The Small Proline-Rich Proteins Constitute a Multigene Family of Differentially Regulated Cornified Cell Envelope Precursor Proteins

Daniel Hohl, Pierre A. de Viragh, Françoise Amiguet-Barras, Susan Gibbs,* Claude Backendorf,* and Marcel Huber

Laboratory of Skin Biology, Department of Dermatology, University Hospital, Lausanne, Switzerland; and *Laboratory of Molecular Genetics, University of Leiden, The Netherlands

Loricrin, involucrin, small proline-rich protein (SPRR)1, SPRR2, and SPRR3 genes are located within a cluster of 1.5 Mbp on chromosome 1q21 and most likely evolved from a common ancestor. Monospecific polyclonal antibodies and cDNA probes were produced to investigate SPRR transcripts and proteins. SPRR expression was restricted to terminally differentiating squamous cells, preferentially located at the cell periphery, and immunoreactivity was greatly reduced in cells with a mature cornified cell envelope. Furthermore, detectable SPRR2 and SPRR3 levels were strongly increased in differentiating keratinocyte cultures after addition of LTB-2, a specific inhibitor of transglutaminases, suggesting that they are precursor proteins of the cornified cell envelope. In normal epidermis, SPRR1 was restricted to appendageal areas, SPRR2 was expressed coherently, and SPRR3 was completely absent. In the upper digestive tract, SPRR1 was expressed in sublingual and tongue epithelium, SPRR2 was mostly restricted to lingual papillae, and SPRR3 was abundant in oral and esophageal epithelium. In psoriatic epidermis, SPRR1 and SPRR2 were expressed at much higher levels than in normal epidermis. Addition of 10^{-7} M retinoic acid to cultured differentiating keratinocytes significantly down-regulated the expression of SPRR2 and SPRR3 transcripts and slightly decreased that of SPRR1. Thus, SPRR1, SPRR2, and SPRR3 are differentially expressed *in vivo* and *in vitro*, suggesting that the SPRR multigene family evolved to serve as highly specialized cornified cell envelope precursor proteins in stratified epithelia. *Key words: keratinocyte/epidermis. J Invest Dermatol 104:902–909, 1995*

The cornified cell envelope (CE) is the most insoluble component of stratified squamous epithelial cells and appears as an electron-dense and homogeneous "marginal" band replacing the plasma membrane in the uppermost cell layers of squamous epithelia in vertebrates [1,2]. CE formation occurs as a complex but highly orchestrated sequence of events involving the sequential deposition of distinct proteins, with a gradual progression of envelope thickness and rigidity [1,2]. These proteins are cross-linked into an insoluble mesh by the formation of N^{ϵ}-(γ-glutamyl)lysine isodipeptide and disulfide bonds [1–3]. Acyltransfer during N^{ϵ}-(γ-glutamyl)lysine cross-linkage is catalyzed by transglutaminases TGK (synonym TG1) and TGE (synonym TG3) [4]. Several proteins including involucrin, loricrin, sciellin, pancornulins, small proline-rich proteins (SPRR), cornifin, cystatin A (synonym keratolinin), the 210-kD and 195-kD proteins, and others have been implicated as CE precursor proteins [1–3].

SPRR are small and proline-rich proteins expressed in terminally differentiating human keratinocytes [5,6]. They contain a variable number of repeating elements in their central portions with a common sequence motif (XKXPEPXX) [7] and end domains having significant homology with the N- and C-terminal domains of loricrin and involucrin [8]. SPRR genes constitute a multigene family clustered within a 300-kb DNA segment on chromosome 1q21 [7] close to the loricrin and involucrin genes [9]. This cluster contains approximately eight SPRR2 genes, two SPRR1 genes, and a single SPRR3 gene [7]. SPRR gene structure is similar to that of loricrin and involucrin [10,11], consisting of two exons with the second exon containing a complete open reading frame [7]. On the basis of these similarities and the fact that mature CEs have a relatively high proline content of 7.7% to 13.7% [1,2], we hypothesized [8] that SPRRs might be identical to the proline-rich CE constituent postulated 3 decades ago by Matoltsy and Matoltsy [12]. In fact, a protein expressed in cultured tracheal epithelial cells from rabbits [13], pigs [14], and monkeys [15], also termed cornifin, has recently been reported to be the homologue of human SPRR1 [16–18] and was suggested to be a CE precursor protein [16].

In the present study, we provide biochemical evidence that SPRR2 and SPRR3 are also CE precursor proteins, and we detail the expression characteristics of the SPRR gene family in human keratinocytes.

MATERIALS AND METHODS

Tissues Tissues were snap-frozen in liquid nitrogen or fixed immediately after excision either for 48 h in freshly prepared phosphate- and saline–

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Reprint requests to: Dr. Daniel Hohl, Laboratory of Skin Biology, Department of Dermatology, CHUV, CH-1011 Lausanne, Switzerland.

Abbreviations: CE, cornified cell envelope; SPRR, small proline-rich protein.

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buffered 4% paraformaldehyde for *in situ* hybridization or for 24 h in Teller-Nitzky solution (formaldehyde 2%, acetic acid 5%, ethanol 65% in H_2O) at room temperature for immunohistochemistry. After fixation, the tissues were dehydrated and embedded in paraffin.

Cell Culture Normal human keratinocytes obtained from breast epidermis were grown submerged in modified MCDB 153 medium (KGM; Clonetics Corp., San Diego, CA) on plastic (Falcon Labware, Oxnard, CA) essentially as described previously [19,20]. Retinoic acid (RA; Hoffmann-LaRoche Inc., Nutley, NJ) and LTB-2 (Syntex Research, Palo Alto, CA) were added to the medium just before use. All experiments were carried out at least in triplicate.

Keratinocytes were grown to 90% confluence in 0.05 mM Ca^{++} medium, shifted to 0.35 mM Ca^{++} medium, and harvested 8 d after the Ca^{++} shift. To test the effects of LTB-2, an inhibitor of epidermal transglutaminases [21], on SPRR expression, we grew keratinocytes to 45% confluence in 0.05 mM Ca^{++} medium, shifted them to 0.25 mM Ca^{++} medium with or without 0.1 mM LTB-2, and harvested the cells after 5 d. To test the effects of RA on SPRR expression, keratinocyte cultures grown to 90% confluence in 0.05 mM Ca^{++} were shifted to 0.35 mM Ca^{++} medium containing 0.1 mM LTB-2 and either 0.01% dimethylsulfoxide (solvent control) or 10^{-7} M RA, and harvested after 8 d. To test the time course of RA-mediated suppression of SPRR mRNA after differentiation had been initiated, we grew keratinocytes in 0.15 mM Ca^{++} to 80% confluence; shifted them to 0.35 mM Ca^{++} with or without RA, added to the medium to 10^{-7} M at day 0, 3, 5, 7, or 7.5; and harvested all cells at day 8.

Antibodies SPRR1 antibodies were those produced by Kartasova *et al* [6] and were used as described previously. Monospecific polyclonal antibodies to synthetic peptides of SPRR2 (PKCPEPCPPPKCPQPCPP) and SPRR3 (IKVPDQGFIKFPEPGA) coupled to keyhole limpet hemocyanin were prepared in white New Zealand rabbits essentially as described previously [22,23]. Antisera 1B anti-SPRR2 and 4A anti-SPRR3 were selected because of their monospecificity and were used at a dilution of 1:200 or 1:500 for immunostaining and 1:100 or 1:200 for immunoblotting.

Construction of HeLa Cells Expressing SPRR cDNAs SPRR1, -2, and -3 cDNAs were subcloned into expression vector pECV24 [24]. The neo resistance gene on pECV24 was excised with *Kpn*I and *Xho*I and replaced by the respective SPRR cDNA sequences [5,7], generating pESPR1, pESPR2, and pESPR3. The SPRR1 cDNA was excised from clone 15B [5] with *Kpn*I (5′) and *Hind*III (3′) and subcloned into pIC-20R [25], generating pIC-15B. From this construct, the SPRR1 cDNA was excised with *Kpn*I (5′) and *Xho*I (3′) and introduced in correct orientation into pECV24. The SPRR2 cDNA was excised from clone 930 [5] with *Kpn*I (5′) and *Eco*RI (3′) and subcloned into pIC-20H, generating pIC-930. From this construct, the SPRR2 cDNA was excised with *Kpn*I (5′) and *Xho*I (3′) and introduced in correct orientation into pECV24. The SPRR3 cDNA was excised from λ-SPR3 [7] by *Hind*III and further digested with *Sau*3A. The *Sau*3A (5′) *Hind*III (3′) fragment was subcloned into pIC-20R, generating pIC-spr3-2. From this construct, the SPRR3 cDNA was excised with *Kpn*I (5′) and *Xho*I (3′) and introduced in correct orientation into pECV24. The pESPR1, pESPR2, and pESPR3 constructs were transfected into HeLa cells with calcium phosphate [26], and positive clones were selected with hygromycine [24].

Extraction of Proteins and Immunoblotting Cells were harvested by scraping into lysis buffer containing 0.25 M Tris HCl (pH 7.5), 5% sodium dodecylsulfate (SDS), and 20% β-mercaptoethanol and heated at 95°C for 5 min. After separation on SDS-polyacrylamide gels (12% or 13.5%), the proteins were either stained with Coomassie blue or transferred electrophoretically to nitrocellulose membranes, stained with Ponceau S, destained, incubated with antibodies, and finally stained by the alkaline phosphatase technique according to the manufacturer's instructions (Biorad, Richmond, CA).

Immunohistochemistry On fixed tissue sections, antibodies were routinely incubated for 16 h without prior protease treatment, visualized using the immunogold-silver–enhanced staining technique according to the manufacturer's protocol (Auroprobe LM; Amersham International, UK), and usually counterstained with hematoxylin. Alternatively, antibodies were visualized using fluorescein-isothiocyanate–labeled secondary antibodies (Dako, Denmark) for indirect immunofluorescence microscopy.

Riboprobes, *In Situ* Hybridization, and Slot Blot Analysis Fragments of SPRR1, -2, and -3 and loricrin cDNAs [5,7,27] were subcloned into pGEM-7Zf+ (Promega, Madison, WI). For SPRR1, a 440-bp *Eco*RI fragment from pIC-15B (see above) was then subcloned into the corresponding site of pGEM-7Zf+. For SPRR2, a 700-bp fragment was excised from clone 930 [5] with *Rsa*I and cloned into the *Sma*I site of pGEM-7Zf+.

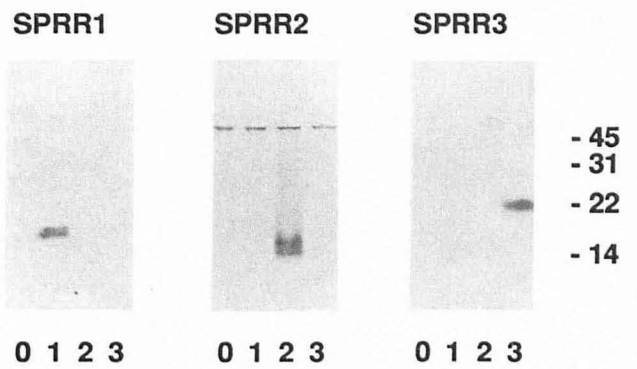

Figure 1. SPRR antibodies show monospecificity. Immunoblots of total protein extracts with antibodies directed against SPRR1, SPRR2, and SPRR3. *Lane 0*, HeLa cells; *lane 1*, HeLa cells expressing SPRR1; *lane 2*, HeLa cells expressing SPRR2; *lane 3*, HeLa cells expressing SPRR3.

For SPRR3, a 320-bp *Hae*III-*Hin*fI fragment from λspr3A [7] was subcloned blunt-ended into the *Sma*I site of pIC-19H [25]. From the resulting plasmid, a 340-bp *Hind*III fragment was isolated and subcloned into pGEM-7Zf+. The orientation of the pGEM-7Zf+ constructs was verified by restriction analysis. The SPRR1 and SPRR2 probes contain the whole cDNA insert, and the SPRR3 probe consists of the repeating region from nucleotides +257 to +597 [7]. To obtain anti-sense probes and sense controls, plasmids were linearized and transcribed with SP6 and T7 RNA polymerase using digoxigenin-labeled UTP (Boehringer Mannheim, Germany) as described [28]. *In situ* hybridization was performed as described [29] and modified [28].

For slot blot analysis, 1 μg plasmid DNA of pGEM-7Zf+ containing full-length SPRR1, SPRR2, or SPRR3 fragments or pGEM-7Zf+ alone was denatured, neutralized, and applied to a Zetaprobe membrane (Biorad) according to the manufacturer's protocol. Hybridization was performed as described for Northern blot analysis.

RNA Extraction and Northern Blot Analysis Total RNA was isolated [30], separated, transferred, and fixed to membranes according to established techniques [31]. Equal loading and quality of 13 μg of RNA each were assured by measurement of optical density at 260 nm before loading and by ethidium bromide staining after separation. cDNA fragments were excised from the pGEM-7Zf+ constructs used for riboprobe synthesis, isolated, and labeled with ^{32}P-dCTP by random priming [32]. Prehybridization and hybridization were performed at 65°C in 7% SDS, 0.25 M sodium phosphate pH 7, 1 mM ethylenediamine tetraacetic acid, and salmon sperm DNA 100 μg/ml. The probe was added to a concentration of 1–3 × 10^6 cpm/ml. After 18 h of hybridization, the filters were washed at 42°C in 1% SDS, 0.2 × sodium citrate/sodium chloride buffer for 10 min, and at 65°C in 0.1% SDS, 0.2 × sodium citrate/sodium chloride buffer for 30 min, and autoradiographed.

RESULTS

SPRR Antibodies Are Monospecific Antibodies against SPRR1 detected a band of approximately 17 kD in HeLa cells transfected with pESPR1 (**Fig 1**, SPRR1, *lane 1*) but not in HeLa cells transfected with pESPR2 or pESPR3. SPRR1 ran slower than expected from sequence analysis, which predicts a protein of 9.8 kD. This aberrant migration has been observed previously [6] and is probably due to the high proline content of the protein. Antibodies against SPRR2 detected a band of approximately 15 kD in HeLa cells transfected with pESPR2 (**Fig 1**, SPRR2, *lane 2*) but not in HeLa cells transfected with pESPR1 or pESPR3. Again, SPRR2 ran slower than expected from sequence analysis, which predicts a protein of 7.8 kD. Clean resolution of the SPRR2-specific band was a consistent problem in over 50 immunoblots of HeLa cell and keratinocyte extracts. One could interpret the minor bands in keratinocytes (**Fig 2**, *lanes 1–3*) as a sign of concomitant expression of several slightly different SPRR2 protein products. However, smearing also occurred in HeLa cells expressing a single SPRR2 gene (**Fig 1**, SPRR2, *lane 2*), indicating that it is caused at least partly by abnormal electrophoretic properties of SPRR2. Minor cross-reactivity with a single protein of approximately 50 kD

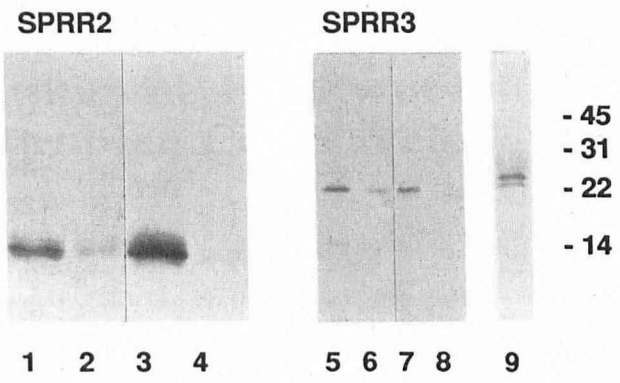

Figure 2. Transglutaminase inhibitors increase the accumulation of soluble SPRR2 and SPRR3. Immunoblots of total protein extracts with antibodies directed against SPRR2 (*lanes 1–4*) and SPRR3 (*lanes 5–9*). *Lanes 1,5*, differentiating keratinocytes cultured with LTB-2; *lanes 2,6*, differentiating keratinocytes; *lanes 3,7*, differentiating keratinocytes cultured with LTB-2; *lanes 4,8*, differentiating keratinocytes cultured with LTB-2 and 10^{-7} M RA; *lane 9*, differentiating keratinocytes from a different individual. Note the additional anti-SPRR3–positive band of similar molecular weight.

was observed in all HeLa cell extracts but was absent in keratinocytes *in vitro* (**Fig 2**, *lanes 1–4*) and *in vivo* (D. Hohl *et al*, unpublished data). Antibodies against SPRR3 detected a band of approximately 22 kD in HeLa cells expressing pESPR3 (**Fig 1**, SPRR3, *lane 3*) but not in HeLa cells transfected with pESPR1 or pESPR2. SPRR3 also ran slower than expected from its sequence analysis, which predicts a molecular weight of 18 kD. Preimmune sera for these antibodies revealed no reactivity at all (data not shown).

SPRR2 and SPRR3 Increase in the Presence of Transglutaminase Inhibitors, and Evidence for an Allelic Polymorphism of SPRR3 Low levels of both SPRR2 and SPRR3 were noted in keratinocytes grown to 45% confluence in 0.05 mM Ca^{++} medium and shifted to 0.25 mM Ca^{++} for 5 d (**Fig 2**, *lanes 2,6*). To establish whether SPRR2 and SPRR3 are potential substrates of transglutaminase, we added LTB-2 to the medium under identical culture conditions. Markedly increased levels of SPRR2 and SPRR3 (**Fig 2**, *lanes 1,5*) were detected, indicating an accumulation of these proteins in a soluble form in the absence of active transglutaminase. This result is compatible with their being substrates of transglutaminase. An interesting finding was an additional band of approximately 24 kD by anti-SPRR3 in extracts of keratinocytes originating from some individuals only (**Fig 2**, *lane 9*). This indicates a possible allelic polymorphism of the SPRR3 gene.

SPRR1, SPRR2, and SPRR3 Are Differentially Expressed *In Vivo* SPRR1 was observed in the appendageal regions of normal human epidermis from the upper spinous to the upper granular layers (**Fig 3*A***) and in the upper layers of the outer hair root sheath (data not shown). SPRR1 expression in palmoplantar and especially in foreskin epidermis was often patchy (**Fig 3*B,C***). Analysis of the squamous epithelia of the upper alimentary tract revealed moderate staining in the upper spinous and granular layers of lingual epithelium but strong expression in the tips of lingual papillae (**Fig 4*A***). In sublingual epithelium, SPRR1 expression was restricted to the uppermost living layers (**Fig 4*B***). No SPRR1 was found in esophageal epithelium (**Fig 4*C***) except for rare suprabasal areas (data not shown). SPRR2 was expressed in the granular layers of appendageal epidermis at high levels and moderately in the granular layers of interappendageal and palmoplantar epidermis (**Fig 3*D,E***). In foreskin epidermis, patchy SPRR2 staining was detected from the upper spinous layers to the stratum corneum (**Fig 3*F***). Squamous epithelia of the upper alimentary tract expressed little SPRR2, mainly restricted to the suprabasal areas of lingual tips (**Fig 4*D–F***). Neither sublingual nor esophageal mucosa expressed SPRR2. SPRR3 was not detected at all in epidermis (data not shown). In contrast, squamous internal epithelia lining the tongue and esophagus strongly expressed SPRR3 suprabasally (**Fig 4*G–I***). The unusual strong staining for SPRR3 appeared to be due to high constitutive levels of expression, because it was restricted to the epithelial compartment and was not present in the dermis (**Fig 4*G–I***) or in other tissues (data not shown). For all antibodies used, cellular staining was peripheral or both cytoplasmic and peripheral on paraffin-embedded material (**Figs 3,4**) but mostly peripheral on frozen sections (data not shown). Minor nuclear staining was sometimes observed with SPRR1 and SPRR2 antibodies and was mainly restricted to the basal layer (**Fig 3*A*; Fig 4*B***) and to the granular layer of palmoplantar epidermis (**Fig 3*E***), respectively. Preimmune sera 1B and 4A and secondary antibodies alone revealed no reactivity at all (data not shown).

SPRR1, -2, and -3 Transcripts Are Abundant in Cultured Keratinocytes Slot blot analysis revealed monospecificity of the SPRR1, -2, and -3 cDNA fragments used in this study (data not shown). Northern blot analysis of cultured differentiating keratinocytes revealed abundant expression of SPRR1, -2, and -3 transcripts and minor cross-reactivities with other RNA species (**Fig 5*C***). RNA from dissected fractions of scalp epidermis showed very low levels of SPRR1 transcripts in the epidermal fraction (**Fig 5*E***) and more abundant expression in the follicular fraction (**Fig 5*F***). SPRR2 transcripts were abundant in both the epidermal and follicular fractions (**Fig 5*E,F***). No SPRR3 transcripts at all were detected in RNA isolated from epidermal keratinocytes *in vivo* (**Fig 5*E,F***). These results are consistent with the distribution patterns revealed by *in situ* hybridization (see below) and by immunohistochemistry. As expected, identical blots hybridized with a loricrin cDNA probe revealed the highest expression in the epidermal fraction *in vivo* and the lowest in cultured keratinocytes (**Fig 5*E,F,C***) [20,27,33].

In situ hybridization of normal human epidermis showed a distribution of transcripts very similar to the immunohistologically detected expression of SPRR1, -2, and -3. In the scalp, SPRR1 transcripts were detected in the upper spinous and granular layers, mostly restricted to the follicular (**Fig 6*A***) and acrosyringeal epidermis. SPRR2 transcripts were found coherently expressed in the epidermis but were restricted to the uppermost spinous and granular layers (**Fig 6*D***). Dramatic differences were noted in psoriatic epidermis, in which both SPRR1 and SPRR2 transcripts (**Fig 6*C,F***) and proteins (data not shown) were strongly expressed from the middle spinous to the upper granular layers. Probing with SPRR3 anti-sense and SPRR1, -2, and -3 sense transcripts did not show any hybridization either in normal or in psoriatic epidermis (data not shown). Control experiments with the loricrin riboprobe (data not shown) revealed the expression pattern as described before [27,33].

SPRR2 and SPRR3 Transcription *In Vitro* Is More Sensitive to RA than Is SPRR1 Previous experiments have indicated that the synthesis of CE precursors is differently affected by retinoids [34]. Involucrin expression remained unchanged by RA [35] or was affected only at high concentrations [36], whereas loricrin, filaggrin [20], and cornifin [16] expression was clearly reduced. To assess the influence of RA on SPRR2 and SPRR3 protein synthesis, we cultured keratinocytes under conditions allowing strong SPRR protein accumulation (i.e., with LTB-2). Addition of 10^{-7} M RA blocked SPRR2 and SPRR3 protein synthesis (**Fig 2**, *lanes 4,8*). To determine the influence and time course of RA on SPRR mRNA levels, we used Ca^{++} concentrations permissive for high transcriptional activity of the SPRR genes during the whole experiment. RA added at 10^{-7} M at various times to cultures already exposed to Ca^{++} resulted in a decrease of SPRR mRNA expression within 72–120 h (**Fig 7**, *lanes 2–6*). RA inhibited SPRR2 and SPRR3 mRNA accumulation much more strongly than the expression of SPRR1 (**Fig 7**, *lanes 2,3*).

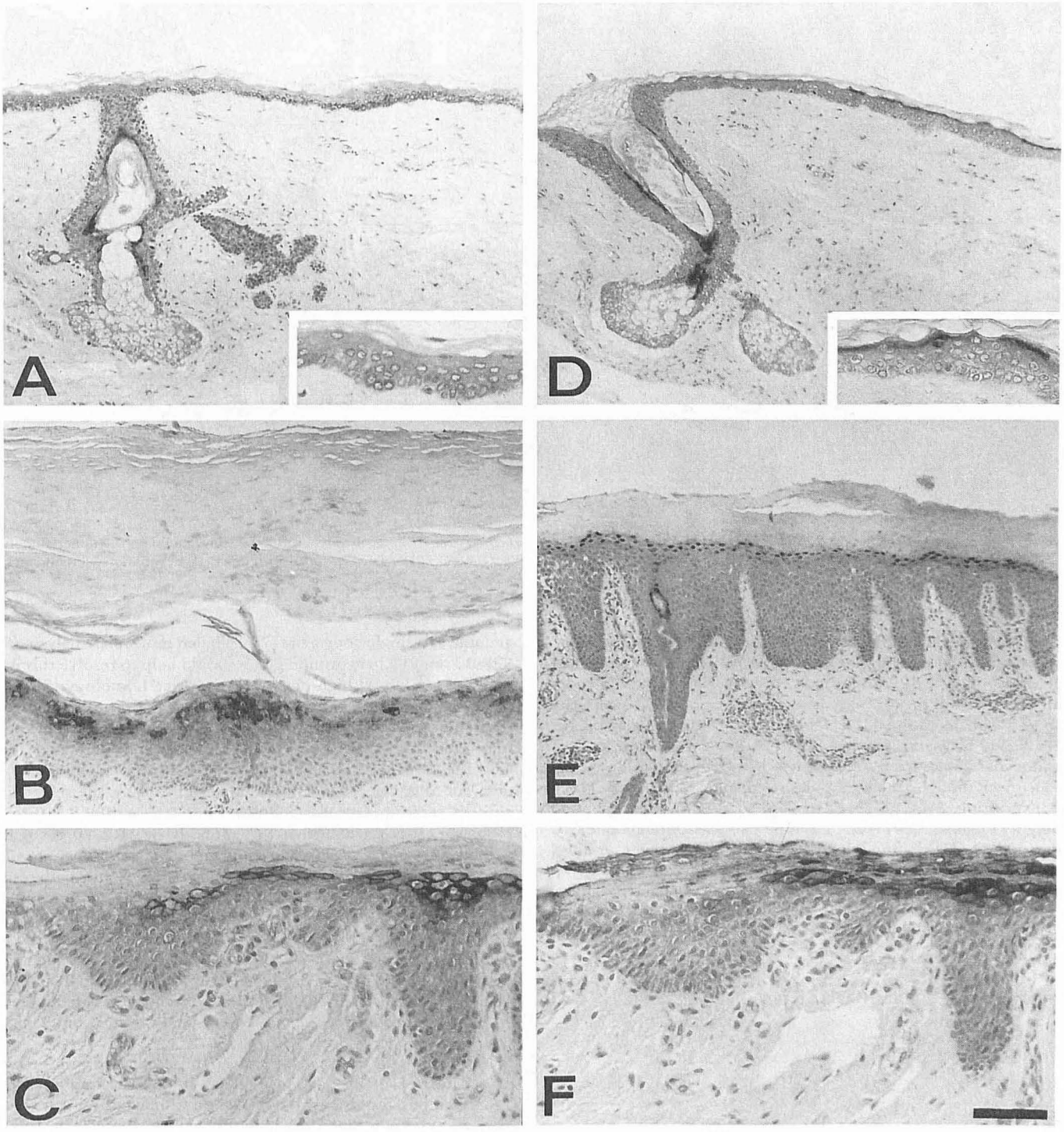

Figure 3. SPRR1 expression is concentrated in appendageal epidermis. Immunohistochemistry of normal human scalp (*A,D*), plantar epidermis (*B,E*), and foreskin epidermis (*C,F*) with antibodies against SPRR1 (*A,B,C*) and SPRR2 (*D,E,F*). *Bar:* *A,D*, 125 μm; *B,C,E,F*, and *inserts* in *A,D*, 50 μm.

DISCUSSION

In this study, we provide strong evidence that SPRR2 and SPRR3 are CE precursor proteins, as postulated previously [8] and as shown for another member of the SPRR multigene family, cornifin, the rabbit homologue of human SPRR1 [16]. First, SPRR2 and SPRR3 are potential transglutaminase substrates in cultured human keratinocytes. Second, they are always located at the cell periphery and in some cells also in the cytoplasm of terminally differentiated keratinocytes. Further proof that SPRRs are CE precursor proteins should come from immunoelectron microscopy and from comparison of the known amino acid sequence of SPRRs with peptides containing a cross-link derived from purified mature CE [27].

Our results corroborate a recent report† suggesting that SPRRs are identical to pancornulins, a family of small, soluble, and basic CE precursor proteins [37,38]. In fact, SPRRs and pancornulins not

† Greco MA, *et al*: The pancornulins are members of the Spr-1 multigene family (abstr). *J Invest Dermatol* 100:501, 1993.

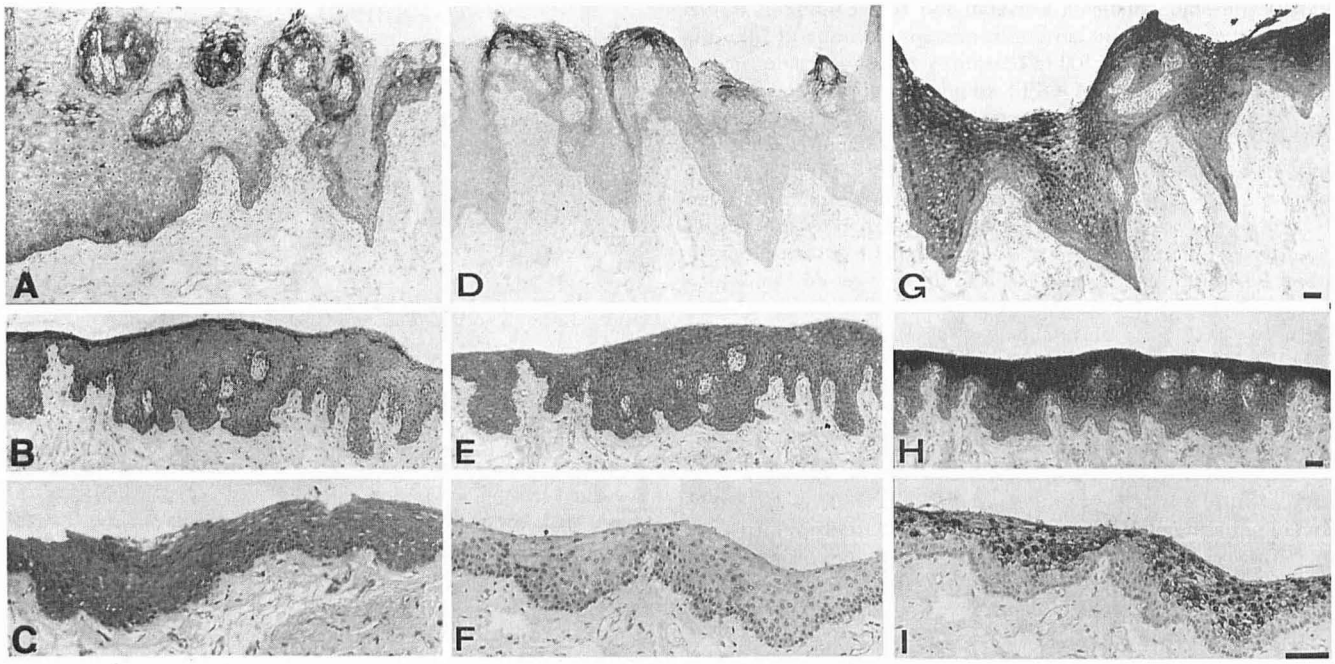

Figure 4. SPRR3 is strongly expressed in epithelia lining the upper digestive tract. Immunohistochemistry of epithelia lining the upper alimentary tract with antibodies directed against SPRR1 (*A,B,C*), SPRR2 (*D,E,F*), and SPRR3 (*G,H,I*). *A,D,G*, dorsal tongue; *B,E,H*, sublingual mucosa; *C,F,I*, esophagus. *Bar*, 50 μm.

only exhibit similar molecular weights as estimated by SDS-polyacrylamide gel electrophoresis, but they also show an identical tissue distribution if SPRR1 is equated with the 16.9-kD pancornulin, SPRR2 with the 14.9-kD pancornulin, and SPRR3 with the 22/24.8-kD pancornulin [39]. Moreover, an additional band for SPRR3 (**Fig 2**, *lane 9*) was consistently noted on immunoblots of keratinocyte samples from some individuals, but not in others **(Figs 2,5–7)**, even though our antibodies were monospecific in HeLa cells expressing SPRR3 **(Fig 1)** and showed specific peptide binding by the enzyme-linked immunosorbent assay technique (M. Huber and D. Hohl, unpublished observation). Similar findings were reported previously for the 22/24.8-kD pancornulins [39]. This protein doublet most likely corresponds to an allelic polymorphism, as already described for keratins [40], involucrin [41], and loricrin [11], because the SPRR3 gene is represented in the human genome as a single copy gene [7]. Detailed molecular analysis of the SPRR3 and the pancornulin genes should help to resolve this issue.

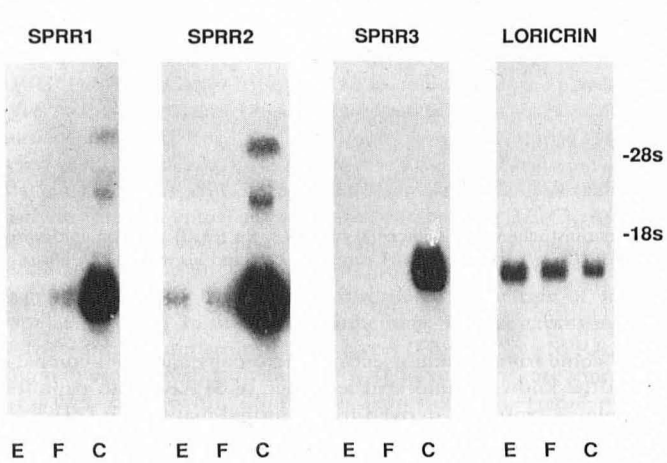

Figure 5. SPRR3 transcripts are absent in epidermis *in vivo*. Northern blot analysis from the epidermal fraction (E) and follicular fraction (F) of dissected normal scalp epidermis and from differentiating, cultured keratinocytes (C) hybridized with probes for SPRR1, SPRR2, SPRR3, and loricrin.

SPRRs exhibit a high degree of sequence homology [7]. Thus, the production of monospecific probes was of utmost importance for a thorough analysis of the expression patterns of the known transcripts and proteins encoded by the SPRR multigene family. The use of HeLa cell lines expressing vectors encoding for the different SPRRs proved to be a powerful tool for the selection of antisera monospecific for SPRR1, SPRR2, or SPRR3 **(Fig 1)**. However, it is possible that the antibody SPRR2-1B recognizes several protein products of the eight known SPRR2 genes. This should be taken into account when interpreting results with respect to gene regulation, i.e., SPRR2 expressed in follicular, interfollicular, or psoriatic epidermis and tongue epithelium could be generated by transcripts from different SPRR2 genes. Similarly, the two known and transcribed SPRR1 genes might not be identically regulated. Thus, sequence-specific polymerase chain reaction of reverse-transcribed SPRR1 and SPRR2 transcripts is being developed and might be necessary to understand the full complexity of their expression.

The results for SPRR1 expression are in conflict with previous observations [16,42] claiming that cornifin was expressed in the upper spinous and granular layers of interfollicular human epidermis, although the authors could not detect appreciable mRNA expression by *in situ* hybridization [42]. We provide three consistent lines of evidence—immunohistochemistry, *in situ* hybridization, and Northern blot analysis of RNA fractions from dissected scalp epidermis—that this is not the case. SPRR1 is expressed at low levels almost exclusively in the intraepidermal parts of appendages in normal human epidermis. The discrepancy might be explained by cross-reactivity of their anti-cornifin antibodies [16,42] with other proteins containing similar epitopes. In fact, several bands of higher molecular weight on immunoblots were interpreted as polymers containing cornifin (see **Fig 4**, *lane B11* in [16]); however, they could also correspond to other cross-reactive protein products expressed during squamous differentiation.

In conclusion, our results suggest that the SPRR genes encode for a family of CE precursor proteins, most likely identical to the pancornulins. The differential expression of its members indicates

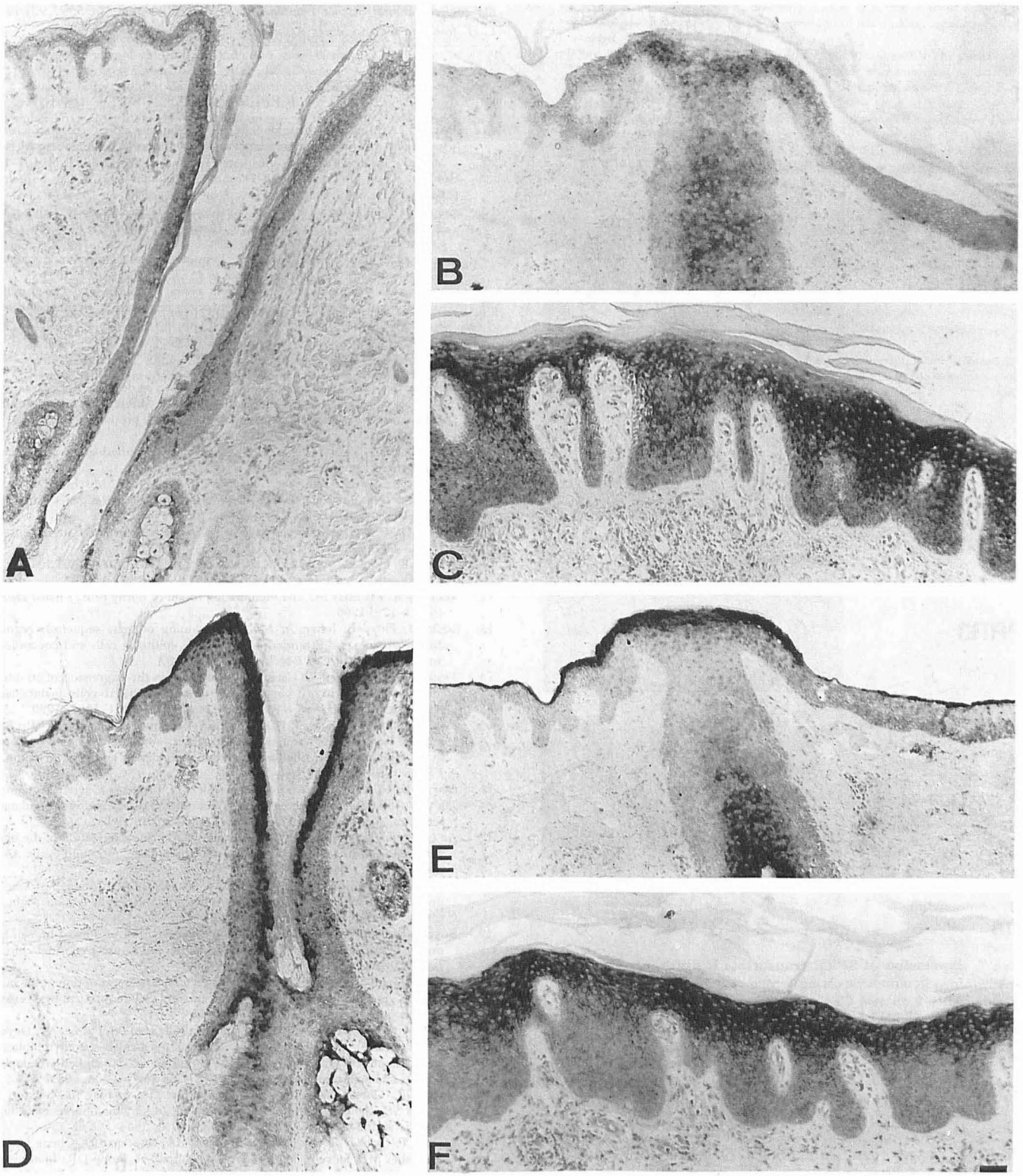

Figure 6. SPRR1 transcripts are not expressed in interappendageal epidermis of the scalp. *In situ* hybridization with anti-sense riboprobes for SPRR1 (*A,B,C*) and SPRR2 (*D,E,F*). *A,B,D,E*, normal scalp epidermis; *C,F*, psoriatic epidermis. *Bar*, 50 μm.

that this family has evolved to serve highly specialized functions of the CE. It appears that regulation of the SPRR genes is very complex. SPRR1 expression is concentrated in follicular structures, whereas SPRR2 is also expressed in interappendageal epidermis, and both are strongly induced in psoriatic hyperproliferation. SPRR3 is expressed in all stratified epithelia of the upper digestive tract. The expression patterns of SPRRs are somewhat reminiscent of those for keratins K2 and K6. Like K2p [43,44], SPRR3 is expressed in oral epithelium and is encoded by a different gene than are epidermally expressed SPRRs. Much like K6 [45], epidermal SPRR expression is concentrated in follicular structures, is abundant in hyperproliferative epidermis, and is encoded by two or

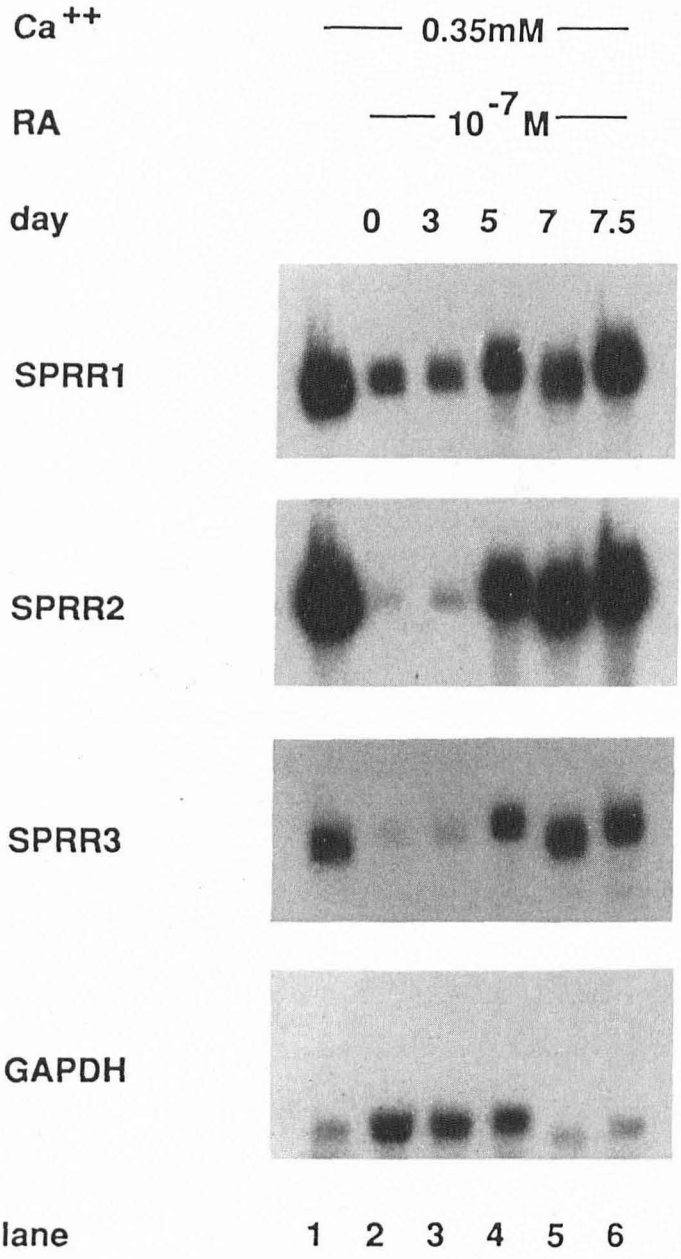

Figure 7. Expression of SPRR transcripts is differentially sensitive to RA. Human keratinocytes were grown to 80% confluence in 0.15 mM Ca^{++}, shifted to 0.35 mM Ca^{++} (day 0), and harvested 8 d later (*lane 1*). RA at 10^{-7} M was added simultaneously (*lane 2*) or 3 d (*lane 3*), 5 d (*lane 4*), 7 d (*lane 5*), or 7.5 d (*lane 6*) after the Ca^{++} switch. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

more genes (or two gene subfamilies). Whether these similarities point to linked functions in terms of serving special programs of differentiation is hypothetical and needs further experiments. As for the keratins, meaningful information on the function of SPRRs is likely to come from mutational analysis of their respective genes either *in vitro* or *in vivo*.

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