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Cloning and Characterization of the Bovine MHC Class I-Like Fc Receptor^{1,2}

Imre Kacs Kovics,^{*†} Zhen Wu,[‡] Neil E. Simister,[‡] László V. Frenyó,^{*} and Lennart Hammarström^{3†}

In the cow, maternal immunity is exclusively mediated by colostrum Igs, but the receptor responsible for the IgG transport has not yet been identified. The role of an IgG-Fc receptor (FcRn) that resembles a class I MHC Ag in transporting IgGs through epithelial cells was recently shown in selected species. We now report the cloning and characterization of the bovine FcRn (bFcRn). The cDNA and deduced amino acid sequences show high similarity to the FcRn in other species, and it consists of three extracellular domains, a hydrophobic transmembrane region, and a cytoplasmic tail. Despite the high similarity of the extracellular domains with other species, the bovine cytoplasmic tail is the shortest thus far analyzed. Aligning the known FcRn sequences, we noted that the bovine protein shows a 3-aa deletion compared to the rat and mouse sequences in the $\alpha 1$ loop. Furthermore, we found a shorter transcript of the bFcRn reflecting an exon 6-deleted mRNA, which results from an inadequate splice acceptor site in intron 5 and produces a transmembrane-deficient molecule, as was previously demonstrated in the related MHC class I gene family in mouse and humans. The presence of bFcRn transcripts in multiple tissues, including the mammary gland, suggests their involvement both in IgG catabolism and transcytosis. *The Journal of Immunology*, 2000, 164: 1889–1897.

The transfer of passive immunity from the cow to the calf involves passage of Igs through the colostrum (1, 2). Upon ingestion of the colostrum, Igs are transported across the intestinal barrier of the neonate into its blood. Whereas this intestinal passage appears to be somewhat nonspecific for types of Igs, there is a high selectivity in the passage of these proteins from the maternal plasma across the mammary barrier into the colostrum (2–5), and only IgG1 is transferred in large amounts (6). There is a rapid drop in the concentration of all lacteal Igs immediately postpartum (7), and the selectivity of this transfer has led to the speculation that a specific transport mechanism across the mammary epithelial cell barrier is involved. Preferential binding of IgG1 to mammary cells was previously demonstrated (8–12).

Although, the identity of the receptor that transports IgG1 across the mammary epithelial cells is not known, several IgG-binding proteins have been isolated from bovine myeloid cells. The cattle homologue of the human Fc γ RI, which was isolated from a bovine genomic library, exhibited similarity to the three extracellular domain exons of human Fc γ RI, but the binding specificity was not determined (13). The bovine Fc γ RII, isolated from alveolar macrophages, binds complexed IgG1 but not IgG2 (14), while the more recently described

boFc γ 2R, isolated from the same source, represents a novel class of mammalian Fc γ R and binds aggregated IgG2 (15).

The protein responsible for transfer of maternal IgG in humans, mouse, and rat, the FcRn,⁴ consist of a heterodimer of an integral membrane glycoprotein, similar to MHC class I α -chains, and β_2 -microglobulin (16). IgG has been observed in transport vesicles in neonatal rat intestinal epithelium (17). Studies have shown that FcRn is also expressed in the fetal yolk sac of rats and mice (18), in adult rat hepatocytes (19), and in the human placenta (20, 21). More recently, Cianga et al. (22) have shown that the receptor is localized to the epithelial cells of the acini in mammary gland of lactating mice. They have suggested that FcRn plays a possible role in regulating IgG transfer into milk in a special manner in which FcRn recycles IgG from the mammary gland into the blood. In addition to these transport functions, current evidence suggests that the FcRn is involved in the homeostasis of serum IgG (23–26). FcRn is expressed in a broad range of tissues and shows different binding affinity to distinct isotypes of IgG, and the correlation among serum half-life, materno-fetal transfer, and affinity of different rat IgG isotypes for the mouse FcRn was recently demonstrated (27).

Because the isotype-specific receptor involved in the colostrum transport of IgG1 in the cow is located in the alveolar epithelial cells of the mammary gland, we decided to clone and characterize the α -chain of the bovine FcRn (bFcRn) receptor to try to understand its possible role in the IgG transport process. We now report the isolation of two forms of cDNA encoding the bovine homologue of the rat, mouse, and human IgG transporting Fc receptor, FcRn.¹

Materials and Methods

Cloning of a bFcRn cDNA fragment

RT-PCR. A bFcRn cDNA fragment was first cloned using RT-PCR. Total RNA isolated from liver by TRIzol Reagent (Life Technologies, Gaithersburg, MD) was reverse transcribed using a First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). A segment spanning the $\alpha 1$, $\alpha 2$, and $\alpha 3$ domains was amplified by PCR using two degenerate primers

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² The sequence data have been submitted to the NCBI Nucleotide Sequence Databases under the accession number: AF139106.

³ Address correspondence and reprint request to Dr. Lennart Hammarström, Division of Clinical Immunology, Karolinska Institute at Huddinge Hospital, SE-14186 Huddinge, Sweden.

⁴ Abbreviations used in this paper: FcRn, IgG-Fc receptor; bFcRn, bovine FcRn; RACE, rapid amplification of cDNA ends; UT, untranslated; CYT, cytoplasmic; TM, transmembrane; CKII, casein kinase II.

(B3, 5'-CGCAGCARTAYCTGASCTACAA-3'; B2, 5'-GATTCCSACCACRRGCAC-3') that were designed based on the sequence homology of the published rat, mouse, and human FcRn sequences (16, 18, 20).

Southern blot hybridization. The amplified cDNA was size fractionated on a 1% agarose gel, blotted on a Hybond-N nylon membrane (Amersham, Arlington Heights, IL), and hybridized with a ³²P-labeled human FcRn cDNA probe. This probe was generated by RT-PCR from placental RNA using primers (HUFC2, 5'-CCTGCTGGGCTGTGAACGTG-3'; HUFC3, 5'-ACGGAGGACTTGGCTGGAG-3') and encompassed a 549-bp fragment containing the $\alpha 2$, $\alpha 3$, and transmembrane (TM) regions (20). Blots containing the fractionated PCR-amplified product of bovine cDNA was hybridized in 5 \times Denhardt's solution, 5 \times SSC, 0.1% SDS, and 100 μ g/ml salmon sperm DNA at 60°C for 6 h and then washed in 2 \times SSC and 0.5% SDS twice for 15 min at room temperature, followed by a wash in 0.1 \times SSC and 0.1% SDS for 15 min at 60°C.

Cloning and sequencing. Based on the expected size and Southern blot verification, the proper *Taq* polymerase-generated fragment was cloned into the pGEM-T vector (Promega, Madison, WI). In general, preliminary sequencing was done by *fmol* DNA Sequencing System (Promega) in the laboratory, whereas *TaqFS* dye terminator cycle sequencing was performed by an automated fluorescent sequencer (ABI, 373A-Stretch; Perkin-Elmer, Norwalk, CT) in the Cybergene (Huddinge, Sweden) to achieve the final sequence data.

Cloning of the full-length of bFcRn cDNA

To obtain the full length of bFcRn cDNA we used rapid amplification of the cDNA ends (RACE) technique (28) to isolate and clone the unknown 5' and 3' ends.

3'-RACE. A total of 5 μ g of total RNA was reverse transcribed by using Superscript II (Life Technologies) with the (dT)₁₇-adapter primer (5'-GACTC GAGTCGACATCGA(T)₁₇-3'). The resultant cDNA was then subjected to 3'-RACE PCR amplification using the adapter primer (5'-GACTC GAGTCGACATCG-3') and a bFcRn-specific primer (B3).

5'-RACE. The remaining 5' end portion of the bFcRn was isolated using the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Life Technologies). Briefly, total RNA was reverse transcribed using a FcRn-specific oligonucleotide (B4, 5'-GGCTCCTCCACTCCAGGTT-3'). After first strand synthesis, the original mRNA template was removed by treatment with the RNase mix. Unincorporated dNTPs, primer, and proteins were separated from cDNA using a GlassMax Spin Cartridge (Life Technologies). A homopolymeric tail was then added to the 3' end of the cDNA using TdT and dCTP. PCR amplification was accomplished using *Taq* polymerase, a nested FcRn-specific primer (B5, 5'-CTGCTGCGTC-CACTTGATA-3'), and a deoxyinosine-containing anchor primer. The amplified cDNA segments were analyzed by Southern blot analysis, cloned, and sequenced as described above.

Cloning of a bFcRn genomic DNA fragment

Bovine genomic DNA was purified from liver based on the method of Ausubel et al. (29). To analyze exon-intron boundaries of the $\alpha 3$ transmembrane-cytoplasmic region, we PCR amplified a genomic DNA fragment using the B7 (5'-GGCGACGAGCACCACACTAC-3') and B8 (5'-GATTCCCGGAGGTCWCACA-3') primers. The amplified DNA was then ligated into the pGEM-T vector (Promega) and sequenced as described above.

Tissue distribution

Northern hybridization. Different bovine tissue samples (mammary gland, parotis, liver, jejunum, kidney, and spleen) were collected at slaughter from a lactating Holstein-Friesian cow and frozen immediately in liquid nitrogen. Total cellular RNA purified from these tissues and from the Madin-Darby Kidney (MDBK) cell line (TRIZol Reagent, Life Technologies) (10 μ g/lane) was run on a denaturing agarose gel and transferred to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany). The blots were hybridized with a ³²P-labeled probe, which was generated by Prime-A-Gene kit (Promega) containing the B7-B8 generated cDNA of the bFcRn. The final wash was 0.1 \times SSC and 0.1% SDS at 60°C.

Targeted amplification of the exon 6-deleted bFcRn transcript. Total RNA isolated from different tissues by TRIZol Reagent (Life Technologies) was reverse transcribed using a First-Strand cDNA Synthesis Kit (Pharmacia Biotech). A segment of exon 7 was amplified by PCR. From the two primers, which were involved in this reaction, one (B12, 5'-CTCACGGTGGAGCTGG/CT-3') was designed to anneal to the joining region of exon 5 and exon 7 in the case of the exon 6-deleted transcript. It had a longer 5' region annealing to exon 5 and a short 3' region annealing to exon 7. The

other primer (B11, 5'-GAGGCAGATCACAGGAGGAGAAAT-3') annealed to a segment further downstream of exon 7. The condition of the PCR was an initial 2-min denaturation at 94°C and then 35 cycles of 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C, and a final extension of 5 min at 72°C. The amplified cDNA was size fractionated and analyzed on a 1.5% agarose gel.

Expression and binding assay

The full length of bFcRn cDNA was amplified by B10 (5'-CTGGGGC CGCAGAGGGAAGG-3') and B11 (5'-GAGGCAGATCACAGGAGGAGAAAT-3'). This segment was then cloned into the pCI-neo eucaryotic expression vector (Promega). A total of 10 μ g DNA was transfected into one 10-cm plate of IMCD cells using a CaPO₄ method (30). Cells were diluted and placed under G418 selection. Individual G418-resistant colonies were expanded for binding assays. The presence of the bFcRn in these cells was confirmed by Western blots.

Bovine IgG (Chemicon International, Temecula, CA) was labeled with Na¹²⁵I to a specific activity of ~0.5 Ci/ μ mol using Iodogen (Pierce, Rockford, IL). pH-dependent Fc binding and uptake was analyzed according to the protocol of Story et al. (20). Briefly, cells expressing the bFcRn were first washed with DMEM (pH 6 or 7.5). Then, bovine-[¹²⁵I]IgG in DMEM (pH 6.0 or 7.5) with or without unlabeled bovine IgG was added. The cells were allowed to bind and take up IgG for 2 h at 37°C; then unbound ligand was removed with washes of chilled PBS (pH 6.0 or 7.5). Bound radioligand was measured in a gamma counter.

Western blot

A clone (B1) of IMCD cells transfected with cDNA encoding the bFcRn α -chain, IMCD cells transfected with cDNA encoding the rat FcRn α -chain (31), untransfected IMCD cells, 293 cells transfected with cDNA encoding the human FcRn α -chain (20), and untransfected 293 cells were extracted in 5% SDS. Protein extracts were resolved on gradient polyacrylamide denaturing Tris-glycine gels (Novex, San Diego, CA) and transferred onto polyvinylidene difluoride (Novex). Blots were probed with affinity-purified anti-FcRn peptide Ab, a rabbit antiserum against the peptide LEWKEPPSMRLKARP representing aa 173-187 (bovine residues) of the $\alpha 2$ - $\alpha 3$ domains (31), and bound Ab was detected with HRP-conjugated goat anti-rabbit Ab and enhanced chemiluminescence (Renaissance Chemiluminescence Reagent; NEN Life Science Products, Boston, MA).

Bio-computing

Sequence comparison was completed by using the BLAST programs (32). Sequence pair distances of bFcRn compared to other published FcRn sequences was analyzed by Megalign, Lasergene Biocomputing Software for the Macintosh (DNASTar, Madison, WI) using the Hein method (33) with PAM250 residue weight table.

Results

Isolation of bFcRn cDNA

To isolate a fragment of the bFcRn, we first synthesized cDNA from the RNA isolated from bovine liver, as this tissue was previously demonstrated to express FcRn in other species (19, 20). PCR amplification with two degenerate primers (B3 and B2) yielded a DNA fragment of about 750 bp. The degenerate primers were designed based on two conserved segments of rat (16), mouse (18), and human FcRn (20) sequences. Based on its expected size and the Southern blot verification with a cloned human FcRn fragment, this amplified DNA was ligated into a pGEM-T vector, and one of the clones (clone 15/3) was completely sequenced. The data were compared to other GenBank sequences using the BLAST programs and showed high homology to the human, rat, and mouse FcRn cDNA. The insert of clone 15/3 started in the middle of the $\alpha 1$ domain (exon 3) and ended in the transmembrane region (exon 6).

We then performed 3'-RACE using B3 and the adapter primer which generated a DNA fragment of ~1.3 kbp. Several of the clones obtained were completely sequenced. One of these (clone 4) started in the middle of the $\alpha 1$ domain (exon 3) and ended with a 38-bp-long poly(A) tail. The insert contained a segment of the $\alpha 1$, the full length of the $\alpha 2$ and $\alpha 3$ domains, the TM domain, the cytoplasmic (CYT) domain, and ended with the 3'-untranslated

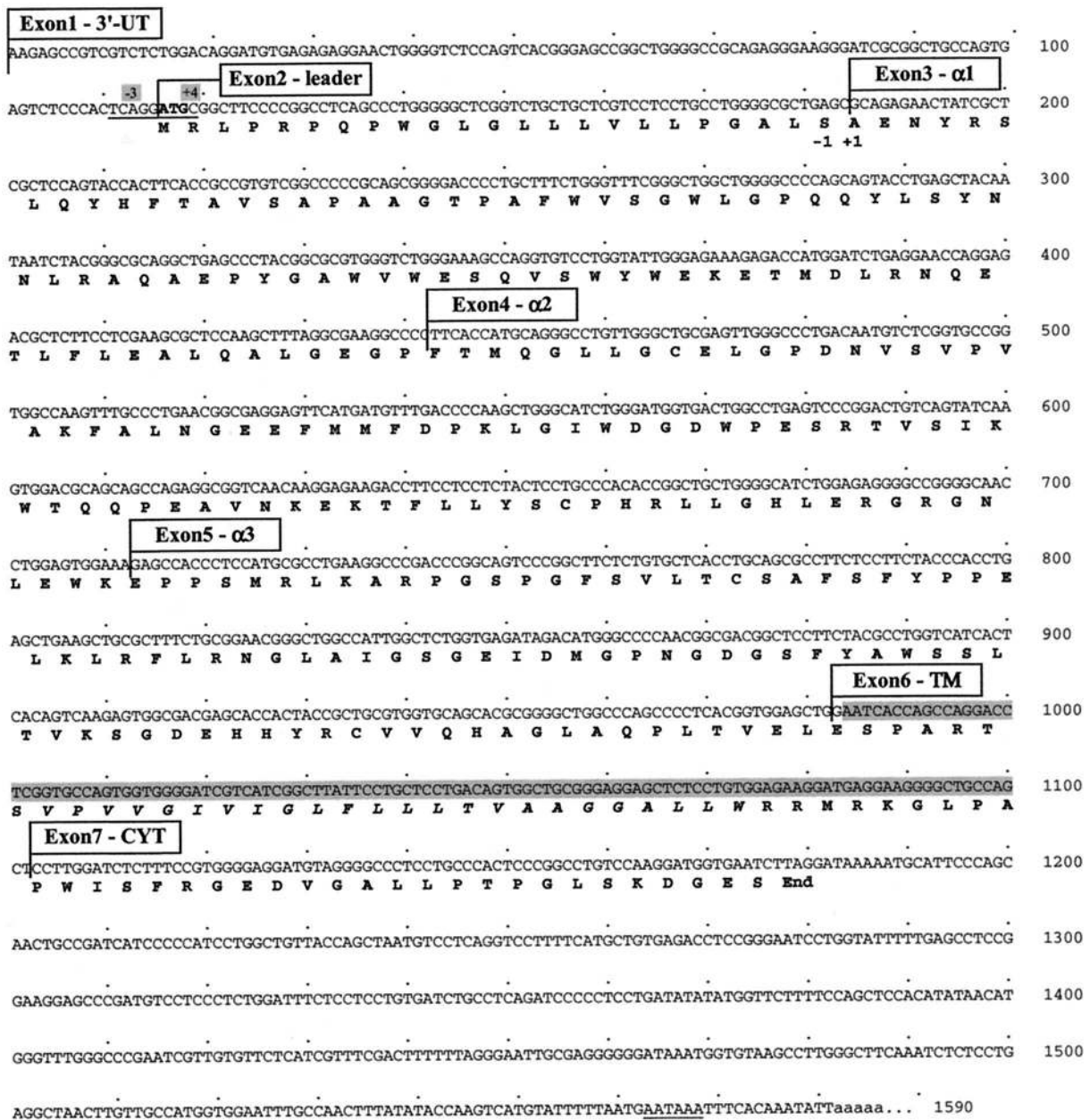


FIGURE 1. The nucleotide sequence and deduced amino acid sequence of the two forms of bFcRn α -chain. The exon 6-deleted form lacks the shadowed nucleotides. The potential ATG start is marked by bold characters, while the segment that refers to the consensus initiation site is underlined; shaded numbers in this motif represents important residues ($-3-A$; $+4-C$) of the translation signal. The predicted NH_2 -terminal after signal peptide cleavage is indicated by $+1$ under Ala. The hydrophobic membrane-spanning segment is marked by italic characters while the polyadenylation signal AATAAA in the 3'-UT is underlined.

(3'-UT) region. The total length of the insert was 1304 bp excluding the poly(A) tail. Another clone (clone 1) revealed complete sequence homology to clone 4 but showed a 117-bp-long deletion between the $\alpha 3$ domain and the CYT region. The total length of the insert was 1187 bp excluding the poly(A) tail.

The 5' portion of the bFcRn was obtained by applying a 5'-RACE technique. The amplification, in which we used B5 and the adapter primers, produced an ~600-bp DNA fragment, which then was ligated into the pGEM-T vector, and one of the clones (clone 5) was sequenced. The insert of clone 5 contained 567 bp, which included the missing $\alpha 1$ signal and the 5'-UT regions. Clones 5 and 4 had an overlap of 335 bp; therefore, it was possible to obtain a composite DNA sequence of 1582 bp, encompassing the entire region of the bFcRn cDNA (Fig. 1).

Characterization of bFcRn cDNA

The sequenced and merged clones from 5'-RACE and 3'-RACE included a 116-bp-long 5'-UT region followed by an ATG initiation codon. This motif is flanked by nucleotides that are important in the translational control in the Kozak consensus, CC(A/G)CAUGG, the most important residues being the purine in position -3 and a G nucleotide in position $+4$ (34). The bFcRn cDNA shows TCAGGATGC, which is different from the optimal Kozak sequence. Although, bFcRn shows a purine base in position -3 , we found C instead of G in position $+4$ in all the clones we have sequenced from this animal (Fig. 1). To exclude the possibility of a *Taq* error during RT-PCR, we checked this motif from two other animals and found the same sequence.

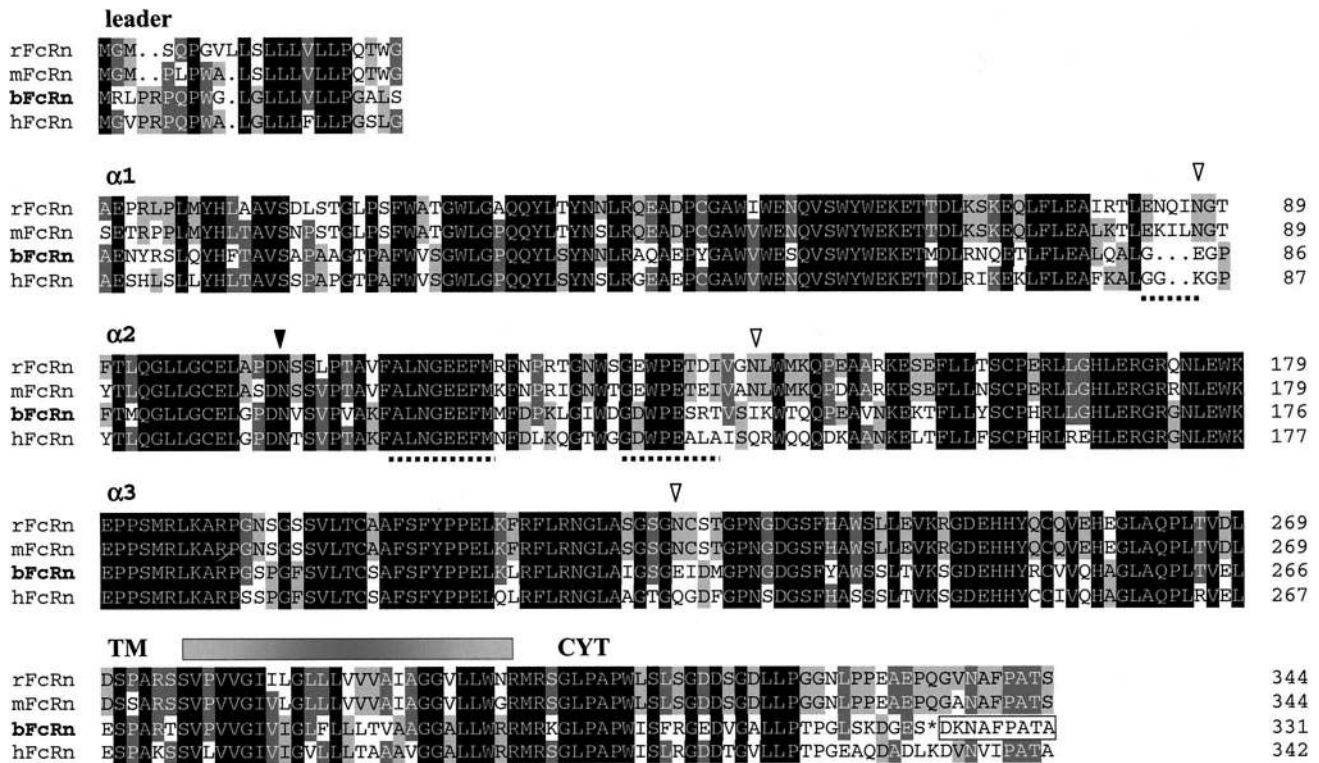


FIGURE 2. Domain-by-domain alignment of the predicted amino acid sequences for rat, mouse, bovine, and human FcRn α -chains. The N-linked glycosylation site, which is found in all the sequences is indicated by a filled triangle, while open triangles indicate additional sites in the rat and the mouse sequences. Dashed underline indicates residues that potentially interact with the Fc. The gray bar indicates the hydrophobic TM region, and the asterisk represents the stop signal in the bovine sequences. Residues in an open box following the stop signal shows the conserved carboxyl end of the bovine CYT domain. Consensus residues are assigned based on the number of occurrences of the character in the column, emphasizing the degree of conservation. The higher the conservation in a column the darker the background of the character (65).

The initiation codon was followed by a 1180-bp- or a 1063-bp-long open reading frame in case of the full-length or the exon 6-deleted form, respectively. The exon-coded segment was followed by a 392-bp-long 3'-UT sequence including the conserved polyadenylation signal (AATAAA).

Fig. 2 shows the deduced amino acid sequence of the bFcRn as compared to those of the human, rat, and mouse. Previous studies indicate that the structure of the characterized FcRn molecules resembles that of the MHC class I α -chain (16, 35). The full-length transcript of the bFcRn α -chain we isolated is also composed of three extracellular domains (α 1- α 2- α 3), a TM region, and a CYT tail. An exon 6-deleted transcript, though, lacks the putative TM region. Except for this missing domain, the two molecules are identical at the DNA as well as at the protein level (Fig. 1).

Comparing the deduced bFcRn amino acid sequence to its human, rat, and mouse counterparts, we found the highest overall similarity to the human FcRn (Table 1). Among the extracellular domains, the α 3-chain turned out to be the most conserved, whereas the cytoplasmic tail reflected the highest dissimilarity.

Table I. Sequence pair distances (in percent similarity) of bFcRn compared to published FcRn sequences, using the Hein method (33) with PAM250 residue weight table

	α 1	α 2	α 3	TM	CYT	Total
Human	75.6	74.4	85.6	74.4	61.5	77.1
Mouse	61.6	66.7	78.9	66.7	46.2	65.9
Rat	59.3	68.9	78.9	66.7	46.2	65.4

The high similarity of the bFcRn as compared to the human FcRn was further emphasized by analyzing the end of the α 1 domain. This segment, which forms a loop in the vicinity of the IgG binding site, shows a 3- or 2-aa residue deletion in the bovine and the human molecules, respectively, compared to the rat and mouse sequences. Another common feature in these two molecules is that they show only one potential N-linked glycosylation site at amino acid residue 124, based on the bFcRn numbering system, compared to the rat or mouse counterparts where there are 3 additional sites (α 1 domain, position 109; α 2 domain, position 150; α 3 domain, position 247 based on the rat FcRn numbering system).

In contrast to the known FcRn sequences, we found an unusually short CYT tail in the bFcRn where this segment is composed of 30- rather than 40-aa residues as in all other FcRn molecules so far analyzed. Despite its shortness, the CYT tail of the bFcRn shows the di-leucine motif (aa 319–320), which was previously identified as a critical signal for endocytosis but not for basolateral sorting (36), although, similar to the human molecule, it lacks the casein kinase II (CKII) phosphorylation site, which is found in the rat FcRn upstream of the di-leucine motif (36).

Interestingly, the nucleotides which follow the stop signal represent codons for similar amino acid residues which are found at the 3' end of the human, rat, and mouse molecules (Fig. 2, residues in rectangle in the bovine sequence), although it lacks the stop signal at the end of this segment which is shared in the other FcRns. Finding this sequence in all the clones we have analyzed and the lack of the common stop signal in the expected conserved position exclude the possibility of a *Taq* error due to the 3'-RACE

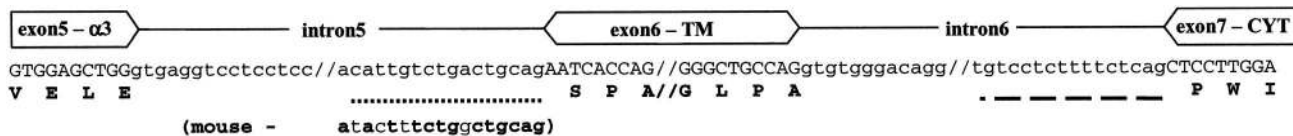


FIGURE 3. Scheme depicting a partial genomic DNA sequence of the bFcRn, which was PCR cloned applying the B7 and B8 primers. Capital letters indicate exons verified by cDNA sequence data. Exons and introns are numbered based on the genomic structure of the mouse FcRn (37). Diagonal breaks are added where segments of the sequence have been deleted for reasons of space. The dotted line indicates the splice acceptor site of intron 5, which carries the conserved AG dinucleotide but lacks the proper PPyT, while the consensus splice acceptor site of intron 6 is highlighted by a dashed line. The splice acceptor site of intron 5 of mouse FcRn is in parenthesis under the bovine sequence, indicating similarities between the two segments. Underlined letters in the mouse sequence indicate homology to the bovine splice acceptor site of intron 5 of the bovine gene.

(RT-PCR) process and suggests that a mutation has occurred in this part of the gene.

Genomic DNA segment of bFcRn

The two different transcripts of the bFcRn were compared to the published mouse genomic sequence (37). Analysis of the mouse exon-intron boundaries around α 3-TM-CYT domains suggested that exon 6 is completely eliminated from the bovine transcript representing clone 1. To verify this hypothesis, we cloned the genomic segment of the region of interest which contained part of exon 5, exon 6, and a short fragment of exon 7 and the two introns (intron 5 and intron 6). The B7/B8-amplified DNA was then cloned and sequenced. The nucleotide sequences surrounding the exon-intron boundaries revealed that the bovine splicing sites agree with their mouse counterparts (Fig. 3).

Analyzing the 5' splice site (donor site) and the 3' splice site (acceptor site) of intron 5 and intron 6, we found that intron 5 has a conserved splice donor site (GT) while its 3' splice site differs from the consensus splice acceptor sequence, which is composed of a polypyrimidine tract (PPyT) followed by an AG dinucleotide (38). Although the acceptor site of intron 5 has the conserved AG dinucleotide, it lacks the conserved PPyT. This nonconserved splice acceptor site of intron 5 shows similarity to the same gene segment of the mouse FcRn since it shows only 4 differences from the 15 nt preceding the AG dinucleotide motif (Fig. 3). Despite this similarity, though, there is a 14-nt-long conserved PPyT in the mouse intron, followed by 24 nt and then the AG dinucleotide (37). A similar sequence was not detected at the 3' end of bovine intron 5 (5'-...ctgtctggat ctctgtgtgga ggaactgacc ccactcctg cctgactcag atctgcgagg ccctaaata tctcaaca ttgtctgact gcagAATCAC CAGCC...), whereas the splice donor and splice acceptor sites of intron 6 shows conserved boundary sequences.

Tissue distribution of the two forms of bFcRn α -chain transcript

We then examined the tissue distribution of the two forms of the bFcRn α -chain mRNA by using Northern blots and RT-PCR. Based on the Northern blot analyses, a 1.6-kb transcript was present in RNA from mammary gland, liver, jejunum, kidney, and spleen from a normal lactating Holstein-Friesian cow and the MDBK cell line (Fig. 4A) at different levels of expression, whereas we did not find expression in parotis. The signal could not represent cross-hybridization with class I MHC mRNA because it was detected with a probe from the TM-CYT-3'-UT region, which is dissimilar from the class I sequences. Although, this probe is able to detect both forms of the bFcRn, we were unable to detect the shorter TM-exon-deleted form, probably because of its low expression level or due to the low resolution of the gel electrophoresis.

To analyze the expression of the alternatively spliced, exon 6-deleted transcript in the tissues listed above, we performed a targeted PCR amplification (39) in which we used primers B11 and B12. B12 corresponds to the 5' boundary conserved region of exon

7 juxtaposed with two conserved nucleotides in 3' boundary region of exon 7. This amplification detected exon 6-deleted transcripts in all tissues tested (Fig. 4B).

Expression and IgG binding of bFcRn α -chain in transfected cells

FcRn-transfected cell lines were assessed by Western blot using rabbit antipeptide antisera raised against an epitope of human FcRn heavy chain (aa 174–188). Because this epitope is common in the human, rat, and bovine FcRn molecules, we used this Ab to detect the expressed bFcRn, as well as its human and rat counterparts, as controls. We detected an ~45-kDa band in the human FcRn-transfected human embryonic kidney 293 cell line, an ~40-kDa band in the bFcRn-transfected IMCD cell lines, and two bands (~50 kDa and ~55 kDa) in the rat FcRn-transfected IMCD cell line. The 45-kDa and the 50- and 55-kDa bands detected of the human and rat FcRn-transfected cells are consistent with the known molecular weight of the human and the rat FcRn α -chains (19, 40), respectively. The lower band in the rat FcRn-transfected IMCD cell line is the high mannose form of FcRn usually found in endoplasmic reticulum, whereas FcRn in the upper band contains complex-type oligosaccharide chains modified in the Golgi. Consistent with this interpretation, the upper band in the same lane is greatly enriched at the cell surface compared with the lower band

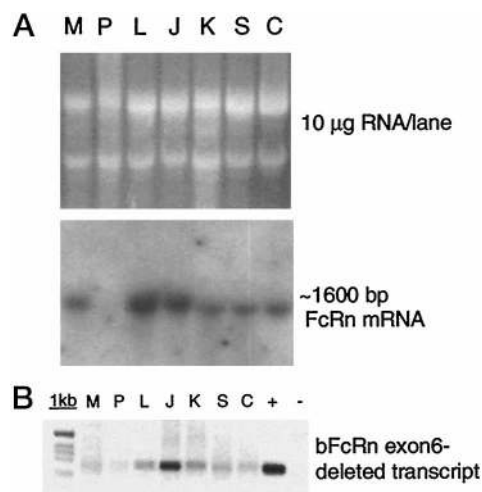


FIGURE 4. Tissue distribution of the two forms of bFcRn α -chain transcripts. *A*, Northern blot analysis of a 1.6-kb transcript in 10 μ g RNA from mammary gland (M), parotis (P), liver (L), jejunum (J), kidney (K), spleen (S), and from MDBK cell line (C) detected using a 32 P-labeled probe from the bFcRn TM-CYT region. *B*, RT-PCR analysis of the exon 6 deleted form of bFcRn transcript. Targeted PCR for exon 6 deleted cDNA amplification using B11/B12 primers.

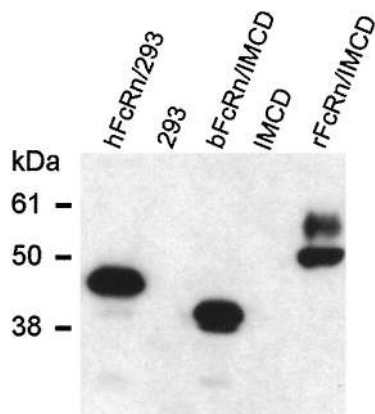


FIGURE 5. Functional expression of FcRn of different species in transfected cell lines. hFcRn/293 represents human FcRn-transfected 293 cell line (20), 293 represents untransfected cells, B1 represents bFcRn-transfected rat IMCD cell lines, IMCD represents untransfected cells, and rFcRn/IMCD represents rat FcRn-transfected IMCD cell line (31). Western blots of total cellular protein (10 μ g/lane) were performed by using affinity-purified rabbit antisera raised against aa 173–187 (bovine residues) of the α 2– α 3 domains.

(K. M. McCarthy, Z. Wu, and N. E. Simister, unpublished observations). There was no hybridization in the untransfected 293 and IMCD cells (Fig. 5).

The nearly 40-kDa band we detected in the bFcRn-transfected IMCD cell line indicates that the cDNA we isolated as bFcRn is intact and can be fully translated. The lower molecular weight of the bFcRn compared to the human and rat molecules is probably due to its shorter CYT region and/or different glycosylation.

To determine whether the bFcRn clone encoded an Fc receptor, we measured the binding of radiolabeled bovine IgG on the bFcRn-transfected rat IMCD cell line (B1). Cells that expressed bFcRn bound IgG specifically at pH 6.0 but not at pH 7.5; untransfected cells showed little or no specific binding at either pH

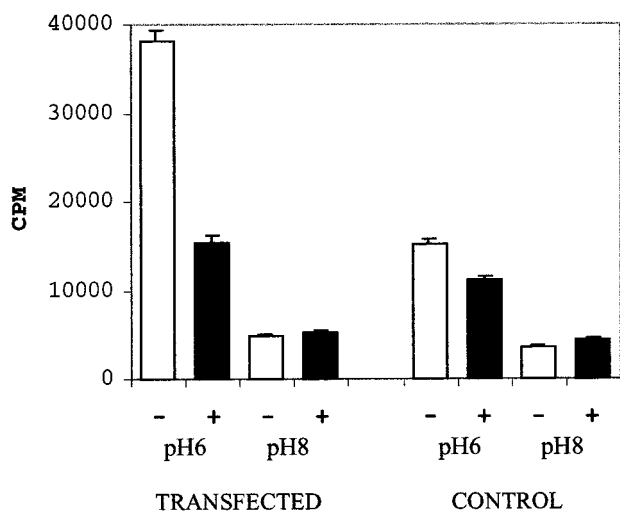


FIGURE 6. Bovine [125 I]IgG binding by bFcRn-transfected IMCD cell line. Assay were done at 37°C with (■) and without (□) competing unlabeled bovine IgG, at pH 6.0 or 8.0. Each column represents the mean cell-associated radioactivity in three replicates; bars show the SEM.

(Fig. 6). A similar pH dependence of binding has previously been observed for human (20) and rat FcRn (41).

Discussion

The predominance of IgG1 in lacteal fluid, intestinal secretions, respiratory fluid, and lacrimal fluid supports the concept of a special role for IgG1 in mucosal immunity in cattle. The higher ratio of IgG1:IgG2 in these secretions when compared to serum could represent a combination of selective IgG1 transport and local synthesis (7). Ig transmission through the mammary epithelial cells is by far the most studied, since in the cow maternal immunity is exclusively mediated by colostral Igs. The receptor responsible for the IgG transport has not been identified yet, although previous studies have indicated that specific binding sites exist on bovine mammary epithelial cells near parturition that are presumably involved in the transfer of IgG1. The purpose of our investigation was to isolate and characterize a cDNA encoding a bovine homologue of the human, rat, and mouse IgG transporting Fc receptor, FcRn.

Sequence analysis

Translation initiation site. Sequences flanking the AUG initiation codon influence its recognition by eukaryotic ribosomes. Compared to the consensus sequence (CC(A/G)CCAUGG) (34, 42), we found that in the bFcRn sequence, a C substitutes the crucial G at position +4, which maybe less significant where consensus motifs upstream from the ATG codon are present (43) as is the case of bFcRn.

Extracellular backbone and the FcRn/Fc interaction site. The bovine cDNA and its deduced amino acid sequence were similar to the rat, mouse, and human FcRns (Fig. 2) (16, 18, 20). Among these sequences, the bovine α -chain shows the highest overall similarity to its human counterpart (Table 1).

Based on the crystal structure of a 2:1 complex of FcRn and the Fc fragment of rat IgG (35), the approximate binding region on each molecule could be localized. The first contact zone of the heavy chain of the rat FcRn molecule can be found at the end of the α 1 domain involving residues 84–86 and 90. The second contact zone involves residues 113–119, while the third contact zone encompasses residues 131–137; both segments are part of the α 2 domain.

The close relationship between the human and bovine FcRn molecules was further emphasized by analyzing the end of the α 1 domain, which was suspected to form the first contact zone in the rat FcRn/Fc interaction. Both the bovine and human FcRns are 3- and 2-aa residues shorter, respectively, compared to their rodent counterparts. It is interesting that these deletions eliminate an *N*-linked glycosylation site found in their rat and mouse counterparts and which is ubiquitous in MHC class I proteins.

The second contact zone, which is part of the α 2 domain, is well conserved, emphasizing its importance in IgG binding. Another difference of the bFcRn compared to the rat molecule is a radical amino acid substitution at the third contact zone (Arg¹³⁴) in the α 2 domain. These observations suggest critical importance of the second and third contact zones, while those residues that make up the first contact zone are probably less crucial in the IgG/FcRn interaction in the cow and also in humans, further supporting the conclusion of Vaughn and Bjorkman (44) who applied site-directed mutagenesis to analyze the role of the predicted contact residues of the rat FcRn. They found that replacement of residues 84–86 of the α 1 domain, which was thought to be the first contact zone, did not significantly alter binding affinity.

We found that the critical residues of the $\alpha 3$ domains (amino acids 216L, 242K, 248H, 249H), which also influence the FcRn/Fc interaction, are conserved among the different species thus far analyzed. The bFcRn, similarly to its human counterpart, has an absence of the *N*-linked glycosylation site in the $\alpha 3$ domain, which is of interest, since for rat FcRn this has been suggested to mediate FcRn dimerization via a carbohydrate handshake (45).

Cytoplasmic domain. The lowest degree of similarity was detected in the bovine cytoplasmic domain, which is 10-aa residues shorter than in other species, although it still contains the element which is thought to constitute a potential signal for endocytosis (36). In the rat molecule, there is a CKII site upstream of this di-leucine motif that is subject to phosphorylation (unpublished observations referred in Stefaner et al. (36)) and which possibly plays a direct role in basolateral sorting or indirectly regulates the activity of a basolateral signal. The human FcRn also lacks the CKII phosphorylation site, but is still transported IgG in a bidirectional fashion (40). Similar to the human molecule, the bFcRn does not contain a CKII phosphorylation site, suggesting that this motif might not be a crucial regulatory factor in the transcytosis process.

It is an interesting observation that the nucleotides which follow the stop signal of the bFcRn represent codons for similar amino acid residues which are found at the 3' end of the human, rat, and mouse FcRn molecules (Fig. 2, residues in rectangle in the bovine sequence). It also lacks the stop signal at the end of this segment, which is shared by the other FcRns. The finding of this sequence in all the clones we have analyzed and the lack of the common stop signal in the expected conserved position in the bFcRn sequence excludes the possibility of a *Taq* error in the 3'-RACE (RT-PCR) process we used to clone this segment. The fact that the stop signal in the cow was encoded by TAG which differs by a single nucleotide from AAG and CAG observed in the human and rat sequences at the same position, respectively, suggests that this critical nucleotide underwent a mutation during evolution. This raises the question whether it still contains all the important motifs necessary for endocytosis and transcytosis. Recently, Stefaner et al. (36) deleted the C-terminal half of the cytoplasmic tail of the rat FcRn to analyze the kinetics of endocytosis and transcytosis of the mutant Fc γ RII/FcRn chimera in the Madin-Darby canine kidney (MDCK) cell line and found that this mutant molecule was still capable of internalization of IgG, suggesting that the bFcRn would still be fully functional in this respect.

Alternative splicing

Most mammalian pre-mRNAs contain several introns, which poses a special problem for the splicing machinery. In the simplest case, where there is a single intron, the primary problem is to locate the splice sites within the pre-mRNA. In more complicated cases, where there are multiple introns, there is the added difficulty of pairing the proper 5' and 3' splice sites so as to avoid exon skipping; this difficulty is compounded with pre-mRNAs that are alternatively spliced (46). The only highly conserved sequences in introns are those required for intron removal, which are found at or near the ends of an intron and very similar in all known intron sequences. They generally cannot be altered without affecting the splicing process. The conserved boundary motif at the 5' splice site (donor site) is a GT dinucleotide, while the consensus sequence at the 3' splice site (acceptor site) is composed of a PPyT followed by an AG dinucleotide (38).

The two forms of the bFcRn we observed differ with regard to the TM region which is responsible for anchoring the molecule to the lipid membrane (Fig. 2). The exon 6 deletion does not cause a frame shift or an aberrant transcript, but rather it retains its original

amino acid sequence. Genomic sequence data showed a lack of the PPyT at the acceptor site of intron 5, which precedes the TM domain (exon 6) (Fig. 3). Previous studies have indicated that the PPyT is one of the important *cis*-acting sequence elements directing intron removal in pre-mRNA, and progressive deletions of the PPyT abolish correct lariat formation, spliceosome assembly, and splicing (47). Our data suggest that the shorter form of FcRn is generated by alternative splicing, where the segment of intron 5-exon 6-intron 6 is treated as an intron and thus the splicing process eliminates exon 6 from the transcript. Alternative splicing generating TM-deleted molecules was previously described in the related MHC class I family in mouse (48, 49) and human (50) where both membrane-anchored and secreted (or soluble) forms have been described. It is noteworthy, from an evolutionary standpoint, that alternative splicing was previously also demonstrated to generate different transcripts of the bovine (51) and sheep (52) MHC class I proteins.

When analyzing the expression of the two transcripts in different tissues using Northern blot and RT-PCR (Fig. 4), we found different expression levels suggesting some degree of tissue specificity. Although these methods do not provide quantitative results, the data raise the question as to the possibility of physiological regulation. The function of the alternative splicing could be to regulate the level of the functional, full-length form, or making a soluble molecule for an as-yet-unknown function. Alternatively, the shorter form may not have a physiological function, but it is merely a biological side product. The possibility of the controlled splicing is supported by a previous study, which indicated that a weak splice site is required for alternative splicing (53). The distribution of these two forms and the physiological functions of the secreted form need to be further analyzed.

Bovine IgG metabolism

In addition to mediating the transfer of maternal IgG to young rodents (16, 54) and to the human fetus (20), it was shown that the FcRn plays a crucial role in regulating serum IgG levels (23–26), and mutated Fc fragments that binds with a higher affinity to FcRn show longer serum persistence (55). In further support of these functions, FcRn mRNA was detected in tissues that are responsible for the materno-fetal transfer (16, 20, 54) and also in tissues that may play a role in IgG homeostasis (19, 26). Since we have detected bFcRn expression in the mammary gland, among other tissues, we analyzed the possibility of the FcRn involvement in the maternal IgG transport, based on the literature.

Most recently, Cianga et al. (22) identified and analyzed the function of the mouse FcRn in mammary gland of lactating mice. They localized the receptor to the epithelial cells of the acini and found that the transport of the IgG subclasses into milk showed an inverse correlation with their affinity to the FcRn, indicating that the FcRn in the lactating mammary gland plays a role in recycling IgG from the milk gland back into the circulation. They also hypothesized that similar mechanisms might control IgG transport into milk, in ruminants, as well.

Three subclasses of bovine IgG have been described previously (56, 57), and two of these, IgG2 (58) and IgG3 (59), occur in two allelic forms. The half-life of the two major IgG isotypes (IgG1, IgG2) have been reported in several studies but the values are extremely divergent between publications (for a review, see Butler (56)). However, the data indicate that they both fall in the range of 10–22 days (60, 61), with a longer half-life for IgG2 (62, 63). Based on the positive correlation between binding affinity and half-life, these data may suggest that IgG2 binds more effectively to the FcRn than IgG1. The 10:1 ratio between IgG1 and IgG2 in the colostrum (7) further supports the mouse model (22); i.e., that

IgG2 is recycled back to the circulation more effectively by an FcRn-mediated transport mechanism.

Because previous studies have shown the presence of IgG1-specific receptors in the mammary gland around parturition (9–12), we cannot exclude the possibility of an alternative explanation regarding to the role of the bFcRn. The receptor would then have to fulfill at least two requirements: 1) it should prefer IgG1 in the binding or in the transport process, and 2) it should mediate basolateral to apical IgG transport in these cells. The controversial data in the literature regarding the half-life of the two major subclasses prevent us from concluding an IgG1 preference from the literature alone. Concerning the second criterion, most of the studies in rodents and in humans on the role of the FcRn in maternal IgG transport have described apical to basolateral transport. Finally, Stefaner et al. (36) analyzed the intracellular routing of the rat FcRn and demonstrated nonvectorial surface transport and bidirectional transcytosis, although they noted that apical to basolateral and basolateral to apical transcytosis were differently regulated. Bidirectional FcRn-dependent IgG transport was also demonstrated in a polarized human intestinal epithelial cell line (40). As a consequence, it seems that cells of different origin, expressing FcRn, have different protein sorting mechanisms related to this receptor. In this context one might hypothesize that in the cow, the mammary epithelial cells are able to carry IgG via FcRn-mediated transcytosis from the blood into their secretory fluid, although none of the studies indicated pH-dependent IgG binding, which we found in analyzing IgG binding to the bFcRn (Fig. 6).

Finally, we cannot exclude the possibility that there is an as-yet-unidentified Fc receptor in the mammary gland, which selectively transports IgG1 into the colostrum, whereas the FcRn may play a part in recycling preferentially IgG2 from the milk gland into the circulation.

In summary, our data indicate that the FcRn transcripts are expressed in different tissues, including the mammary gland in cattle, and strengthens their suggested involvement in IgG catabolism and transcytosis (for a review, see Junghans (64)). It will be of interest to investigate the bFcRn binding affinity or the transport efficiency mediated by this receptor of the bovine IgG subclasses. Analyses of the localization and the expressional level of the bFcRn in the mammary gland at different times during the lactation period may also help to clarify its function in the transport of IgG into the colostrum.

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