATCGTGCTCTTCCTCCTGAGCCTCTTCTACAGCACCACGGTCACCCTGTTCAAGGTGAAGTGATG 200
 F  E  N  L  N  T  M  A  S  T  F  I  V  L  F  L  L  S  L  F  Y  S  T  T  V  T  L  F  K  V  K  *

GCCAGCCAAGAACATGGGGCACCGGAGACGGAACACGAGGGGCTGCCTTGGGGCCGGGTCCCTGGCCTATGTGGCTTGTCCGCTTGTACTGAAATTTTCC 300

CTGCGTCCTCTCCAGCTTCAAGCTGTAAGAAACTGGCTTTTCTCGGAGCAGCTGAGTGCCATGGCCAAGCATGGAGCCCGCAGTAATAGGCTCCACCTGG 400

CCCTGCTTTGCAATGTCGCATTTGTGGCCTTGAAATAAA 439
```

Figure 3. The 3′ portion of the cDNA Cμm. The exons encoding the two C-terminal transmembrane domains (TM1 and TM2) and part of the CH4 are indicated.

```
        1                                                   47
Bovine  ......EGEV SAEEEGFENL NTMASTFIVL FLLSLFYSTT VTLFKVK
 Sheep  ......---- ---------- ---------- ---------- -------
   Pig  ......---- ---------- ---------- ---------- -------
 Human  .......--- --D------- WAT------- ---------- -------
 Mouse  .......--- N--------- WTT------- ---------- -------
Rabbit  .......--- G--------- WTT------- ---------- -------
 Shark  ...SSDHIWI EDN--ESG-I WTT------- ----I---AA -------

        |_______________________________________________|_|
                             TM 1                      TM 2
```

Figure 4. Pile-up alignment of vertebrate Cμ amino acid sequences of the two C-terminal transmembrane domains (TM1 and TM2). Dots indicate gaps inserted to maximize homology and dashes indicate amino acid identity to bovine Cμ.

Pulsed-field gel electrophoresis

High molecular weight DNA was prepared from bovine peripheral blood as described previously.[31] The DNA sample blocks were equilibrated three times for 30 min with the restriction enzyme incubation buffer (Boehringer Mannheim buffer H for *Mlu*I) and digested with 75 U *Mlu*I (Boehringer Mannheim, New England Biolabs (Beverly, MA) and Amersham). The digested samples were electrophoresed for 40 hr at 400 V with a pulse time of 30 seconds in a 1% agarose gel, 0·5 × Tris Boric acid, EDTA (TBE) buffer using a TAFE apparatus (Beckman Instruments, Palo Alto, CA). *Saccharomyces cerevisiae* chromosomes (Bio-Rad, Richmond, CA) were used as size markers.

Phylogenetic analysis

To construct the phylogenetic tree, we used the complete amino acid sequences of the IgM heavy-chain constant region of 23 species (for name and references see Fig. 5). The analysis was performed using CLUSTAL W with bootstrap 1000.[32]

RESULTS

In order to sequence the bovine μ gene, we screened a bovine recombinant phage cDNA library. Ten different clones containing Cμ were identified and one clone was selected for sequencing. Comparison of the sequence with a previously described, partial sequence of bovine Cμ cDNA[26] revealed a membrane form of Cμ (Fig. 2). To determine the nucleotide sequence of the secretory form of the Cμ and exon–intron boundaries, genomic DNA contained in a phage clone was sequenced. The gene contained four constant region domain-encoding exons (CH1 to CH4) and two exons encoding the transmembrane domain (TM1, TM2). Comparison of the sequence of the complete cDNA and genomic Cμm revealed a 59-base pair (bp) secretory tail. The distance between the Cμs and the TM1, was approximately 2·0 kilobases (kb) (Fig. 2). The TM1 exon was spliced into a cryptic site within the CH4 exon (CG/GTAAAC) (Fig. 3) which is highly conserved among mammals.[33,34] Deduced amino acid sequence of the bovine Cμ transmembrane domains (TM1 and TM2) and comparison with its counterpart in other vertebrates, revealed a significant degree of conservation among different species (Fig. 4). The TM2 exon was 250 bp long but only six bases were translated into amino acids and the rest formed the 3′ untranslated region. This correlates well with data from other species. The complete amino acid sequence of bovine Cμs, showed a high similarity to sheep Cμs (88%) and a lower degree of similarity to pig (62%), rat (62%), rabbit (58%), human (56%), hamster (55%), mouse (54%), chicken (28%), shark (22%) and rainbow trout (20%) Cμs, which suggests an evolutionary tree as depicted in Fig. 5.

Southern blot analysis of different bovine genomic DNA samples digested with *Bam*HI, *Hin*dIII and *Taq*I, respectively, revealed multiple hybridizing bands in each sample. In some samples, as exemplified by the *Taq*I-digested samples in Fig. 6, there were two major bands of two sizes, either (4·3 and 7·0 kb) or (4·3 and 6·1 kb), whereas in some other samples, there were at least three bands (Fig. 6, Table 2). The 7·0-kb band was allelic to the 6·1-kb band and corresponded to our sequenced gene and previously published restriction maps[30] suggesting that it constitutes the functional μ gene. The 4·3-kb band was a polymorphic allele to the 5·0- or 8·3-kb bands which appeared to constitute a second Cμ gene, possibly representing the previously described ψCμ gene.[35] We double-digested five selected samples with *Bam*HI–*Hin*dIII, *Xba*I–*Bam*HI, *Hin*dIII–*Taq*I *and* *Xba*I–*Taq*I, respectively (Fig. 7). In the samples digested with *Bam*HI–*Hin*dIII and *Xba*I–*Bam*HI, there were three constant bands (5·5, 3·7, 2·9 kb) and (2·6, 3·5, 3·7 kb), respectively, of which the 5·5-kb band of the first and the 2·6-kb band of the second digestion corresponded to our sequenced gene and previously published restriction maps,[29] suggesting that it also constituted the functional μ gene and two other remaining bands in each digestion, represented alleles of the ψCμ gene. In the samples digested with *Taq*I–*Hin*dIII, we observed one major band (3·2 kb) and two weaker bands (Fig. 7, Table 2) where we

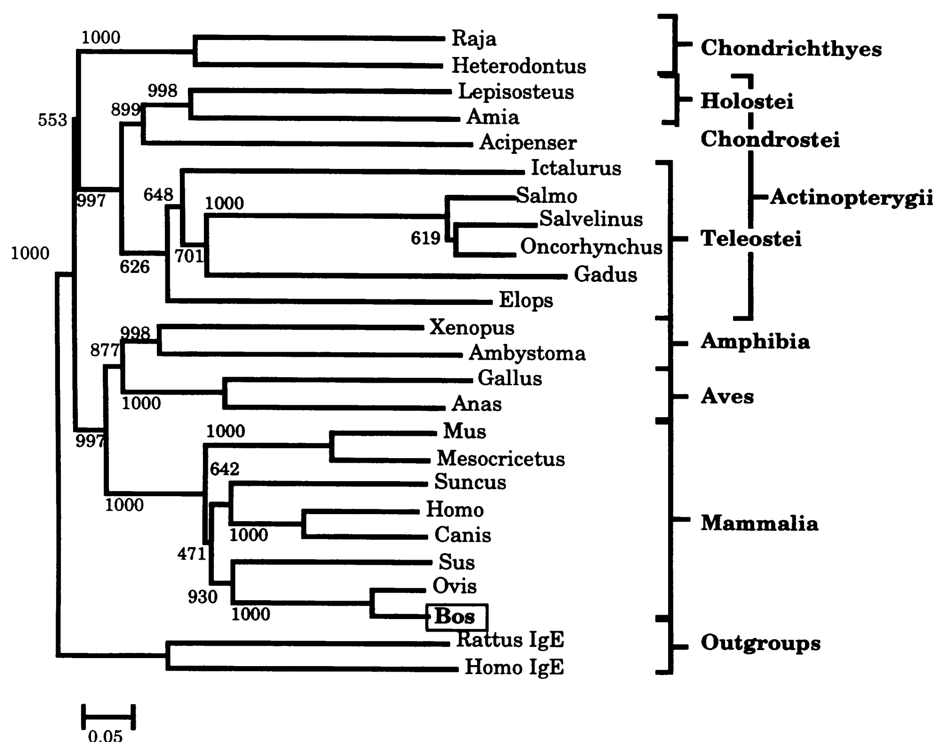

Figure 5. Cμ gene phylogeny in vertebrates. Homology searching, based on the complete amino acid sequences of IgM heavy-chain constant region of 23 species. The name and accession number of these data are: *Raja erinacea* (little skate, M29679), *Heterodontus francisci* (horned shark, S01853), *Lepisosteus osseus* (gar, U12455), *Amia calva* (bowfin, U12456), *Acipencer baeri* (Siberian sturgeon, unpublished results from L.P.'s laboratory), *Ictalurus punctatus* (channel catfish, M27230), *Salmo salar* (Atlantic salmon, S48652), *Salvelinus alpinus* (Arctic charr, X83373), *Oncorhynchus mykiss* (rainbow trout, X65262), *Gadus morhua* (Atlantic cod, X58870), *Elops saurus* (lady fish, M26182), *Xenopus laevis* (clawed frog, M20484), *Ambystoma mexicanum* (axolotl, X68700), *Gallus gallus* (chicken, X01613), *Anas platyrhynchos* (Duck, U27213), *Mus musculus* (mouse, P01872), *Mesocricetus auratus* (golden hamster, A02168), *Suncus murinus* (house shrew, X13920), *Homo sapiens* (human, A02162), *Canis familiaris* (dog, P01874), *Sus scrofa* (pig, S42881), *Ovis aries* (sheep, X59994), *Bos taurus* (cow, this paper), *Rattus norvegicus* IgE (rat, X00923), *Homo sapiens* IgE (human, V00555).

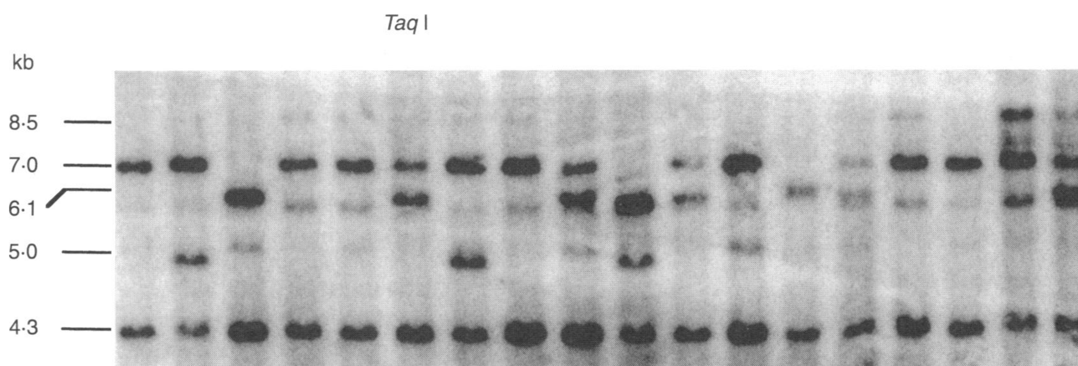

Figure 6. Southern blot analysis of 18 different bovine genomic DNA samples digested with *Taq*I and hybridized with Cμ-probe.

cannot determine which bands belong which gene. In the samples double-digested with *Taq*I–*Xba*I, we could clearly observe the existence of the two bovine Cμ genes. In this combination, two major bands (2·4 and 2·2 kb) were visualized with different densities. The weaker band (2·4 kb) might be the ψCμ gene, which shows less homology to our probe (data not shown), and the stronger band (2·2 kb) is the functional Cμ gene (Fig. 7, Table 2).

Furthermore, we digested the bovine high molecular weight DNA with *Mlu*I and ran pulsed-field gel electrophoresis (PFGE). Hybridization with bovine Cμ revealed only one 400-kb band which could also be identified with other bovine immunoglobulin probes (data not shown). Thus the ψCμ gene could not be adequately accounted for. As we could not observe fragments smaller than 150 kb in the PFGE, they might have run off the gel and thus not been detected. Alternatively, the ψCμ gene may not have been completely digested and may therefore remain in the high molecular weight DNA or located in the same position as the band containing the functional genes. Taken together these data

Table 2. Southern blot analysis of bovine genomic DNA with different combinations of restriction enzymes

Gene	*Xba*I–*Bam*HI	*Bam*HI–*Hind*III	*Hind*III–*Taq*I	*Taq*I–*Xba*I	*Taq*I
n	5	5	5	5	18
μ	**2·6**	**5·5**	*3·2*	**2·2**	**6·1** **7·0** **6·1, 7·0**
ψμ	3·5, 3·7	3·7, 2·9	*4·4, 7·0* *4·4, 6·4,* *4·0, 4·4, 7·0* *4·4, 4·8, 7·0* 4·4, 6·4, 7·0	2·4	4·3 4·3, 5·0 4·3, 8·3

The fragments of the bovine functional Cμ gene are indicated by bold figures and ψμ gene by normal figures. The underlined figures indicate the number of occurring shape of bovine Cμ genes. The *n* indicates the number of the analysed samples. The italic figures are not determined whether they belong to the functional Cμ gene or the ψμ gene.

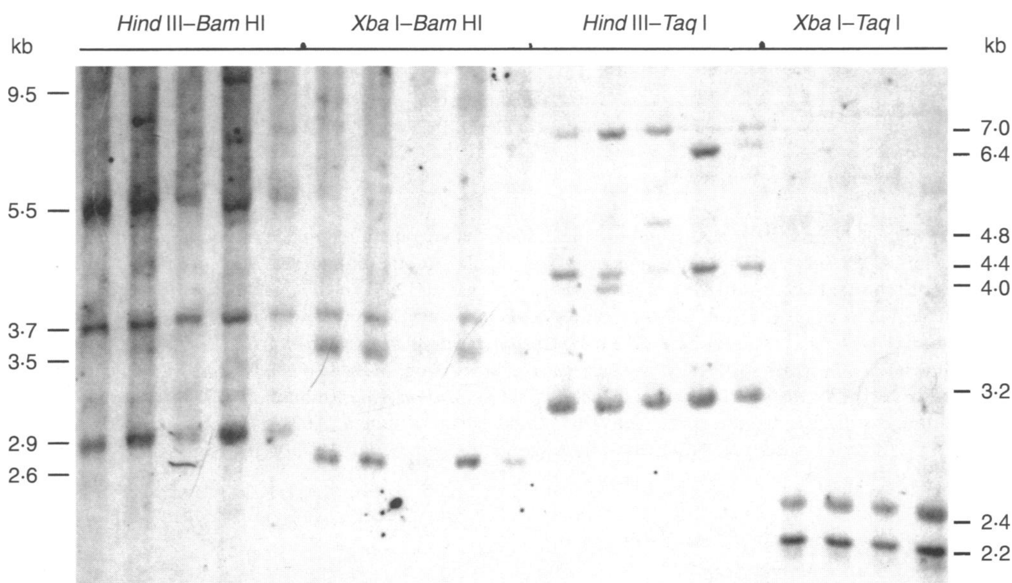

Figure 7. Southern blot analysis of four different bovine genomic DNA samples digested with different combination of restriction enzymes and hybridized with Cμ-probe.

suggest that there is more than one Cμ gene in the bovine genome with an allelic restriction fragment length polymorphism (RFLP).

DISCUSSION

Given orally to patients, immunoglobulins from immunized bovine colostrum and milk have been shown to protect against gastroenteritis induced by several human pathogens such as *Shigella flexneri*[36] and rotavirus.[37,38] They have also been used successfully as oral prophylaxis in newborn calves.[39] The determination of the level of immunoglobulin classes and subclasses in these products require standardized reagents. Currently available reagents have difficulties in distinguishing between bovine immunoglobulins of different classes or subclasses in diagnostic tests. The availability of sequence data on the μ gene permits synthesis of IgM-specific peptides such as IATAEVLS (at position 575) and ISEGQVETVQ (at position 830), which can be used for production of anti-bovine IgM antibodies. The mentioned specific peptides did not show any sequence similarity with rat, rabbit, or sheep IgM nor with other classes of bovine immunoglobulin.

Furthermore, these data allow the determination of the phylogeny of the bovine Cμ gene. Amino acid sequence comparison of the bovine Cμs with other known Cμ genes revealed a marked homology to sheep Cμs as do other bovine immunoglobulin genes such as Cγ,[40–42] Cα[43] and Cε.[44]

One characteristic structural feature of Cμ genes is the evolutionarily retained CH4 internal splice site for joining CH4 to TM1.[33,34] The donor splice site in the bovine sequence appears to be *CG/GTAAAC* which is well conserved in some vertebrates, such as human, sheep, rabbit and hamster, while in other vertebrates, such as chicken, rat, rabbit and shark, the conserved donor splice site, is *G/GTAAA*. The alternative splicing takes away only the secretory tail which is similar to other species of mammals but differs from those of Actinopterygii, such as channel catfish, rainbow trout and Atlantic cod. In these species, the 3′ portion of the CH3 exon

is joined directly to the membrane exon TM1 with the omission of the entire CH4 exon.[6,24]

The cysteine residue of the C-terminal peptide of the μ-chain is necessary for assembly of IgM polymers[45] and the penultimate cysteine residue at position 624, and lack of proline at position 621, seem to be required for pentamer formation.[46] These features are well conserved in the bovine IgM, but differ from those of Actinopterygii, such as bowfin[3] channel catfish,[4] rainbow trout[5] and Atlantic cod,[6] where IgM forms a tetramer (for details see ref. 46).

The mammalian immunoglobulin heavy-chain constant region (CH) gene cluster has evolved through multiple gene duplications, resulting in an increasing number of immunoglobulin genes in mammals such as human, cow, rabbit and mink.[47–51] Except for the Atlantic salmon,[52] and cow,[35] no other species has been reported to carry two μ genes. Tobin-Janzen and Womack's suggestion[35] on the presence of two bovine μ genes was based on Southern blot analysis of bovine–hamster hybrid somatic cell DNA, digested with *Hin*dIII. One of the Cμ genes is located on chromosome 21 together with the other immunoglobulin genes[35,53] and the other Cμ-related gene (IgML-gene) is located on chromosome 9.[35] Our Southern blot analysis of 18 different bovine genomic DNA samples, digested with *Hin*dIII, *Eco*RI and *Taq*I, supports the suggestion of two μ genes in the cow. Our sequence data of the Cμ gene are also identical to the previously described, partial cDNA sequence of the bovine Cμ[26] with the exception of four point mutations and two insertions of TG and A nucleotides, respectively, in the CH4–TM1 intron which indicates that our sequence is indeed derived from the functional Cμ gene is located on chromosome 21.[35]

The sequences of the bovine Cμ, Cε,[44] Cγ,[49] Cγ2,[49] Cγ3[42] and Cα[43] have all been characterized. It would now be interesting to determine the physical map of these genes to allow evolutionary comparison with other mammals and to determine the number of different classes and subclasses in the bovine immunoglobulin locus. In order to determine the sequences of these genes in the germline, we have previously screened a bovine cosmid library. Only one clone containing two genes, ε and α, was found in the cosmid library, suggesting that the distances between the other immunoglobulin genes may be larger than 30–40 kb. Based on the restriction sites for *Mlu*I within the μ and α genes, they would be expected to be the most 5′ and 3′ genes within the locus with the ε gene located approximately 14 kb upstream of the α gene. However, the order of the three γ genes is still unknown and currently ongoing screening of bovine heterohybridomas, may yield insight into this problem and thus help us to create a physical map of the entire locus.

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