Sequence analysis of porcine polymeric immunoglobulin receptor from mammary epithelial cells present in colostrum

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The composition of colostrum is quite different from that of mature milk. In particular, colostrum contains relatively high concentrations of proteins responsible for the immunological defence of newborns, such as lactoferrin, lactoperoxidase, lysozyme and immunoglobulins (Farkye, 1992; Cals *et al.* 1994; Telemo & Hanson, 1996; Wang *et al.* 1997). Whereas lactoferrin and lactoperoxidase are synthesized in mammary epithelial cells (Cals *et al.* 1994; Molenaar *et al.* 1996), the immunoglobulins are derived from blood serum (Larson, 1992). Immunoglobulin A (IgA), predominantly found in milk, participates in the development of the gastrointestinal system and the immune system in newborn infants. It is transported by a system that involves formation of a complex with the polymeric IgA receptor (pIgR) exposed on the basolateral aspect of mammary gland epithelial cells, followed by internalization and release into milk as secretory IgA (Rosato *et al.* 1995; De Groot *et al.* 1999). Consequently, expression of pIgR in mammary epithelial cells also contributes to the development of the immune system. These proteins specifically expressed in the early stage of lactation are of interest. However, few studies have been carried out compared with those focusing on the major proteins in mature milk, in part owing to problems of mammary gland availability.

Imamura *et al.* (1996) reported the isolation of mRNA from bovine mammary epithelial cells derived from colostrum and showed that it is possible to detect α_{s1}-casein mRNA, predominantly expressed in the bovine mammary gland, through the application of reverse transcriptase polymerase chain reaction (RT-PCR). We have applied this procedure to porcine colostrum, and successfully analysed the coding sequence of pIgR. In this paper we describe the possibility of detecting gene expression in mammary epithelial cells present in colostrum and the porcine pIgR sequence obtained is compared with those of other animal species.

MATERIALS AND METHODS

Isolation of mRNA from porcine colostrum and subsequent RT-PCR

Porcine colostrum was obtained by hand milking within 36 h of parturition and collected into test tubes containing portions of Trizol reagent (Gibco, Rockville, MD

20877, USA). Total RNA was isolated according to the manufacturer's instructions. Extraction of mRNA from the total RNA was performed using magnetic beads (Dynal, N-0212 Oslo, Norway) according to the manufacturer's instructions. For RT-PCR, first-strand cDNA was synthesized from the mRNA using M-MLV reverse transcriptase (Promega, Madison, WI 53711, USA) in the presence of random primers (Gibco). The resulting cDNA was used for amplification by PCR. After an initial denaturing step (2 min at 95 °C) each sample was subjected to 40 PCR cycles, each consisting of 1 min at 95 °C, 1 min at an appropriate annealing temperature and 1 min at 72 °C, with a final extension at 72 °C for 10 min. The resulting PCR products were subjected to electrophoresis through an agarose gel in the presence of ethidium bromide and photographed under u.v. illumination. The primer sets used in this study were as follows: 1F (5′-TGTGGAAAGCCTTTCAAGCAG-3′) + 1R (5′-CAGTCAATTCAAAAGTGAGGAG-3′), 2F (5′-GCACAGAATATGGACTCTTCC-3′) + 2R (5′-CTCACAGAGCCACTGATCCAG-3′), 3F (5′-GAGGGCGACCTGGAGATCC-3′) + 3R (5′-GGGTCCCAGGAGGGGTG-3′) and 4F (5′-TATCTACACTGCGGGCAAGTG-3′) + 4R (5′-CAGGCCCATGGGGATGTTCC-3′), designed to evaluate the expression of porcine β-casein (nucleotide accession no. X54974 in EMBL and Genbank), α-lactalbumin (M80520), β-lactoglobulin (X54976) and lactoferrin (M81327) respectively. Other primer sets, 5F (5′-ACGGGAGAGAAGGAAGTAGC-3′) + 5R (5′-GCAGTAGCCGTTGGCTCC-3′), 6F (5′-CAAAGCCCCATATTTGGTCC-3′) + 6R (5′-GTTAGCAGGATCCTGCCTTCA-3′), 7F (5′-CTGTGCCAGCTAAAAAATGGG-3′) + 7R (5′-CCTTCTGTTCCACTGCCACG-3′), 8F (5′-GGCTGGTACTGGTGTGG-3′) + 8R (5′-GCTTCCTCCTTGGAT GACC-3′) and 9F (5′-GGTTCACCACCACTACCGAG-3′) + 9R (5′-ACCAGCACACC TCTGGGCACAG-3′) were used for amplification of a partial sequence of pIgR with reference to that from other animal species such as human (nucleotide accession no. M24559 in EMBL and Genbank), mouse (U06431), rat (X 15741), rabbit (X00412) and bovine (X81371).

Cloning and sequence analysis of PCR products using primers specific for the pIg receptor

The PCR products obtained by amplification of pIgR were separated on agarose gels. Nucleotides were extracted, the gel placed in a dialysis tube and electroelution carried out to obtain the fragment. The isolated fragment was ligated with the TA cloning vector pGEM-T Easy (Promega) and the ligation products were used to transform *Escherichia coli* JM109. DNA manipulations were carried out using standard procedures (Maniatis *et al*. 1989).

The DNA sequencing reaction was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA 94404, USA). The sequencing product was purified using a Centri-Cep spin column (Princeton Separation Inc., NJ 07710, USA) and analysed with an ABI Prism Model 377-18 DNA sequencer (PE Applied Biosystems). The sequence results were assembled using Auto Assembler (PE Applied Biosystems). The amino acid sequence determined in the present study was compared with other published sequences in various databases through the DNA databank of Japan (DDBJ, Mishima 411-8540, Japan).

Multiple alignment of amino acid sequences was carried out using CLUSTAL W v. 1.7 (Thompson *et al*. 1994). Other sequence analyses were performed using Genetyx-Mac v. 9.0 (Software Development Co., Tokyo 150-0002, Japan). The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession no. AB032195.

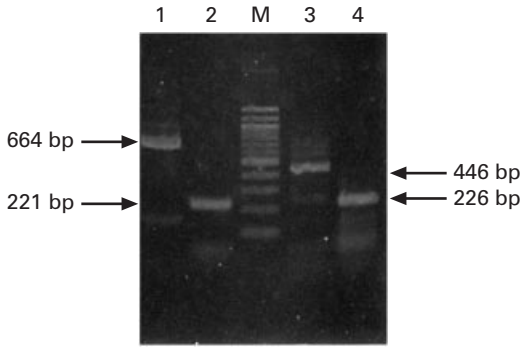

Fig. 1. Analysis of the expression of milk proteins by reverse transcriptase polymerase chain reaction using mRNA isolated from porcine colostrum as the template. Lane l, β-casein; lane 2, α-lactalbumin; lane 3, β-lactoglobulin; lane 4, lactoferrin; M, 100 bp ladder marker.

RESULTS AND DISCUSSION

A volume of colostrum of 1–2 ml was sufficient to obtain mRNA for analysis of gene expression by RT-PCR. Since the sequences of the genes encoding porcine milk proteins are unknown, we referred to the structures of those of other animal species and designed each set of primers to correspond to a different exon region to exclude amplification of genomic DNA contaminants. It is well known that the organization of caseins, α-lactalbumin and β-lactoglobulin has been conserved during evolution (Mercier & Vilotte, 1993). Fig. 1 shows the electrophoresis profile of the PCR products amplified by means of various primer sets. The sizes of the amplified fragment in the case of β-casein, α-lactalbumin, β-lactoglobulin and lactoferrin were predicted to be 664, 221, 446 and 226 bp respectively. All samples showed the expected migration and it was confirmed that the mRNA isolated from porcine colostrum was suitable to detect gene expression in porcine mammary epithelial cells at an early stage of lactation. Since limited data were available on the sequence of porcine pIgR (nucleotide accession no. F14851 in EMBL and Genbank), we tried to determine the entire coding sequence. Taking into account sequence information for other animal species, we designed five primer sets for amplification of porcine pIgR cDNA. After arrangement to eliminate overlapping of the amplified regions, the sequence obtained was found to include a single open reading frame consisting of 2277 nucleotides encoding a protein of 758 amino acids (Fig. 2). Comparisons between the nucleotide sequence of porcine pIgR and those of pIgR from other species revealed identities of 77·9, 80·4, 66·0, 72·0 and 70·9% for human, bovine, rabbit, rat and mouse respectively. For the amino acid sequences, comparisons showed identities of 68·2, 71·4, 48·8, 60·7 and 59·7% respectively (Fig. 3). Based on the results of a comparative study (Bakos *et al.* 1991), the signal sequence was predicted to be MTRFFYACLLAIFPVVSM. A motif in the C-terminal region, H^{659}–R^{660}–X–X–V^{663}, was recognized which contains residues proposed to be critical for the intracellular transit of the synthesized pIgR to the basolateral surface (Aroeti & Mostov, 1994). A serine residue serving as a phosphorylation site is often situated between two tyrosine residues in the C-terminal region and these residues are thought to be involved in internalization of pIgR (Hunziker & Kraehenbuhl, 1998). In porcine pIgR, Ser^{729} and Tyr^{671} are candidates in this context; however, the second tyrosine seems to be absent, as is the case with bovine pIgR.

In spite of the agricultural importance of such studies, little attention has been paid to gene expression and synthesis of milk proteins in the porcine mammary

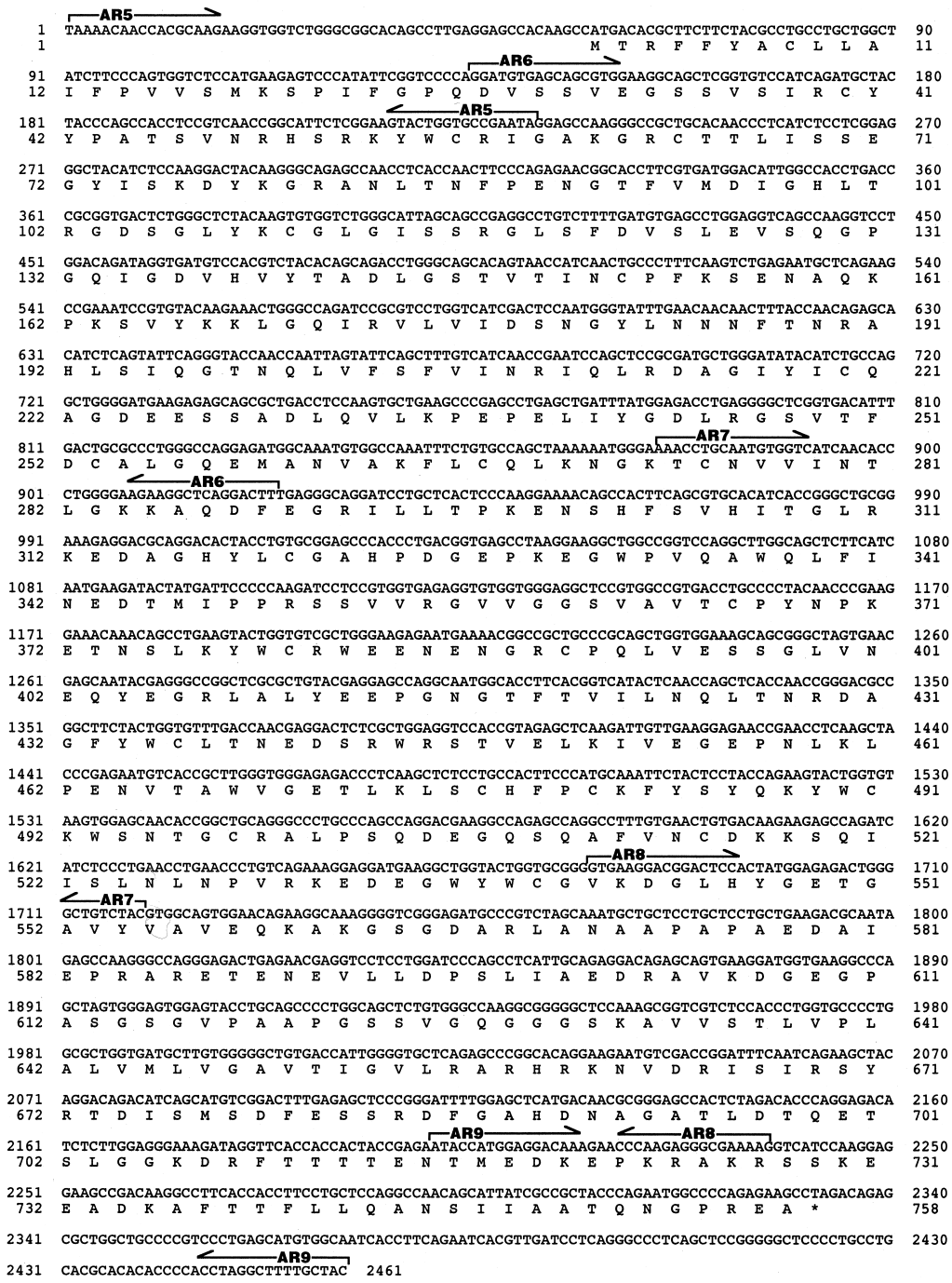

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the porcine polymeric IgA receptor. The amplified region (AR) lacking its primer nucleotide sequence is also shown; AR5, AR6, AR7, AR8 and AR9 were obtained by polymerase chain reaction using the primer sets 5F + 5R, 6F + 6R, 7F + 7R, 8F + 8R and 9F + 9R respectively.

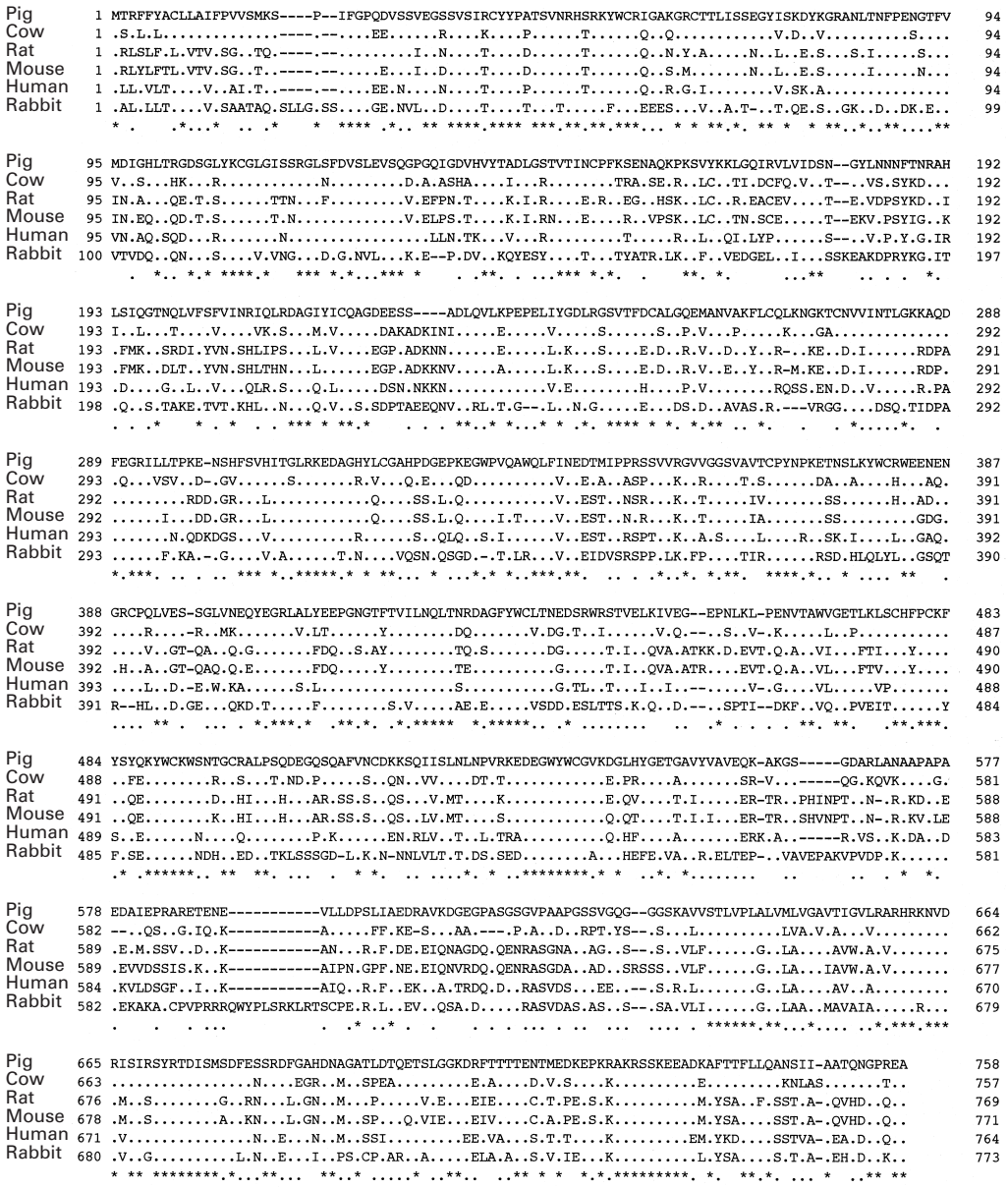

Fig. 3. Amino acid sequence alignment of the corresponding regions of the polymeric IgA receptors for the pig (the present study), human, cow, rabbit, rat and mouse. The receptor sequences referred to in the case of the last five species are those with the respective nucleotide accession nos M24559, X81371, X00412, X 15741 and U06431 in EMBL and Genbank. The dots (.) in the sequence indicate that the amino acid residue is the same as that in the porcine sequence. The asterisks (*) and the dots (.) below the sequence indicate that the amino acid residue is conserved in all or in more than half of the molecules respectively.

gland. In this study, we have determined the nucleotide sequence of porcine pIgR using mRNA from mammary epithelial cells present in a small quantity (1–2 ml) of porcine colostrum. In our experience, the quantity of colostrum or mature milk available depends on the individual pig to be milked. However, the strategy applied

in this study will allow investigation without slaughter of the expression of other milk proteins, transcription factors and so on during lactation, with material of the same origin as in studies focusing on cells in the mammary gland.

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