Identification of two cDNA clones encoding small proline-rich proteins expressed in sheep ruminal epithelium

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Small proline-rich (SPRR) proteins are markers frequently associated with squamous cell differentiation. They have been proposed to be a novel group of precursor polypeptides for the cornified envelope in epidermal keratinocytes. A plus/minus screening procedure was used to identify cDNA clones expressed in mature but not in neonatal sheep ruminal epithelium. Two clones encoding SPRR proteins were identified and are reported here. Clone 27 encodes an ovine SPRR protein corresponding to the human type-II SPRR protein. Clone 26 encodes an ovine SPRR protein similar to human type-II SPRR protein, but which also contains an N-terminal His-Pro repeat similar to the paired repeats found in the *Drosophila* paired proteins. The unique combination of a paired domain and an SPRR protein has not been reported prior to this study. The tissue distribution indicates that specific expression of the genes corresponding to these two clones occurs in the epithelium of the ruminant forestomach, and to a lesser extent in skin epithelium. *In situ* hybridization demonstrated that the SPRR mRNA for both clones were localized in the stratum granulosum, in support of their putative physiological function, i.e. formation of the cornified envelope. Based on Northern blot analysis, mRNA complementary to the two clones appears in the ruminal epithelium by 1 week of age, corresponding to the formation of the stratum granulosum during ruminal epithelial development. The different patterns of changes in amount of mRNA corresponding to these clones during rumen epithelial development indicate that they play different roles in rumen epithelial development.

INTRODUCTION

The small proline-rich (SPRR) proteins are markers frequently associated with squamous cell differentiation, such as in epidermis and airway epithelium [1]. Initially, SPRR genes were identified as UV- and TPA (tissue-type plasminogen activator)-inducible genes in human keratinocytes after differential screening of keratinocyte cDNA libraries [2]. Later it was shown that the expression of SPRR genes was significantly increased during the normal differentiation process of human keratinocytes both *in vivo* and *in vitro* [3,4]. Based on chromosomal organization and nucleotide sequence data of individual SPRR genes, three SPRR subfamilies termed type I, II and III (SPRRI, SPRRII and SPRRIII) were identified, with two different SPRRI loci, seven different SPRRII loci, and one SPRRIII locus, all residing on a 300-kb genomic fragment [5]. The different SPRR genes show a diversity in regulation in response to UV irradiation and phorbol ester treatment. In human keratinocytes, SPRRII gene is both UV- and TPA-inducible, SPRRI gene shows only TPA induction, and SPRRIII gene only UV induction [5]. The function of these SPRR proteins is still not clear. The N- and C-termini of SPRR proteins are similar to the corresponding domains in loricrin and involucrin, suggesting that SPRR proteins constitute a new class of cornified envelope precursor proteins [6]. Marvin et al. [7] characterized cornifin, a proline-rich protein similar in structure to SPRR proteins, demonstrating that cornifin seems to function as a component of the cross-linked envelope in differentiating squamous cells. However, further studies are needed to identify all of the members in each SPRR subfamily (e.g. only three out of seven potential members of the human SPRRII subfamily have been identified), and to clarify the functional diversity of the SPRR gene families and their functions in the differentiation of other types of squamous epithelia.

Mature ruminal epithelium is a stratified keratinized squamous epithelial tissue, with a structure similar to skin, albeit with a higher degree of cornification and keratinization. Despite a structure that resembles a barrier or protective epithelium, the rumen in mature ruminants functions as a major site of nutrient absorption. Papillae on the luminal surface of the rumen serve to increase the surface area for absorption of volatile fatty acids (VFA) produced by the microbial fermentation within the rumen [8,9]. Postnatal ruminal epithelial development could be adopted as a useful model to study the differentiation of stratified keratinized squamous epithelia. The rumen in neonatal ruminants is a non-functional sac that has neither a stratum corneum nor a distinct continuous stratum granulosum [10]. The postnatal development of ruminal epithelium is characterized in part by tissue keratinization, marked growth of papillae on the luminal surface of the rumen, and acquisition of a ketogenic phenotype by the ruminal epithelium. These physical and metabolic developmental changes occur at specific times during the first few weeks of life in lambs [11], resulting in a mature rumen at weaning.

The factors responsible for these rumen developmental changes have not been well characterized. On a gross level ruminal development is known to begin after the initiation of solid feed intake by the neonatal animal and the concomitant establishment of the microbial fermentation and VFA production [12–14]. At present it is not known whether ruminal development occurs in response to a direct or indirect action of the VFA on the ruminal epithelium. Genes which can apparently be regulated by butyrate have been found in several non-ruminant species [15,16], which

Abbreviations used: SPRR(I, II and III), (type I, type II and type III) small proline-rich; PRD-SPRRII, type-II small proline-rich with paired repeat; IFN, interferon; TPA, tissue-type plasminogen activator; VFA, volatile fatty acids; GCG, genetic computer group; RT-PCR, reverse transcriptase-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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suggests that direct action of butyrate (one of the major VFA in the rumen fermentation) on gene expression in the rumen is a possibility. The physical and metabolic changes occurring during rumen development presumably result from changes in the expression of specific genes within the ruminal epithelium. As a first step in the identification of some of those genes and the determination of the mechanisms regulating their expression, several cDNA clones corresponding to genes undergoing differential expression during rumen development have been isolated and characterized [17]. Based on nucleotide sequence analysis, two of these cDNA clones were found to encode SPRR proteins corresponding to the SPRRII subfamily. The peptide encoded by clone 26 also contains a unique N-terminal domain similar to the paired repeat found in the *Drosophila* paired gene [18], and represents a new structural motif for the SPRRII subfamily. Characterization of the gene expression pattern using Northern blot hybridization and *in situ* hybridization supports the proposed function of the SPRR gene family, and indicates the role the corresponding genes may play in ruminal epithelial development.

EXPERIMENTAL

Materials

Restriction endonucleases, alkaline phosphatase, AMV reverse transcriptase and Nick Translation Kits were purchased from Boehringer-Mannheim, Inc. (Indianapolis, IN, U.S.A.). *Taq* DNA polymerase was obtained from Promega, Inc. (Madison, WI, U.S.A.). SuperScript® II reverse transcriptase, *Escherichia coli* strain Y1090 and the plasmid pSPORT1 were from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.). Sephaglas Bandprep Kits were from Pharmacia (Piscataway, NJ, U.S.A.). The [α-^{32}P]dCTP (111 TBq/mmol) and [α-^{35}S]dATP (55.5 TBq/mmol) were from DuPont-New England Nuclear (Boston, MA, U.S.A.). Sequenase (Version 2) DNA sequencing kits were from USB (Cleveland, OH, U.S.A.). The adipsin cDNA clone was a kind gift from Dr. Howard Green.

Animal care

Dorset ram sheep and lambs were maintained under typical farm rearing conditions, with lambs given free access to their dams in the Rutgers University sheep flock. Lambs had *ad libitum* access to alfalfa-grass hay (8 % crude protein) and crimped barley supplemented with a vitamin premix. At birth, lambs were randomly assigned to various age groups (< 0.5 day, and 4, 7, 14, 28, 42, 49, 56 and 84 days of age). Ruminal epithelium was obtained using a protocol approved by the Rutgers University Institutional Review Board on the Care and Use of Animals.

cDNA library construction and plus/minus screening

A directional cDNA library was synthesized using as the template poly(A)$^+$ RNA isolated from the ruminal epithelium of an 84-day-old lamb and cloned into λgt22a [17]. The cDNA library was plated at a density of approx. 30000 plaques/150-mm-diam. Petri dish. Twelve 150-mm-diam. Petri dishes were screened, and duplicate DNA lifts were taken using PlaqueScreen discs from each plate for plus/minus screening. Complementary DNA preparations were synthesized and labelled with [α-^{32}P]dCTP and SuperScript® II reverse transcriptase using as the template poly(A)$^+$ RNA from ruminal epithelium of either an 84-day-old lamb (different from that used to synthesize the library; the plus probe), or from a newborn lamb ($<$ 12-h-old, the minus probe). Prehybridizations and hybridizations were carried out at 65 °C for 4–6 h and 18–24 h respectively, in a solution containing 50 % formamide, 1 × Denhardt's reagent, 1 % (w/v) SDS, 1 M NaCl, 10 % (w/v) dextran sulphate and 100 μg/ml of denatured salmon sperm DNA. Membranes were washed at a final stringency of 65 °C, 1 × SSC (150 mM NaCl, 15 mM sodium citrate) and 1 % SDS for 2 h. Autoradiographs of the DNA lifts probed with the plus and minus probes were compared with identical plaques that only hybridized to the plus probe. Differentially expressed plaques were subjected to secondary screening by differential hybridization with plus/minus probes as before, but at a lower plating density to allow plaque purification. Confirmation that mRNA complementary to these clones was present in greater amounts in mature sheep rumen than in neonatal rumen was obtained by Northern blot hybridization of the cDNA clones as described below. Following plaque purification two of the cDNA clones for which differential mRNA abundance was confirmed were removed from the λgt22a arms by *Not*I and *Sal*I digestion and subcloned into the plasmid pSPORT1.

DNA sequencing and sequence analysis

The DNA sequencing of the cDNA clones was carried out using the dideoxy chain-termination method with Sequenase. Restriction enzyme mapping identified sites used to subclone fragments of the cDNA clone for sequencing. Where necessary, oligonucleotide primers were synthesized to obtain the complete nucleotide sequence. The complementary DNA strands of each cDNA clone were sequenced a minimum of three times. Both DNA databases (GenBank and EMBL) and peptide databases (SwissProt, PIR, GenPept and GBUpdate) were searched for similarity matches to the cDNA sequences and the predicted peptide sequences using the National Center for Biotechnology Information BLAST network services [19]. Sequence comparisons and protein secondary structure prediction were performed using the programs in the Genetics Computer Group (GCG) software package, version 7.2, on a VAX/VMS computer system [20]. The similarity and identity between sequences were determined by the BESTFIT program in the GCG package (gap weight for protein sequences was 3.0, and for DNA sequences was 5.0; gap length weight for protein sequences was 0.1, and for DNA sequences was 0.3).

Reverse transcriptase-PCR (RT-PCR) and analysis of the amplification product

This procedure, based on a protocol described by Ausubel et al. [21] with modifications, was used to amplify a portion of clone 26. Total RNA (10 μg) isolated from mature sheep ruminal epithelium was used as the template. Reverse transcription was carried out at 42 °C for 60 min in a 20 μl reaction volume containing 50 mM Tris/HCl (pH 8.5 at 20 °C), 10 mM $MgCl_2$, 30 mM KCl, 11 mM dithiothreithol, 2 units of RNasin, 1 unit of AMV reverse transcriptase, 1 μM clone 26 3′-primer (5′-ACACTTCGGATCCCATGG-3′), and 1 mM each of dATP, dCTP, dGTP and dTTP. The reaction mixture was then boiled for 5 minutes, and cooled on ice. The subsequent PCR was carried out in a 100 μl reaction volume which contained 20 μl of reverse transcriptase reaction, 50 mM Tris/HCl (pH 9.0 at 25 °C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 1–2 units of *Taq* DNA polymerase and 1 μM clone 26 5′-primer (5′-AAGCATGCCTCACCATCCGCA-3′). Thermocycler conditions were: 95 °C for 4 min, and 52 °C for 1 min; (95 °C for 1 min, and 52 °C for 1 min) × 35; 95 °C for 1 min, and 52 °C for 1 min. The amplification product (220 bp in length) was eluted from an agarose gel. A convenient restriction enzyme cutting site was designed into each primer (*Bam*HI on the 3′ primer, *Sph*I on the 5′ primer). After digestion with these two enzymes, the

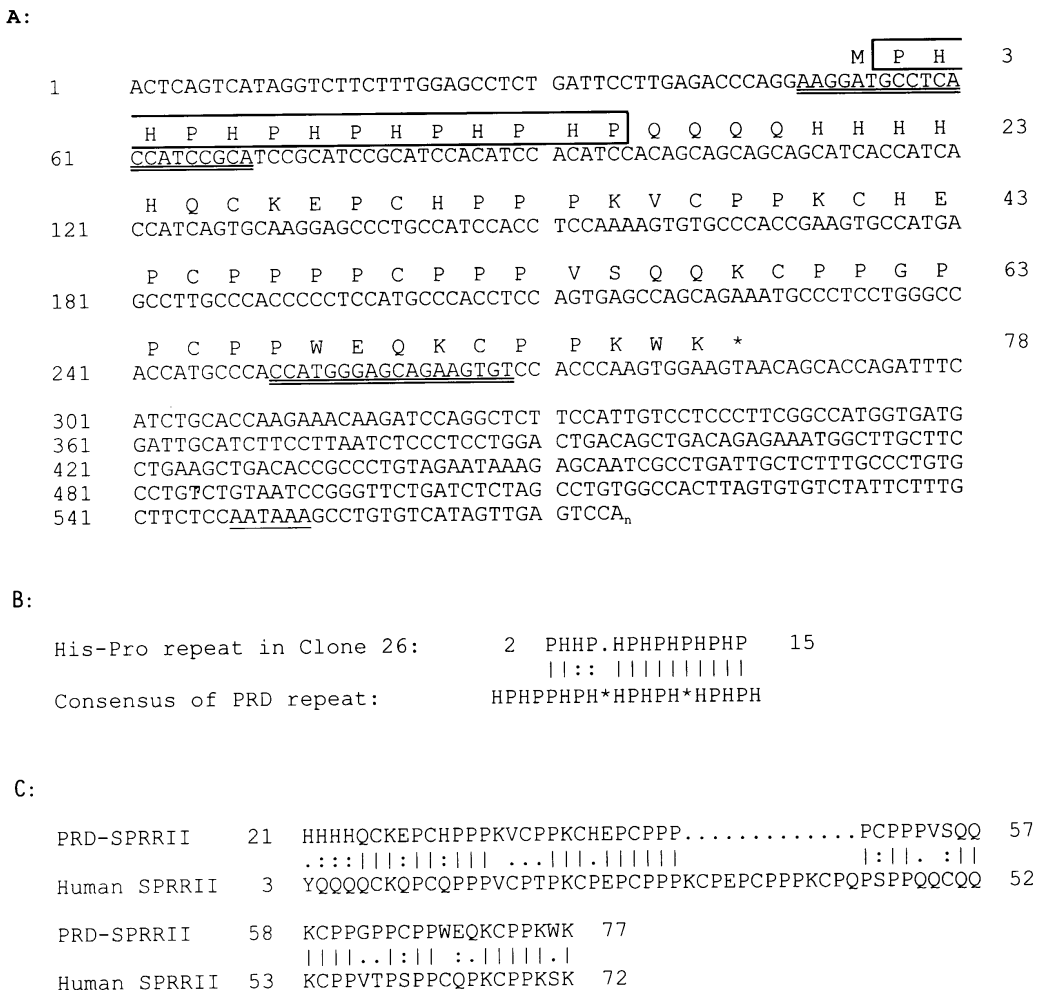


Figure 1 (A) Nucleotide and predicted amino acid sequence of PRD-SPRRII cDNA clone (clone 26) and comparison between (B) the His-Pro repeat of PRD-SPRRII protein and the consensus sequence of PRD repeat(s) and (C) the predicted amino acid sequences of PRD-SPRRII protein and human SPRRII protein

(**A**) The predicted amino acid sequence is presented above the cDNA sequence. Numbers on the left indicate the length of the nucleotide sequence, and numbers on the right indicate the length of the amino acid sequence. The His-Pro repeat which shows high similarity to the PRD repeat of *Drosophila* paired genes is indicated by the box. A possible polyadenylation signal (indicated by the underline) was found 21 nucleotides upstream from the poly(A)$^+$ tail. The design of forward and reverse primers used for RT-PCR are based on the sequences indicated by the double underlines. (**B**) In the consensus sequence of the PRD repeat an asterisk is used to indicate positions where no consensus residues exist among different species of PRD repeats. The similarity between these two sequences is 82%. (**C**) The similarity between these two sequences is 77%.

amplification product was then cloned into pUC119 for sequence analysis. Three clones isolated from independent colonies were sequenced and analysed.

Preparation of total RNA and Northern blot hybridizations

Total RNA was isolated from ruminal epithelium obtained from lambs of different ages (see above), and other tissues (abomasal, omasal and reticular epithelium, perirenal adipose tissue, duodenum, heart, kidney, liver, skin and spleen) obtained from 84-day-old lambs using a procedure described previously [17]. Total RNA was electrophoresed through 1.2% denaturing agarose–formaldehyde gels. Following electrophoresis, gels were routinely photographed before and after blotting. Total RNA was electroblotted on to GeneScreen using a Trans-Blot Cell (Bio-Rad, Hercules, CA, U.S.A.), and cross-linked with a UV Stratalinker (Stratagene, La Jolla, CA, U.S.A.). Blots were probed with cDNA removed from the multiple cloning site of pSPORT1 by *Mlu*I digestion following a procedure described previously [17]. Membranes were routinely stripped and reprobed with a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Clontech (Palo Alto, CA, U.S.A.) to ensure equal loading of RNA. Both autoradiographs and the 18 S rRNA band on the negatives of photographs of agarose gels used for Northern blot analysis were quantified by scanning laser densitometry using an Ultroscan XL laser densitometer and the GSXL2 software package (Pharmacia). A minimum of three blots were analysed, each blot containing RNA from different neonatal and mature animals. The RNA molecular mass markers were co-electrophoresed with the experimental RNA samples. The sizes of RNA transcripts were determined by comparison with an RNA size marker.

In situ hybridization

Rumens were dissected from sheep and lambs as described previously [17]. The rumen wall was dissected into 1 cm × 1 cm squares, frozen in a solid CO_2–ethanol bath within 2 min of

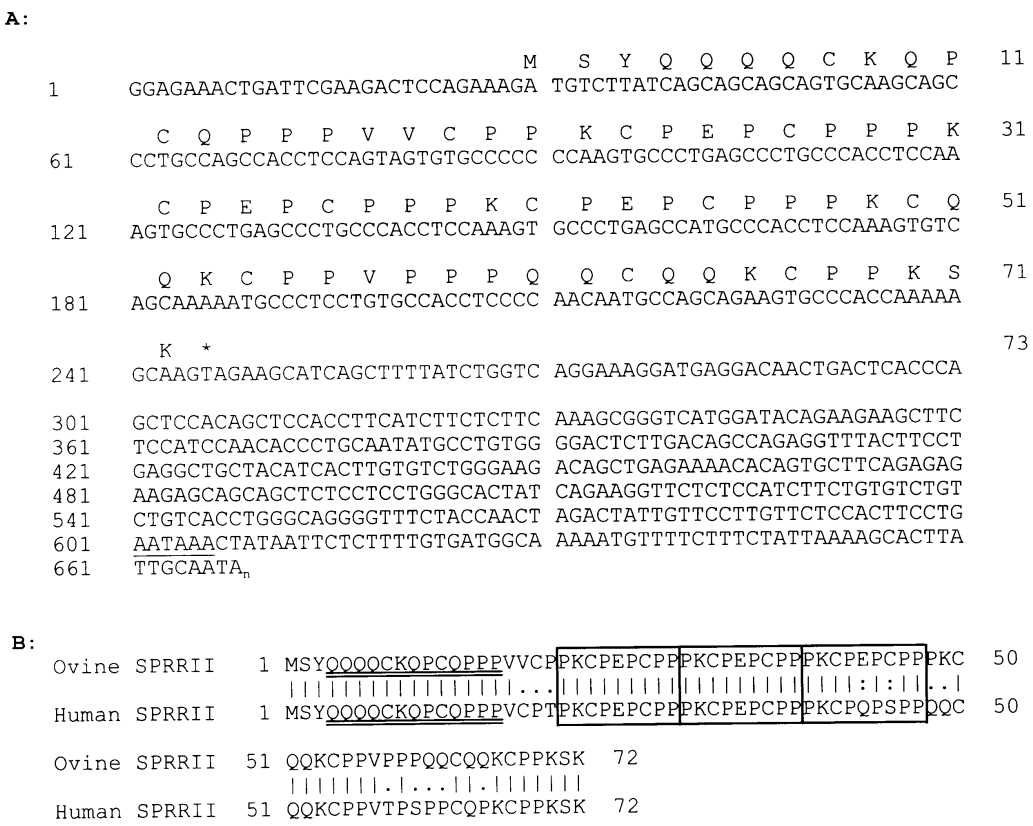


Figure 2 (A) Nucleotide sequence and predicted amino acid sequence of ovine SPRRII cDNA clone (clone 27) and (B) comparison between the predicted amino acid sequences of ovine and human SPRRII protein

(**A**) The predicted amino acid sequence is presented above the cDNA sequence. Numbers on the left indicate the length of the nucleotide sequence, and numbers on the right indicate the length of the amino acid sequence. A possible polyadenylation signal (indicated by the underline) was found 61 nucleotides upstream from the poly(A)$^+$ tail. (**B**) The similarity between the two sequences is 86%. Two characteristic sequences for human SPRRII protein, 'QQQQCKQPCQPPP' (indicated by the double underlines) and a triplicate tandem repeat of 'PKCPEPCPP' (indicated by the boxes), were also found in ovine SPRRII protein.

collection, and kept at −70 °C until cryostat sectioning. Frozen 10 μm sections were thaw-mounted on to gelatin-coated slides and stored at −20 °C until use. *In situ* hybridization was essentially as described by [22]. After fixation in 4% formaldehyde/PBS (0.05 M) for 10 min, slides were rinsed twice in PBS (3 min each) and treated with 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride/0.9% NaCl (pH 8) for 10 min at room temperature. Slides were then dehydrated in sequential ethanol baths: 70% (1 min), 80% (1 min), 95% (2 min) and 100% (1 min), followed by chloroform (5 min), and ethanol 100% (1 min) and 95% (1 min). Slides were subsequently air-dried. The cDNA probes were then labelled by nick translation, using a protocol modified for [^{35}S]dATP incorporation [21]. Reactions were carried out at 15 °C for 1.5 h in a 20 μl volume containing 50 ng of DNA, 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithioerythritol, 2 units of *E. coli* DNA polymerase I, 1.6 m-units of DNase I, 0.02 mM each of dCTP, dGTP and dTTP, and 100 μCi of [α-^{35}S]dATP. The ^{35}S-labelled probes [specific radioactivity: (5–9) × 10^7 c.p.m./μg] were denatured in boiling water for 10 min, immediately cooled on ice, and mixed with hybridization buffer containing 4 × SSC, 50% formamide, 1 × Denhardt's solution, 10% dextran sulphate, 500 μg/ml denatured sheared salmon sperm DNA, 250 μg/ml yeast tRNA and 100 mM freshly made dithiothreitol. Hybridization mixture (50 μl with 4×10^5 d.p.m. of probe) was applied to each section. Hybridization was carried out in a humidified chamber at 37 °C for 18–20 h. The slides were washed in 1 × SSC at 55 °C four times (15 min each), and twice at room temperature (30 min each), then dehydrated with 70% ethanol and air dried. The slides were exposed to Amersham Hyperfilm overnight to determine the proper exposure time for emulsion autoradiography. Slides were coated with Amersham LM-1 emulsion, and exposures were carried out for 2 to 4 weeks at 4 °C in boxes containing drying agent. After development, slides were counter-stained with Toluidine Blue (0.2%) and photographed under both bright- and dark-field illumination. To check the specificity of hybridization, an adipsin cDNA probe, which is not expressed in ruminal epithelium, was used as a negative control.

RESULTS

Identification of clones 26 and 27 by sequence analysis and RT-PCR

A plus/minus screening procedure was used to isolate cDNA clones corresponding to mRNA which is highly abundant in mature ruminal epithelium but is not abundant in neonatal ruminal epithelium. Characterization of two of those clones, clones 26 and 27, is reported here.

The complete nucleotide sequence (574 nucleotides) and the predicted amino acid sequence of clone 26 are given in Figure 1(A). Northern blot hybridizations indicate that the corresponding mRNA is around 800 nucleotides in length. Thus, the cDNA

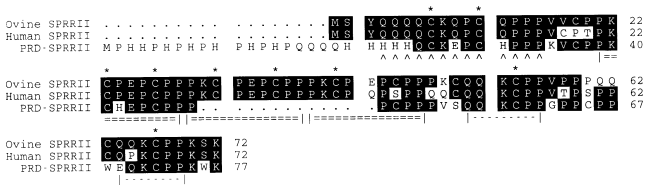
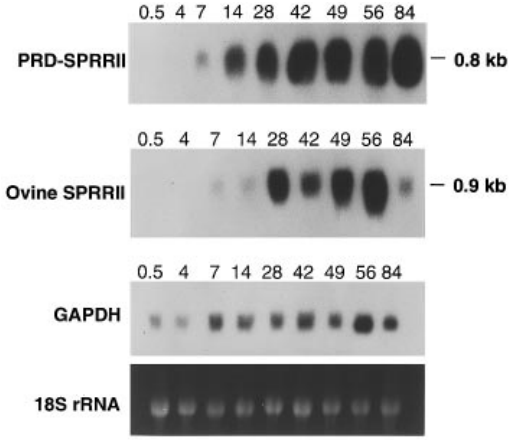


Figure 3 Alignment of amino acid sequences of ovine and human SPRRII, and ovine PRD-SPRRII proteins using the GCG PILEUP and PRETTYBOX programs

Conserved positions for cysteine residues found across the three sequences are indicated by an asterisk. The ' ∧ ' is used to indicate the characteristic N-terminal sequence of SPRR proteins. The C-terminal repeats are indicated by '|−−−|'. Characteristic repeats in SPRRII proteins are indicated by '|===|'.



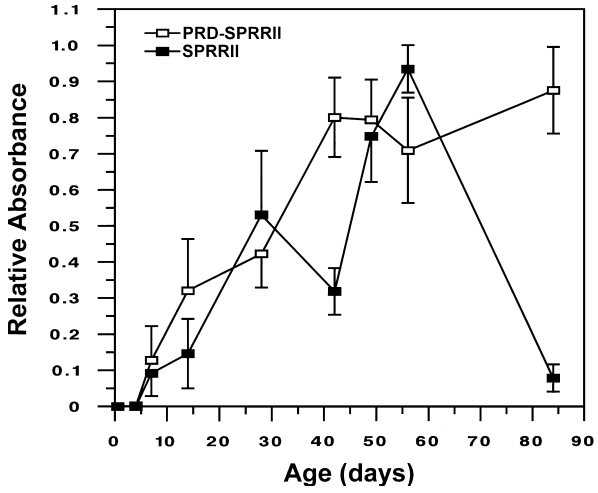


Figure 4 Time-course of appearance of SPRRII and PRD-SPRRII mRNA

Upper panel: triplicate blots containing total rumen RNA from three different animals at each of the indicated ages were hybridized with PRD-SPRRII and ovine SPRRII cDNA probes. A representative autoradiograph for each clone is shown. The estimated length of the mRNA corresponding to each cDNA clone is indicated to the right of the corresponding blot. Each lane contains total RNA (30 μg) isolated from the rumens of lambs of different ages: 0.5, 4, 7, 14, 28, 42, 49, 56 and 84 days. Membranes were routinely stripped and reprobed with a cDNA probe for GAPDH to ensure equal loading of RNA. (One representative autoradiograph is shown here.) The 18 S rRNA band from the corresponding agarose gels was also used to ensure equal RNA loading (one representative photographic negative of an agarose gel is shown here). Lower panel: the relative absorbances of autoradiographs from triplicate blots for each clone were determined using laser densitometry (PRD-SPRRII, □; SPRRII, ■), after normalizing against the absorbance values of 18 S rRNA bands on the photographic negatives of the corresponding agarose gels for each Northern blot. The maximum absorbance value was designated as 1.0. The relative absorbance values for the other lanes were expressed as a percentage of this maximum absorbance value. Standard errors of the cumulative results are also presented.

insert of clone 26 probably represents a near full-length copy of the corresponding mRNA. The predicted start codon ATG (nucleotides 53–55) is preceded by an in-frame stop codon TGA (nucleotides 38–40), which indicates that the ATG is the start of the putative encoded protein. There were no other significant open reading frames encoded by this sequence. A potential polyadenylation signal was found 21 nucleotides upstream from the poly(A)$^+$ tail.

A 14-amino-acid sequence at the N-terminus was found that exhibited 82 % similarity to the paired (PRD) repeat, a histidine-proline repeat of about 18 amino acids (Figure 1B), that typically appears in *Drosophila* paired genes [18,23] which function in the formation of body segmentation during embryonic development in *Drosophila* [23]. The remaining portion of clone 26 encodes an amino acid sequence that resembles (77 %) human type-II SPRR protein [2,5]. The similarity between the peptide encoded by clone 26 and SPRRI protein [1] is only 24 %. The relationship between the predicted amino acid sequence of clone 26 and human SPRRII protein is shown in Figure 1(C).

When two such disparate sequences (PRD repeat and SPRRII sequence) are found together in a cDNA clone, the question arises as to whether or not that clone might not be an artifact of the library construction. To address that question two oligonucleotide primers were synthesized which hybridize to opposite strands of clone 26, as indicated by the double underlined sequences in Figure 1(A). These oligonucleotides served as the 5′ and 3′ primers for RT-PCR using total rumen RNA from a mature sheep as the template. The RT-PCR produced a DNA fragment of the expected size (220 bp), which was then directionally cloned into pUC119 using convenient restriction cutting sites designed into the primers. The sequence of this subcloned RT-PCR product is exactly the same as the corresponding sequence on the cDNA clone, indicating that clone 26 (termed PRD-SPRRII protein) is not an artifact, but is legitimately expressed in the rumen epithelium.

The complete nucleotide sequence and the predicted amino acid sequence for clone 27 are given in Figure 2(A). A possible polyadenylation signal was found 61 nucleotides upstream from the poly(A)$^+$ tail. Clone 27 encodes a polypeptide (72 amino acids) that exhibits 87 % similarity to human SPRRII protein (72 amino acids) across the entire sequence (Figure 2B). Three tandem repeats of the nonamer 'PKCPEPCPP', a typical characteristic of human SPRRII protein, were found in the polypeptide encoded by clone 27. The signature sequence 'QQQQCKQ-PCQPPP' (with some conserved substitutions) which appears in all known SPRR proteins [5] was also located at the N-terminus of the clone 27 polypeptide. All of these findings indicate that clone 27 is an ovine SPRRII protein.

Because clone 26 and clone 27 are both related to SPRRII protein, they were aligned with human SPRRII protein to identify consensus sequences and conserved patterns among these three peptides (Figure 3). The results of the comparison showed a highly conserved cysteine distribution pattern across all three polypeptides: 'C-X_3-C-X_{10}-C-X_3-C-(X_2-C-X_3-C-X_4-C-X_{12})-C-X_{12}-C'. The region within the parentheses is used to indicate the gap area inserted to allow alignment of the PRD-SPRRII

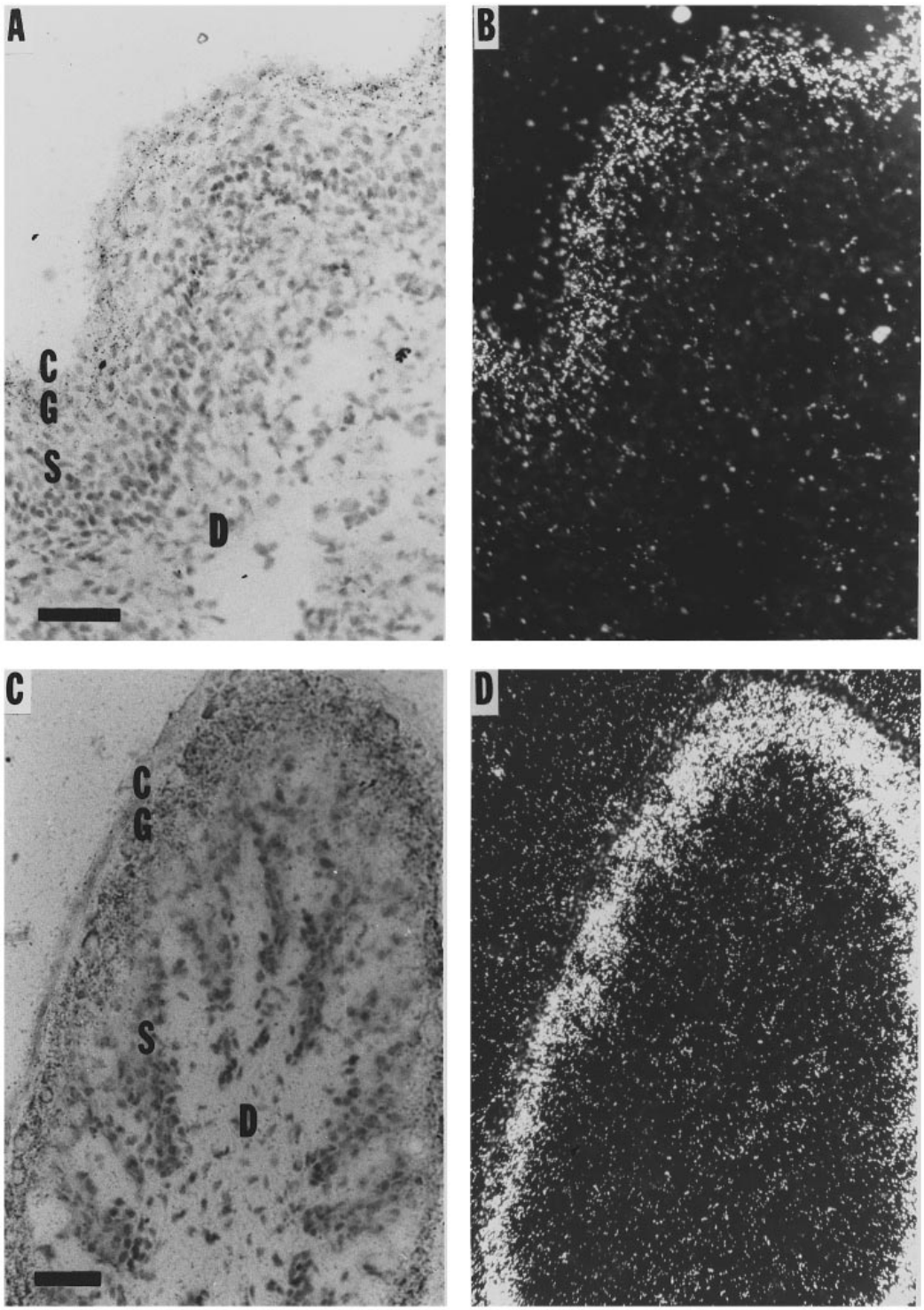


Figure 5 ***In situ*** **localization of PRD-SPRRII mRNA in sheep ruminal epithelium**

Hybridizations to rumen epithelial tissue sections obtained from a 14-day-old lamb (**A** and **B**) and from an 84-day-old lamb (**C** and **D**) are shown here. Bright-field photomicrographs are provided in (**A** and **C**) as an aid to identification of the cell types in the corresponding dark-field photomicrographs (**B** and **D**). The PRD-SPRRII mRNA was only detected in the stratum granulosum. The small bar in the bottom left corner of (**A**) and (**C**) represents 50 μm on the tissue section. Abbreviations: C, stratum corneum; G, stratum granulosum; S, stratum spinosum; and D, dermis.

sequence, and the conserved cysteine distribution pattern in this specific area is determined based on the ovine and human SPRRII sequences. Within the consensus sequence, proline, lysine, glutamine and cysteine are the four major amino acids. A hexamer 'QQKCPP' with certain conservative substitutions (Q to E, and Q to P) was repeated twice at the C-terminus in all three sequences (Figure 3). The characteristic repeat sequence of SPRRII protein 'PKCPEPCPP' [5] was found only once in PRD-SPRRII protein, while it was repeated three times in ovine and human SPRRII protein. The SPRR signature sequence

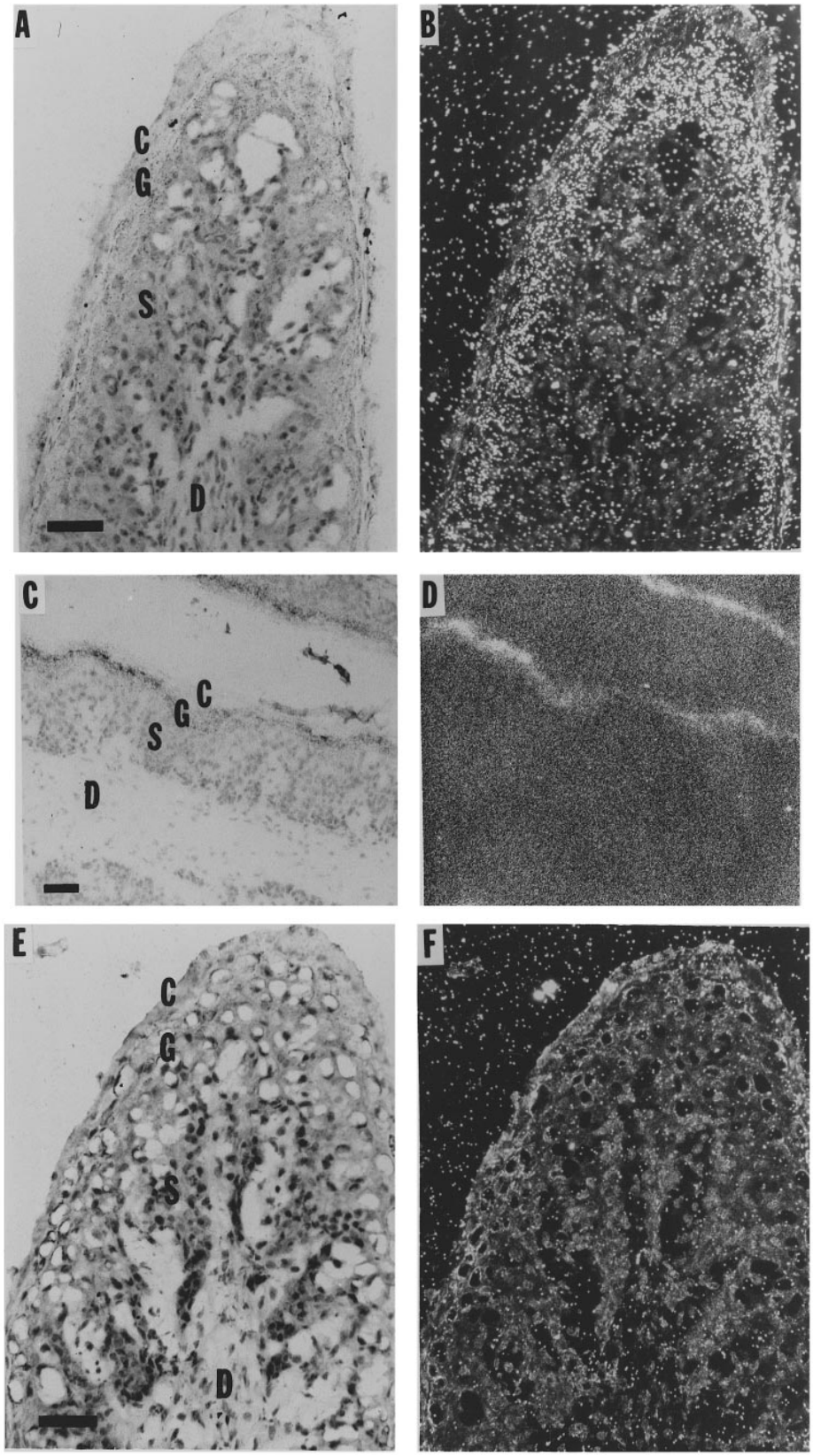


Figure 6 ***In situ*** **localization of SPRRII mRNA in sheep ruminal epithelium**

Hybridizations to rumen epithelial tissue sections obtained from a 56-day-old lamb (**A**, **B**) and from an 84-day-old lamb (**C**, **D**) are shown here. Hybridizations of the negative control probe (adipsin cDNA probe) to tissue sections obtained from an 84-day-old lamb (**E**, **F**) are also presented here. Bright-field photomicrographs are provided in **A**, **C**, and **E** as an aid to identification of the cell types in the corresponding dark-field photomicrographs (**B**, **D**, and **F**). The SPRRII mRNA was only detected in the stratum granulosum. The small bar on the bottom left corner of (**A**), (**C**) and (**E**) represents 50 μm on the tissue section. Abbreviations: C, stratum corneum; G, stratum granulosum; S, stratum spinosum; and D, dermis.

'QQQQCKQPCQPPP' was also located in PRD-SPRRII, but with some conservative substitutions: 'HHHHQCKEPCHPPP'.

Time-course of appearance of PRD-SPRRII and SPRR mRNA

In order to determine the time-course of appearance of the PRD-SPRRII and SPRRII mRNA, Northern blot analysis was conducted using total RNA isolated from sheep ruminal epithelium at birth (< 0.5 day of age), and 4, 7, 14, 28, 42, 49, 56 (weaning) and 84 days of age (Figure 4, upper panel). The average relative absorbance values obtained from three blots probed with the two clones are also presented in Figure 4 (lower panel), after normalizing against the absorbance value of the 18 S rRNA bands on the photographic negatives corresponding to each RNA gel. Messenger RNA complementary to the PRD-SPRRII and ovine SPRRII clones cannot be detected in ruminal epithelium until 4 to 7 days after birth. The amount of mRNA complementary to PRD-SPRRII clone increases to reach a plateau between 42 and 84 days after birth. In contrast, the level of ovine SPRRII mRNA reaches a peak between 49 and 56 days after birth, but decreases dramatically by 84 days of age to a level comparable with that observed at 7 days of age.

In situ localization

In situ hybridization was used to determine the spatial expression patterns of these two SPRR genes within ruminal epithelium. The genes encoding the PRD-SPRRII and SPRRII proteins have a very similar expression pattern across the cell strata during ruminal epithelial development (Figures 5 and 6). In agreement with the Northern blot analysis, the PRD-SPRRII and SPRRII mRNA cannot be detected until 14 days after birth using *in situ* hybridization. The hybridization signal for PRD-SPRRII mRNA is located within the primary stratum granulosum of rumen epithelium obtained from 14-day-old lambs (Figures 5A and 5B) and remains restricted to the stratum granulosum throughout rumen development (Figures 5C and 5D). The hybridization signal for SPRRII mRNA also remains restricted to the stratum granulosum throughout rumen development (Figures 6A, 6B, 6C and 6D). A similar spatial expression pattern of SPRRII within rumen epithelium was observed for 56- and 84-day-old lambs (Figures 6A, 6B, 6C and 6D). Unlike the results of the Northern blot hybridization, the intensity of signal observed with *in situ* hybridization of the SPRRII cDNA to rumen epithelium from 84-day-old lambs (Figures 6A and 6B) does not seem significantly different from that observed with rumen epithelium from 56-day-old lambs (Figures 6C and 6D). However, unlike Northern blot hybridization, it is generally difficult to quantitatively analyse the results of *in situ* hybridization due to the lack of internal control. The negative control probe (adipsin cDNA probe) did not hybridize to rumen epithelium (Figures 6E and 6F).

Tissue distribution pattern of PRD-SPRRII and SPRRII mRNA

Total RNA was isolated from rumen, abomasal, omasal and reticular epithelium, perirenal adipose tissue, duodenum, heart, kidney, liver, skin and spleen, which were obtained from 84-day-old lambs. Only the epithelium of the ruminant forestomach compartments (i.e. rumen, reticulum and omasum) contained high levels of mRNA complementary to the two clones (Figure 7). Small amounts of hybridizable mRNA were also found in sheep skin for the two SPRR cDNA clones, although the amount of mRNA complementary to ovine SPRRII clone was barely detectable (Figure 7). These results suggest that the expression of PRD-SPRRII and SPRRII genes is confined to stratified keratinized squamous epithelia.

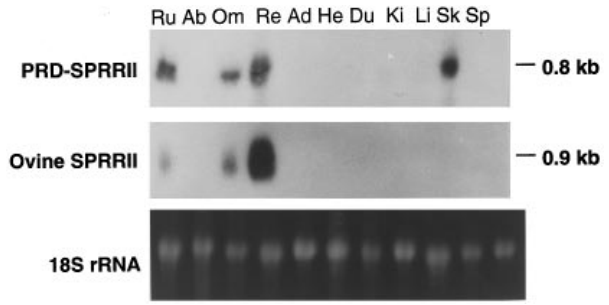


Figure 7 Tissue distribution of SPRRII and PRD-SPRRII mRNA

Triplicate blots containing total RNA isolated from different tissues (Ru, rumen; Ab, abomasum; Om, omasum; Re, reticulum; Ad, perirenal adipose tissue; He, heart; Du, duodenum; Ki, kidney; Li, liver; Sk, skin; and Sp, spleen) obtained from three 84-day-old lambs were hybridized with PRD-SPRRII and ovine SPRRII cDNA probes. A representative autoradiograph for each clone is shown. The estimated length of the mRNA corresponding to each cDNA clone is indicated to the right of the corresponding blot. Each lane contains 30 μg of total RNA. The 18 S rRNA band from the corresponding agarose gel was also used to ensure equal RNA loading (one representative photographic negative of the agarose gel is presented here).

DISCUSSION

The current results suggest that a unique member of the SPRR protein family (PRD-SPRRII protein) has been identified. The SPRR proteins have been suggested to constitute a previously unidentified class of precursor proteins involved in the formation of the cornified envelope found in stratified epithelia [5]. The results in this study support this proposed function; however, the functional diversity of the SPRR gene family needs to be clarified in further studies.

This study demonstrates that the expression of SPRR mRNA is restricted to the stratum granulosum. In human epidermal epithelium, SPRR proteins were located in the stratum granulosum and in several top cell layers of the stratum spinosum [3]. Thus, evidence at both the mRNA level (this study) and the protein level [3] indicates that the expression of these genes is initiated after cells undergo terminal differentiation. Since members of the SPRR protein family were proposed to function as precursors of the cornified envelope [6], it is reasonable to expect expression of SPRR genes to occur after the terminal differentiation of epithelial stem cells, but before the formation of the cornified envelope. This would be in cells lying between the cells of the stratum basale and the stratum corneum. The result of the *in situ* hybridization results in this study supports the hypothesized function of the SPRR family. Since the SPRR mRNAs are expressed only after cells migrate to the stratum granulosum (Figures 5 and 6), these mRNA could be used as markers to identify cells exhibiting squamous-differentiated phenotypes or terminal differentiation. Previous studies suggested that in normal human epidermal keratinocyte cells, irreversible growth arrest precedes the expression of the squamous-differentiated phenotype [24]. Thus, irreversible growth arrest should precede the appearance of SPRR mRNA.

The histological changes occurring in developing ruminal papillae have been well characterized [10]. The appearance of SPRR mRNA during ruminal epithelial development corresponds well to the histological changes of ruminal epithelium. In ruminants younger than 1 week of age, no continuous stratum corneum is observed along the papillae. Granular cells are also not distinctively identifiable. Based on the time course of appearance of PRD-SPRRII and SPRRII mRNA and the results

of the *in situ* hybridizations, the expression of the SPRR genes in the rumen is initiated with the appearance of the granular layer, about 1 week after birth.

Apparently different roles are played by these functionally related SPRR proteins during ruminal epithelial development. For example, the SPRRII protein may be necessary for rapid initial growth of the ruminal epithelium, since the SPRRII mRNA level drops dramatically after allometric growth [25] of the lamb rumen between 28 and 42 days. The PRD-SPRRII protein may function both in ruminal growth and maintenance, since the PRD-SPRRII mRNA level maintains a plateau during and after the period of allometric growth. The different expression pattern of the SPRRII and PRD-SPRRII genes indicates the complexity of individual function of the members of the SPRR family, which will be a challenge for future research.

The function for the PRD-specific His-Pro repeat in paired proteins is unknown. A similar His-Pro repeat pattern was also identified in the proline-rich domain of a rabbit proacrosin-related peptide [26]. A corresponding proline-rich domain in human and boar proacrosin was suggested to be involved in the recognition and binding of the spermatozoa to the zona pellucida of the ovum [27]. This domain also has a potential SH3 binding site [28]. The His-Pro repeat forms a secondary structure containing repetitive turns [29], followed by a hydrophilic region with no turn. This characteristic is true for the His-Pro repeat domain in proacrosin, the *Drosophila* paired protein, and ovine PRD-SPRRII protein (results not shown). Proline residues have been demonstrated to serve in binding or scaffolding roles in protein–protein interactions [28]. The role of the His-Pro repeat domain in protein–protein interactions is beyond the scope of this paper; however, it will be a future challenge to elucidate the function of this domain in the PRD-SPRRII protein.

The conserved distribution pattern of cysteine residues across human and ovine SPRRII protein and PRD-SPRRII protein indicates the important role of cysteine in these proteins. One possible function of cysteine is the binding of metal ions [30]. The four conserved cysteines at the N-terminus ($C\text{-}X_3\text{-}C\text{-}X_{10}\text{-}C\text{-}X_3\text{-}C$) could form a potential tetrahedral co-ordination configuration for metal ion binding. Cysteine can also function in the formation of disulphide bonds, resulting in dimerization or polymerization of proteins [31]. A related protein, SPRRI, has a molecular mass based on SDS/PAGE that is almost double the predicted molecular mass of the protein [3], and dimerization of the SPRRI protein could be responsible for this migration pattern.

The tissue distribution of SPRRII and PRD-SPRRII mRNAs demonstrated specific expression of the corresponding genes in the epithelium of the ruminant forestomach (i.e. rumen, reticulum and omasum) and epidermal epithelium (Figure 7). The luminal surface of the abomasum is lined by simple columnar epithelium instead of the keratinized stratified squamous epithelium which lines the three compartments of the ruminant forestomach [32]. The difference in gene expression between the abomasum and the ruminant forestomach (Figure 7) reflects the differences between their functions and their histological characteristics. At the same time, the consistent expression of these genes in ruminant stomach and skin epithelia is consistent with the histological similarity between these epithelia, which are both stratified keratinized squamous epithelia.

To understand further the regulation of ruminal epithelial development, especially in relation to the expression of SPRR genes, it will be necessary to isolate the corresponding genomic clones and characterize the gene structure and promoter regions. In human keratinocytes, the promoters for SPRRI gene [1] and some members of the SPRRII gene family [4] have been isolated. Protein kinase C and probably protein kinase A play important roles in the regulation of the transcription of the SPRRI gene [1]. Further study on promoters driving the expression of SPRRII and PRD-SPRR will help to elucidate the general mechanism of squamous epithelial differentiation.

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