A Unique Type I Keratin Intermediate Filament Gene Family is Abundantly Expressed in the Inner Root Sheaths of Sheep and Human Hair Follicles

C. Simon Bawden, Clive McLaughlan, Antonietta Nesci,* and George Rogers
Departments of Animal Science and *Biochemistry, University of Adelaide, Adelaide, South Australia

A unique type I keratin intermediate filament group, comprising three highly related proteins and expressed in the inner root sheath of hair follicles, has been identified in both sheep and human. The first members from these species are named oIRSa1 and hIRSa1 and each encodes a protein of 450 amino acids, with compositional characteristics intermediate between those of previously described hair keratin and epidermal cytokeratin type I intermediate filaments. Detection of abundant mRNA transcripts derived from the sheep and human genes by cRNA in situ hybridization only in the inner root sheath and not in the medulla concurs with the findings of earlier ultrastructural analyses that have reported intermediate filaments only in the inner root sheath. Clustering of the IRSa keratin genes is apparent in the genomes of both species. The three hIRSa genes, known to reside on human chromosome 17, are closely linked to three further type I keratin intermediate filament genes of unknown function. This new gene complex, contained almost entirely within a 156 kb BAC (hRPK.142_H_19), is likely to lie near the type I intermediate filament cytokeratin and hair keratin gene loci at 17q12-q21. A phylogenetic analysis including all known human type I intermediate filament cytokeratins, hHa keratins, hIRSa, and hIRSa-linked keratins suggests that origin of the IRSa keratin intermediate filament linkage group preceded origin of most of the epidermal cytokeratins and all hair keratins during emergence of the keratin intermediate filament genes. Key words: IRS differentiation/keratin gene cluster/type I IF domain.


Formation of wool and hair in the skin is directed by a complex differentiation program that results in the formation of eight different cell types within medullated follicles. Within each different follicular compartment, regulatory mechanisms specify coordinated expression of type I and type II keratin intermediate filaments (KIFs) and sequential expression of these and filament–associated proteins to form the structures required for follicle growth and fiber production (for a review, see Powell and Rogers, 1997). In the outer root sheath, expression of KIFs appears limited to the “soft” epidermal cytokeratins. In most other layers, epidermal cytokeratins are expressed but the “hard” keratins of hair and KIF–associated proteins provide structural components of the fiber proper. Many epidermal cytokeratin pairs have been localized to specific follicle cell layers (Heid et al, 1988a; 1988b; Moll et al, 1988; Stark et al, 1990; Schirren et al, 1997), and the expression patterns of many genes for type I and type II IF hair keratins and KIF–associated proteins have been mapped in wool (Powell et al, 1991; Powell and Rogers, 1994; 1997) and hair follicles (Bowden et al, 1994; 1998; Winter et al, 1994; 1998; Rogers et al, 1995; 1996; 1997a; Langbein et al, 1999).

The inner root sheath (IRS), derived from 70%-80% of cells produced in the follicle bulb (Wilson and Short, 1979), surrounds the hardening fiber and plays a crucial role in fiber moulding. KIFs and the filament–associated protein trichohyalin are produced early in IRS cell differentiation (Rogers, 1959) and then become modified via the action of peptidyl-arginine deiminase (Rogers et al, 1997b) and cross-linked by transglutaminase (Rogers et al, 1977; Tarcsa et al, 1997) to form a hardened structure that fills the cells. These cells provide a rigid cylindrical framework within which subsequent hardening of the fiber cortex and cuticle occurs. Though much is known of the trichohyalin molecule (Rothnagel and Rogers, 1986; Fietz et al, 1993; Lee et al, 1993) and although epidermal cytokeratin pairs K6/K16, K1/K10, and K4 and K18 have all been proposed to be expressed in the IRS (Heid et al, 1988a; Stark et al, 1990; Schirren et al, 1997), the identity of the KIF responsible for formation of the immunologically distinct (Lynch et al, 1986) 10 nm filaments in this follicle layer has not been established.

Here we report the finding of a novel group of type I IF proteins that are expressed in the IRS cells of sheep and human hair follicles. We have identified a number of different cDNA and gene clones from the sheep and human, respectively, which encode highly related proteins. cRNA in situ hybridization using gene–specific 3′-noncoding probes applied to sheep and human skin sections was used to determine the site of expression of the different family members from each species. These proteins are likely to represent members of presumed type I–type II IF protein pairs required for KIF formation in the IRS.
Preparation of wool follicle cDNA
Wool follicles were stripped from the skin of Corriedale wethers by epilation (see Rogers et al, 1997b) and mRNA was isolated by the method of Chomczynski and Sacchi (1987). cDNA was prepared for 5'RACE (rapid amplification of cDNA ends) or 3'RACE (Frohman et al, 1988) as previously described (Rogers et al, 1997b). Wool follicle cDNA prepared for 3'RACE was a kind gift from G. Sander.

Cloning of keratin type 1 IF cDNA
For the 5'RACE, a forward 5' primer (25-mer: 5'-TAGAATTCACACCTTCCACTCCGT3'-3') was designed to be specific to the conserved 3' COOH coding sequence (Asn-Pro-Cys-Ser-Thr-Pro; underlined sequence above) found in exon 7 of the mouse and human Ha2 clones just downstream of the IF rod domain. 3' anchor primer (5'-CCTCTGAAGGTTCCAGATCTAGTG-3') and a 3' primer specific to cDNA sequence within the 362 bp ovine clone (29-mer: 5'-CCTCTGAAGGTTCCAGATCTAGTG-3') were produced by linearization of inserts in the 3'-noncoding region (a 287 bp NdeI/EcoRI fragment) and a 3'-noncoding region probe from partial cDNA oIRSa3.1 (238 bp NooI/EcoRI fragment); see sequence in GenBank AF227761).

cDNA in situ hybridization

In situ hybridization using gene-specific 3'-noncoding probes was performed as described by Powell and Rogers (1998) on 7 μm sections of sheep tissue biopsies transferred to TESPa-treated slides (Rentrop et al, 1986). Haematotoxin (Gurr, BDH) or the tripartite stain SACPIC (Auber, 1950) was used to stain sections for the remainder of this cDNA proceeded by 5' Amplifier RACE kit (Clontech, Palo Alto, CA). Use of the 5' anchor primer (5'-CCGGATCCCTGCAGGAATTCGTCGACT(18)(A/C/G)) was determined to be a doublet by quantitative analysis of phosphorimage exposures of the oIRSa1 exon 6/7 fragment-probed filter. The strong band determined to be a doublet by quantitative analysis of phosphorimage exposures of the oIRSa1 exon 6/7 fragment-probed filter.

Total RNA northern/genomic southern hybridization and hybridizations

Northern Ten microgram samples of total RNA isolated from Merino sheep tissue samples according to the method of Chomczynski and Sacchi (1987) were glyoxylated and fractionated in 1% agarose (10 mM sodium phosphate buffer) alongside RNA molecular size markers (M; Gibco-BRL). The method, adapted from McMaster and Carmichael (1977), is described in Sambrook et al (1989). RNA was then transferred to Hybond- XL nylon membrane (Amersham Pharmacia Biotech) in 7.5 mM NaOH by vacuum blotting.

Genomic southern Genomic DNA from human cells (HeLa), mouse liver (CBAXC57), and sheep tail tissue (Corriedale and Merino) was prepared by digestion of samples with 300 μg per ml Proteinase K (Boehringer Mannheim) in 1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris (7.5, 5 mM ethylenediamine tetracetic acid, and then purification of DNA by phenol/chloroform/ethanol precipitation and ethanol precipitation according to the method described in Sambrook et al (1989). Fractionation of 10 μg samples of oIRSa- and HindIII-digested sheep, human, and mouse genomic DNA in 1% agarose (TAE) alongside molecular weight markers (a mixture of HindIII-digested Lambda and EcoRI-digested SP6-1 bacphage DNAs; Geneworks) was followed by transfer to Zetaprobe GT membrane (BioRad) by vacuum blotting in 0.1 × SSC, 0.1% SDS at 100°C. Autoradiography was carried out at –80°C for 16 h and phosphorimaging analysis at room temperature for 4–16 h.

The following should be noted. (i) PCR amplification, cloning, and sequencing of the exons 6/7 region of the oIRSa1 gene from Merino DNA (see legend to Fig 1) was carried out to confirm that weak hybridization of the oIRSa1 cDNA exon 6/7 region coding probe to a fourth fragment in each of the digested sheep DNA samples (also detected specifically by the 3'-noncoding probe for oIRSa1) was due to the presence of EcoRI and HindIII sites within the intron connecting these exons. (ii) The strong band of ~6.3 kb in human HindIII-digested DNA (Fig 4a, lane 2) was determined to be a doubtable quantity by quantitative phosphorimaging exposures of the oIRSa1 exon 6/7 fragment-probed filter.
Isolation and characterization of a cDNA encoding a novel sheep type I IF keratin

In an attempt to isolate a cDNA encoding the COOH-terminal region of a predicted ovine orthologue of the murine and human hair cuticle-specific type I IF, Ha2 (Winter et al., 1994; Rogers et al., 1996), 3′-RACE was performed using a degenerate oligonucleotide and wool follicle cDNA as template. A 362 bp product was cloned, the nucleotide sequence was determined, and then 5′-RACE was carried out to isolate the remainder of this cDNA. The full nucleotide sequence (Fig 1) is predicted to encode a new type I KIF of 450 amino acids (aa) (M, 49,318.6) with a primary structure intermediate between hair keratin and epidermal cytokeratin. This IF has been termed oIRSa1 because it is expressed solely in the IRS of the follicle (see Discussion).

An amino acid sequence alignment of the sheep type I IF cytokeratin K15, the type I IF hair keratin 8c2, and the predicted oIRSa1 protein is given in Fig 2. The size of the coding region is similar to cytokeratin K15 (453 aa; Whitbread and Powell, 1998) but larger than type I KIFs of wool, protein components 8a (413 aa; Dowling et al., 1986), and 8c2 (404 aa; Wilson et al., 1988). Sequencing of a number of clones demonstrated the existence of four types. Some were identical to oIRSa1 but others were from highly related mRNA species (termed oIRSa2, oIRSa3.1, and oIRSa3.2). oIRSa3.2 is so named because nucleotide homology in the 3′-noncoding region (100% identity) suggests it is a recently formed allele of oIRSa3.1. These cDNAs predict type I IF proteins with C-terminal tails of 52, 61, and 39 residues, respectively, including conserved residues at the C-terminal boundary of the 2B rod domain. An alignment of the available C-terminal amino acid sequences of oIRSa2, oIRSa3.1, and oIRSa3.2 with oIRSa1 is shown in Fig 3. The full nucleotide sequence of oIRSa1 includes intronic sequence, from the oIRSa1 gene in Merino sheep genomic DNA. An EcoRI site, part of the oligo-dT-MCS primer used in PCR, is shown (1658 nt; nucleotide numbering given on Fig 1). The presumptive protein sequence (450 aa) is shown beneath the nucleic acid sequence (single letter amino acid code) and the beginning and end of the α-helical rod domain are indicated by solid arrowheads.

The α-helical rod domain of oIRSa1 is 314 amino acid residues in length, with 79 and 56 residue N- and C-terminal head and tail domains, respectively. Amino acid residues in the conserved α-helix termination motif of the 1A (KVTMQNLNDRILASYL) and 2B (EKEIETYCLL) rod domains (Parry, 1997) and in the linker region L2 (NRRDAEAW; Steinert and 2B (EKEIETYCLL) rod domains (Parry, 1997) are additional members that make this a new type I KIF family.

The full nucleotide sequence (450 aa) is shown beneath the nucleic acid sequence (single letter amino acid code) and the beginning and end of the α-helical rod domain are indicated by solid arrowheads.

RESULTS

The nucleotide identity between oIRSa1 and these other cDNA clones (data not shown) and conservation at the putative 2B rod domain terminus, it can be concluded that the oIRSa2, oIRSa3.1, and oIRSa3.2 proteins are additional members that make this a new type I KIF family.

Identification of related ovine, human, and murine genes

Southern analysis of genomic DNA from Corriedale and Merino sheep was performed using oIRSa1 3′-coding (Fig 4a) and 3′-noncoding region probes (Fig 4b, c). Murine and HeLa cell...
DNA was included in the analysis to demonstrate relatedness of coding regions of the proposed mouse and human homologs and gene complexity in these species. An oIRSa1 cDNA coding region probe strongly detected three fragments in both the EcoRI and HindIII sheep DNA digests (Fig 4a). This probe also hybridized to three fragments in each of the human and mouse DNA lanes, for both EcoRI and HindIII DNA digests, indicating the existence of three genes in each species that are highly related to each other and to oIRSa1. Stripping and reprobing with an oIRSa1 3'-noncoding probe detected only single fragments in EcoRI (3.6 kb) and HindIII (9.4 kb) digests of the Corriedale and Merino sheep genomic DNA (Fig 4b), but there were no hybridization products in the mouse or human genomic DNA (data not shown). Finally, when a 3'-noncoding probe from oIRSa3.1 was used to probe the southern filter, a single EcoRI fragment (2.6 kb) and HindIII fragment (9.4 kb) were detected in the sheep lanes (Fig 4c) but no genomic fragments were detected in human or mouse DNA (data not shown).

Use of the full nucleotide sequence of oIRSa1 in a BLASTN search identified a mouse skin-derived IF cDNA clone, Krt1-c29, recently added to the database (Sato et al., 1999), to be related (~78% nucleotide identity). Moreover, a region within a human chromosome 17 BAC genomic clone (hRPK.142_H_19) was highly related, having ~87% identity over presumptive coding exons. Homology was also found to this human clone in similar searches using the oIRSa2, oIRSa3.1, and oIRS3.2 cDNA sequences.

The 156 kb DNA insert contained within the BAC was searched for further type I KIF sequences related to oIRSa1, given the southern result and the clustering of genes previously shown for sheep (Powell et al., 1986; Powell and Beltrame, 1994) and human...
The seven human genes were designated Genes 0–6, the original was used in a BLASTN scan of the clone. Six other potential and potential transcription initiation (TATA) and termination 6/7 region of the first human gene identified, conserved in oIRSa1, (Fig 5) mapped within the BAC (lanes 7), indicating that they share a common orientation. Also, restriction enzyme analysis of the BAC, (lanes 8), with low nucleotide homology in this region (34%–69%), were clearly not detected by the 3′-noncoding region of partial cDNA oIRSa3.1 (c; ovine DNA only) were sequentially applied to the southern filter. The distance of migration and size (kb) of molecular weight markers are indicated on the left and sizes (kb) of fragments detected by the 3′-coding exons are marked on the right (arrows).

KIFs (Rogers et al, 1998; 2000). Nucleotide sequence of the exon 6/7 region of the first human gene identified, conserved in oIRSa1, was used in a BLASTN scan of the clone. Six other potential human type I IF genes related to oIRSa1 were identified. The seven human genes were designated Genes 0–6, the original gene identified being Gene 3. Probable locations of coding exons and potential transcription initiation (TATA) and termination (polyadenylation AATAAA) signals for all seven genes were mapped within the BAC (Fig 5), indicating that they share a common orientation. Also, restriction enzyme analysis of the BAC identified EcoRI and HindIII fragments containing exon 6/7 regions of Genes 3, 5, and 6, respectively, consistent with sizes of human DNA fragments detected in the southern transfer (Fig 4). Fragments from Genes 0, 1, 2, and 4, with low nucleotide homology in this region (34%–69%), were clearly not detected by the probe at the moderate wash stringency used.

A prediction of exon/intron structure in genes of the human BAC clone sequence found their structures to be analogous to those of the human type I IF cytokeratins (Steinert et al, 1985; Fuchs and Weber, 1994) and sheep cytokeratin K15, with eight exons. By comparison, genes for the known human hair type I KIFs (Rogers et al, 1998) and for sheep wool keratin 8c2 (Wilson et al, 1988) usually have seven exons. Given the exon structure of the human genes identified here and the high homology between these human BAC-encoded keratins, the oIRSa1-a3 keratins, and mouse skin Krt1-c29 type I KIF, it is probable that the human gene and ovine cDNAs represent sequences derived from type I IF cytokeratin antecedents. Further evidence for this is that the human BAC is derived from chromosome 17, the same chromosome upon which the human type I KIF gene loci are located (Hediger et al, 1991).

Notwithstanding possible errors in the human BAC sequence, an alignment of C-terminal protein sequences predicted for five of the human genes (hGene 1, 3, 4, 5, and 6) was made with oIRSa1, the partial cDNAs oIRSa2, a3.1, and a3.2, and mKrt1-c29 (Fig 3). This alignment shows that the Gene 3 product (450 aa) is probably the orthologue of oIRSa1 (450 aa; 91% amino acid identity). Likewise, the Gene 6 product is the orthologue of oIRSa2 (90% amino acid identity in the 3′-coding exons 6/7/8), and the Gene 5 product (459 aa) is most likely the orthologue of mKrt1-c29 (448 aa; 89% amino acid identity) and oIRSa3.1 (89% amino acid identity in the 3′-coding exons 6/7/8).

A sequence alignment-based search of the database using oIRSa1 found homology to human, mouse, and rabbit type I IF cytokeratin sequences, especially K10 and K12 (~55–60%). A comparative alignment of the proteins encoded by the oIRSa1 cDNA, human IF Gene 3 identified in the BAC (designated hIRSa1; M, = 49,323; 6; see Discussion), and the mouse Krt1-c29 type I IF protein is included in Fig 2. This reveals that the ovine clone shares greatest amino acid identity with hIRSa1 (91%) and somewhat less with the mouse type I IF (80%), levels similar to those seen among type I KIF homologs from different mammalian species (~80%–90% amino acid identity). A comparison of the amino acid compositions of the protein encoded by oIRSa1 with that of the murine clone Krt1-c29 and with murine hair and cytokeratin type I IF is given in Table I. Whereas the proportions of some amino acids within the ovine protein parallel those typically found in the hair IF (serine and phenylalanine) and others the cytokeratin IF (valine and possibly lysine), there are amino acids (cysteine, glycine, and proline) whose levels are midway between compositions typical for these IF types. The amino acid compositions of IF protein preparations from guinea pig IRS and hair (Steinert et al, 1971) are also shown (Table I). Despite the fact that the amino acid composition for the guinea pig IRS KIF represents analysis of a crude preparation, compositional proportions for many of the amino acids referred to above show the same trends. Like other type I IF proteins, oIRSa1, hIRSa1, and mKrt1-c29 are acidic proteins with predicted pI values of 4.98, 4.93, and 4.90, respectively.

Analysis of the tissue distribution of IRSa by cRNA in situ hybridization and northern transfer analyses. Initial cRNA in situ hybridization analysis of expression of oIRSa1 in sheep skin sections, using a 3′-noncoding probe, localized mRNA to the IRS of the hair follicle (Fig 6a–e). Comparative in situ analysis using a cRNA probe for trichohyalin (thh) expressed in both the IRS and medulla (Fietz et al, 1993), applied to consecutive skin sections, confirmed the localization (Fig 6d–f). Considering probes were of similar size and equivalent specific activity, mRNA from oIRSa1 seems to be synthesized at slightly lower levels than thh in the IRS, below the zone of Aubé (Auber, 1950), with expression coinciding with or beginning just after the onset of thh expression. oIRSa1 mRNA continues to be produced in developing IRS cells and some mRNA persists beyond the zones of hardening of the respective IRS cell layers. Unlike thh (Fig 6e, f, i, j), no oIRSa1 mRNA was detected in cells of the medulla.
A survey of other keratinized sheep epithelial tissues known to express thh, namely adult tongue, esophagus and rumen, and fetal hoof, by cRNA in situ hybridization analysis using the same 3′-noncoding probes, failed to detect oIRSa1 mRNA (Fig 6k for tongue; data not shown for other tissues). In comparison, the thh probe decorated the oesophageal-like (E) region of filiform papillae of the tongue (Fig 6l) and the suprabasal epithelium of the esophageal rumen, and fetal hoof as described previously (data not shown; see Fietz et al, 1993). Hybridization of antisense 3′-noncoding DIG-labeled probes derived from partial cDNAs oIRSa2 and oIRSa3.1 to Tukidale sheep skin sections gave signals restricted to the IRS of the wool follicle in a pattern identical to that seen for the oIRSa1 clone probe with no hybridization to any other cell type in the sections (data not shown).

Expression of the human BAC type I IF genes in human skin sections (hairline scalp) was analyzed following isolation of 3′-noncoding region probe fragments from HeLa genomic DNA. Probes for Genes 3 (hIRSa1), 5 (hIRSa3.1), and 4 were cloned (see Materials and Methods). Genes 3 and 5 because of their proposed relationship to oIRSa1 and oIRSa3.1, and Gene 4 because it encodes a protein different from those encoded by the ovine cDNAs cloned here. When applied to the skin sections, probes for Genes 3 and 5 hybridized specifically to the IRS of human hair follicles, in a manner identical to the ovine probes, with no hybridization signal in the medulla (Fig 6m, n). The control sense probe did not hybridize to any part of the sections (Fig 6o) nor did the antisense cRNA probe for Gene 4 (data not shown).

Relationship of the BAC-encoded keratins to cytokeratins and hair keratins A phylogenetic analysis was performed using protein sequences of the human hair cortical, cuticle, and IRS type I KIFs, hIRSa1, hIRSa2, and hIRSa3.1, epidermal type I IF cytokeratins, and the two other type I KIFs encoded by Genes 1 and 4 of the human BAC hRPK.142_H_19 (Fig 8). Whether whole sequences, 2B rod domains, or the exon 6/7/8 region of these proteins was used, this analysis showed evolutionary relationships between hair and cytokeratin type I IF similar to those reported previously by Rogers et al (1998) for proteins within the groups. Key cytokeratins (K9/K10/K12) emerge prior to the hair keratins, and the grouping of specific cytokeratins (K9/K10; K14/K16/K17; K13/K15) and hair keratins (Ha1/Ha3.1/Ha3.2/Ha4; Ha2/Ha5/Ha6; Ha7/Ha8) was supported in the analysis. Interestingly, it suggests that the human BAC Gene 1, 3, 4, 5, and 6 products were derived from cytokeratin 9. Moreover, whether the 488 amino acid coding region for Gene 1 or an alternative predicted 542 amino acid coding region is included, the analysis suggests that BAC Gene 1 is the genetic precursor of the entire hIRSa keratin group. The phylogenetic tree shows the cytokeratin type I IF, K10, emerging from K9 with the new hIRSa keratin group, followed by K12, which precedes all hair type I KIFs and most of the other type I IF cytokeratins. This explains why K10 and K12 were invariably identified in database searches as being the KIFs next most related to oIRSa1, after the BAC genes and mKrt1-c29. Database searches using exon 6 amino acid sequences predicted for human BAC Genes 3, 5, and 6 individually also found these two cytokeratin sequences to be very closely related. The relationship between cytokeratins K9, K10, and K19 is different from that previously proposed (Rogers et al, 1998), possibly because the new keratin group fills a gap present in the sequence information used for alignment at that time.

**DISCUSSION**

The results describe a new ovine type I KIF cDNA, oIRSa1, and three related partial ovine cDNAs (oIRSa2, oIRSa3.1, oIRSa3.2) each encoding members of a new keratin group expressed in the...
IRS of the wool follicle and with only limited homology to known hair keratins and epidermal cytokeratins. Moreover, human type I KIF orthologues of these ovine cDNAs have been identified. As the new IRS acidic (IRSa) keratins are a distinct type I KIF group, the name oIRSa1 for the new ovine cDNA indicates its origin, expression site, and that it is the first acidic KIF of this type described for the IRS. The other three ovine clones have thus been termed oIRSa2, oIRSa3.1, and oIRSa3.2. Likewise, the human Genes 3 and 5 identified within the BAC clone and shown in this study to be expressed in the IRS are designated hIRSa1 and hIRSa3.1, according to the similarity of their predicted protein sequences to the corresponding ovine clones. Human Gene 6 will be designated hIRSa2 if, like oIRSa2, it is found to be expressed in the IRS. The exact site of expression of the mouse skin cDNA clone Krt1-c29 remains to be determined although the high degree of homology at the protein level strongly suggests this is a true orthologue of oIRSa3.1 and hIRSa3.1 and could be designated mIRSa3.1. The phylogenetic relationship between the IRSa type I KIFs and the previously characterized hair and cytokeratin type I IFs suggest that the new keratin group may be the direct progenitors of the previously described hair IF and some cytokeratin IFs. This could explain characteristics of the new IRSa keratins, with compositional and structural features intermediate between the other two type I KIF groups.

The products of the ovine cDNAs, the human BAC Genes 3, 5, and 6, and mouse cDNA Krt1-c29 share a high degree of homology with each other. The genomic southern result ascertained that there are indeed three highly related IRSa genes in each species. As seen for the hIRSa genes in the BAC, clustering of the oIRSa genes is suggested by hybridization of the gene-specific 3′-noncoding antisense probes to one HindIII fragment in sheep DNA. As the 3′-noncoding region of oIRSa1 is very close to one end of this fragment and the general 3′-coding probe only binds weakly to it, the oIRSa3.1 3′-noncoding region must be near the other end, with transcription of the two genes in a convergent arrangement, as seen for genes in the ovine type II KIF locus (Powell and Beltrame, 1994).

Considering the apparent specificity of expression of the oIRSa and hIRSa genes and the abundance of their mRNA in the IRS, we expect these proteins to play a major role in formation of the 10 nm IFs of the IRS. KIF observed at the extracellular matrix level appear in the IRS just after the trichohyalin granules become visible.

Figure 6. Localization of IRSa type I IF expression by RNA in situ hybridization. RNA in situ hybridization using ovine and human gene-specific cRNA probes to Tukidale sheep skin (a±j) and tongue sections (k, l) and to human hairline scalp sections (m±o). For the sheep sections, a 331 base ovine trichohyalin 3′-noncoding antisense probe (see Bawden et al., 1998) was applied to longitudinally sectioned skin (d±f) and tongue (j) and to transversely sectioned skin (i, h). The corresponding consecutive Tukidale skin and tongue sections were probed with a 259 base oIRSa1 3′-noncoding antisense probe (longitudinal skin, a, b, c; tongue, k; transverse skin, g, h). Parts c, f, h, and j are dark-field photographs of the probed sections (3P-rUTP-labeled probes) in b, e, g, and i, respectively. For the human scalp consecutive sections, 3′-noncoding DIG-labeled antisense probes specific for human Genes 3 (hIRSa1; m) and 5 (hIRSa3.1; n) were applied (for probe information, see Materials and Methods). A negative control (no probe, only anti-DIG-alkaline phosphatase conjugate applied) is shown (o). Sections to which digoxigenin-rUTP-labeled probes and anti-DIG-alkaline phosphatase conjugate (dark blue precipitate signal) were applied are stained with SACPIC stain (a, d, m, n, o) whereas sections probed with 3P-rUTP-labeled probes are stained with hematoxylin (b, e, g, i, k, l). Scale bars: a (b±f; 50 μm); g (h±j; 25 μm); k (l; 100 μm) and o (m, n; 100 μm).
Rogers, 1958a; 1958b; Rogers, 1964; Steinert et al, 1971) and interact with trichohyalin to form the hardened IRS encasing the developing fiber (Rothnagel and Rogers, 1986; Fietz et al, 1993; Rogers et al, 1997b; Tarcsa et al, 1997). Our in situ hybridization data accord with the absence of such filaments in the medulla cells. This is further evidence that oIRSa1/hIRSa1 and the other members of this type I IF group are likely to be the major KIF form present in the IRS. The previous report of K10 expression in the IRS (Stark et al, 1990) might be explained by the close phylogenetic relationship of hIRSa1 and K10, and cross-reaction of the K10 antibodies.

Comprehensive BLAST comparisons of the human BAC clone with known human keratin and other keratin sequences present in the database revealed that, like the oIRSa and hIRSa keratins, the other putative KIFs encoded by genes in this BAC are novel. Transcripts from human Gene 4 were not detected in the IRS of human hair follicles by cRNA in situ hybridization [cf. Gene 3 (hIRSa1) and Gene 5 (hIRSa3.1); see Fig 6] despite the presence of consensus TATA and CAAT sequences in the 5'-untranslated region (sequences also present in Genes 0, 1, 3, 5, 3'-flanking DNA for Gene 6 has a poly adenylation signal). Gene 4 may be expressed in a tissue other than the IRS, being phylogenetically closer to Gene 1 than to the other genes. Gene 2, a suspected pseudogene with no identifiable promoter, Gene 0, predicted to encode either a 301 or 374 amino acid protein, and Gene 1 (encoding a 342 or 488 amino acid protein) each require further characterization. Elucidation of their expression status and true coding regions where appropriate will be important for clarification of the relationship between the IRSa, hair, and cytokeratin IF.

The murine skin type I KIF, mKrt1-c29 (Sato et al, 1999), is the only related sequence currently in the database and as suggested here is probably the orthologue of oIRSa3.1/hIRSa3.1. Sato et al (1999) showed, by analysis of gene order in the mouse type I KIF locus on chromosome 11, that mK10 and mK12 are clustered with mKrt1-c29 (mIRSa3.1) and that this group is part of a bigger locus, with the murine type I IF hair keratin (mHa1, mHa2, mHa4) separating them from the type I IF cytokeratins (mK13, mK15, mK19, mK14, and mK17). mKrt1-c29 is closest to the centromere and spaced 80 kb (or less) from mK10. There is some knowledge of gene arrangement in the type I IF locus on the long arm of human chromosome 17 (Bader et al, 1988; Milisavljevic et al, 1996; Ceratto et al, 1997). The placement of hIRSa3.1 (Gene 5) in the human BAC and absence of K10 or K12 within the new type I IF linkage group identified here, however, provide no clue as to the spatial relationship between these and the other type I KIF genes (Steinert et al, 1985; Fuchs and Weber, 1994; Rogers et al, 1995; 1998) within the human genome. Unfortunately, a recent scan of the database identified no other human sequences that can link the human BAC to other known human type I KIF sequences.

Overall, the data presented here and other recent data suggest that there are many KIFs with specialized functions in the pilosebaceous unit and in other epithelia that remain to be found. Apart from the previously cloned ovine type I KIFs of wool, cytokeratin K15, and the new ovine and human IRSa keratins...
described here, the recent finding of a human type II KIF, Khf6, specifically expressed in the companion layer of the outer root sheath (Winter et al., 1998), indicates that, as in other cellular layers of the hair follicle, there are type I and type II KIFs in the IRS that pair to form IF networks.

The present characterization of the type I IFs in the IRS is an important finding for the understanding of the structure and function of this layer. The insoluble contents of the differentiated cells are similar to that of the fiber cortex in that they contain a protein complex of keratin filament and matrix. Compared to the fiber cortex and according to the type I sequences described here, however, the IRS of the IRS cells have compositional features, especially their relatively low content of cysteine residues, which place them between the hair keratins and the cytokeratins. Furthermore, the matrix is not a complex of two families of proteins but is derived from trichohyalin, a unique protein that undergoes an enzyme-catalyzed modification of conformation followed by isopeptide cross-linking. The difference between cortical and IRS structures may facilitate the main role of the IRS as a physical support for the plastic hair as it grows in the follicle. Progressive hardening of the IRS by isopeptide cross-linking during growth, first in the Henle layer and then in the Huxley layer and IRS cuticle, moulds the fiber and appears to be intricately related to moulding of the flattened cuticle cells on the hair surface. The structural differences between fiber and IRS may also reflect the need for the IRS to be degraded by proteases in the upper reaches of the follicle (Ekholm and Egelrud, 1998) where its cells separate from the fiber and are sloughed. The molecular organization of hair keratin, a material of closely packed KIF and KAP proteins, makes it resistant to such proteolysis. Hence it is likely that proteins of the IRS are in a more open, fibrous organization that is amenable to isopeptide degradation. This is possibly achieved through isopeptide cross-links instead of by intensive disulphide cross-linking.

The authors wish to thank Dr. Robert Sinclair and Dr. Jack Green (Department of Dermatology, St. Vincent’s Hospital, Melbourne, Australia) for providing human scalp samples. We also thank Professor Phil Hynd for the provision of facilities within the Department of Animal Science, University of Adelaide, and Natasha Edwards for the preparation of human and sheep skin sections used in the RNA in situ hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.

REFERENCES


Bader BL, Jahn L, Franke WW: Low level expression of cytokeratins 8, 18 and 19 in hybridization experiments. This work was conducted under the auspices of the Cooperative Research Center for Premium Quality Wool.

Ekholm E, Egelrud T: Expression of stratum corneum chymotryptic enzyme in human IRS are in a more open, fibrous organization that is amenable to isopeptide cross-linking instead of by intensive disulphide cross-linking.


