Structure and Expression of Genes for a Class of Cysteine-rich Proteins of the Cuticle Layers of Differentiating Wool and Hair Follicles

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Abstract. The major histological components of the hair follicle are the hair cortex and cuticle. The hair cuticle cells encase and protect the cortex and undergo a different developmental program to that of the cortex. We report the molecular characterization of a set of evolutionarily conserved hair genes which are transcribed in the hair cuticle late in follicle development. Two genes were isolated and characterized, one expressed in the human follicle and one in the sheep follicle. Each gene encodes a small protein of 16 kD, containing >50 cysteine residues, ranging from 31 to 36 mol% cysteine. Their high cysteine content and in vitro expression data identify them as ultra-high-sulfur (UHS) keratin proteins. The predicted proteins are composed almost entirely of cysteine-rich and glycine-rich repeats. Genomic blots reveal that the UHS keratin proteins are encoded by related multigene families in both the human and sheep genomes. Tissue in situ hybridization demonstrates that the expression of both genes is localized to the hair fiber cuticle and occurs at a late stage in fiber morphogenesis.

All hairs share a common structural organization in which a cuticular layer of flattened cells encases a multicellular cortex. The cuticle usually forms a minor histological component of hairs, often only a single layer of overlapping cells in wool fibers but up to several layers in human hair (Swift, 1977). In hair follicle differentiation many keratin genes are transcribed to produce between 50 and 100 different proteins. These keratin proteins are classified into four groups according to their chemical and physical properties; the intermediate filament proteins, the high-glycine/tyrosine proteins, the high-sulfur (HS) proteins, and the ultra-high sulfur (UHS) proteins (Fraser et al., 1972).

In the development of the hair fiber, the cells in the follicle bulb destined to become cortex or cuticle cells undergo different differentiation pathways (for review see Powell and Rogers, 1986). Not only are there extreme changes in cell shape, but a striking hexagonal structural pattern is revealed in the cortical cells by EM (Rogers, 1959) in which intermediate filament proteins forming 10-nm-diam filaments are surrounded by a matrix composed of the other protein groups. In contrast, the cuticle cells become flattened and there seems to be a synthesis and gradual accretion of granules into two or three amorphous cysteine-rich intracellular layers, aligned parallel to the axis of the fiber (Happey and Johnson, 1962; Roth and Helwig, 1964; Dobb et al., 1972; Woods and Orwin, 1980).

Many sheep wool keratin proteins have been sequenced, but because of their high cysteine content the UHS keratin proteins are very difficult to study and pure proteins have not been isolated. The UHS keratin proteins comprise a small fraction of the total keratin proteins of wool but form a more significant component in mouse and human hair (Gillespie, 1983). The UHS keratin proteins are identified as containing >30 mol% of cysteine (Gillespie and Broad, 1972) as compared with the sequenced HS keratin proteins which contain from 16 to 24 mol% of this amino acid (Crewther, 1976; Swart et al., 1976). Their histological location in the hair fiber is uncertain but there is evidence that they occur both in the fiber cortex and the cuticle (Gillespie et al., 1964; Rogers, 1964; Ley and Crewther, 1980; Marshall and Ley, 1986).

A mouse UHS keratin gene was recently isolated by cross-hybridization using an oligonucleotide probe equivalent to a cysteine-rich repeat in the sheep wool HS keratin proteins (McNab et al., 1989). The predicted mouse protein contains 37 mol% cysteine. Additionally, a partial wool follicle cDNA clone (K4: 75 amino acids, 37 mol% cysteine) has been tentatively identified as an UHS keratin clone on the basis of its high cysteine content (Powell and Rogers, 1986). The sites of expression of these genes have not been identified.

One of the intriguing features of the UHS keratin proteins in sheep is that their level seems to be sensitive to the supply of cysteine. When the supply of cysteine is increased to the

1. Abbreviations used in this paper: HS, high sulfur; UHS, ultrahigh sulfur.
wool follicle by infusion of the sheep per abomasum with this amino acid there is a dramatic increase in the UHS keratin proteins (Reis and Schinckel, 1963; Gillespie and Reis, 1966; Broad et al., 1970). In general the synthesis of the other major components of wool does not vary, although a downregulation of the wool high-glycine/tyrosine keratin proteins has been reported (Gillespie, 1983). To investigate these phenomena we are isolating and characterizing the expression patterns of sheep wool keratin genes. In this report we describe the molecular characterization of related UHS keratin protein encoding genes from the sheep and human genomes. Tissue in situ hybridization has revealed that they are expressed in the hair cuticle cells at a late stage of fiber differentiation.

**Materials and Methods**

**Genomic Library Screening**

The human genomic cosmid library was a gift from Dr. K. H. Choo (Birth Defects Research Institute, Royal Children's Hospital, Parkville, Australia) and was constructed as described by Choo et al. (1986). The library was screened for recombinant clones by the method of Hanahan and Meselson (1983).

The sheep genomic library was a gift from Dr. R. Crawford from the Howard Florey Institute of Experimental Physiology and Medicine (Melbourne, Australia). The library was screened by the plaque hybridization method (Benton and Davis, 1977).

Restriction fragments of interest were subcloned from the genomic clones into pBl-21 vectors. Plasmid DNA was prepared by the method of Birnboim and Doly (1979), using CsCl density gradient equilibrium centrifugation (Radloff et al., 1967).

**DNA Sequencing**

DNA fragments to be sequenced were generated either by digestion with restriction endonucleases or by progressive deletion using BAL31 by the method of Davis et al. (1986) and were subcloned into M13mp8/19 vectors (Norrander et al., 1983). Single-stranded M13 template DNA for DNA sequencing was prepared by the method of Winter and Fields (1980). A kit obtained from Bresatec (Adelaide, South Australia) was used for most sequencing reactions according to the manufacturer's instructions. Some sequencing reactions were conducted using the Sequenase kit (U. S. Biochemical Corp., Cleveland, OH). [α-32P]dATP (3000 Ci/mmol, Bresatec) was used with both kits for labeling of extension products.

**In Vitro Transcription**

All fragments for in vitro transcription were endfilled with Klenow I (Bresatec) and subcloned into a pGEM2 Smal vector (Promega Biotec). The orientation of the inserts was determined by digestion with restriction enzymes that cut asymmetrically in the insert. Transcription templates were linearized downstream of the insert in the polylinker with appropriate restriction enzymes, phenol extracted, and ethanol precipitated before in vitro transcription. Unlabeled RNAs for in vitro translation, and cRNA and RNA labeled to high specific activity using [α-35S]UTP (1,350 Ci/mmol; New England Nuclear, Boston, MA) for in situ hybridization, were synthesized with either T7 or SP6 RNA polymerase by the method of Krieg and Melton (1987), using a kit obtained from Bresatec.

DNA fragments to be cloned for in vitro transcription and translation of the UHS keratin genes were digested with BAL31 exonuclease such that there were no methionine codons in the 5'-flanking sequences before the predicted initiation codon of each gene. The human UHS keratin gene construct (base 162-base 961 in Fig. 2 a) contained 74 bp of 5'-noncoding region, the complete coding region, and 216 bp of 3'-noncoding sequence. The sheep UHS keratin gene construct (base 697-base 1,716 in Fig. 3 a) contained 56 bp of 5'-noncoding region, the complete coding region, and 416 bp of 3'-noncoding sequence.

**In Vitro Translation**

Before in vitro transcription the transcripts were capped at the 5' end by the addition of G(5')ppp(5')G to the reaction mix as described by Konarska et al. (1984). Translation of the in vitro transcription products was conducted by the method of Pelham and Jackson (1976). 15-μl reactions containing 10 μl reticulocyte lysate (Amersham International, Amersham, UK), 5 mM I-(2,3-thi)serine (37 Ci/mmol; Amersham International), 135 mM potassium acetate, 100 mM of 19 amino acids minus serine (Amersham International), and one-tenth of the RNA from a single in vitro transcription, were incubated for 90 min at 30°C. The reactions were adjusted to 0.05 M Tris pH 8.0, 0.05 M Tris pH 8.0, and were incubated for 30 min at 37°C.

The products were S-carboxymethylated by addition of 4 μl of 1 M iodoacetic acid and 2.3 M Tris (pH 8.5). After 10 min at room temperature, the excess iodoacetic acid was added to react with 2 μl of 14.3 M 2-mercaptoethanol.

**Extraction and S-Carboxymethylation of Protein Samples**

Wool samples were obtained from Border Leicester-Merino cross and Lincoln wethers and human head hair was obtained from an adult Caucasian male. Extraction, 14C-labeling, and S-carboxymethylation were conducted as described by Marshall (1981) using iodo[2-14C]acetic acid (56 Ci/mmol; Amersham International).

**Two-dimensional PAGE**

Two-dimensional PAGE was conducted by a modification of the method of Marshall (1981). Alkaline (pH 8.9) electrophoresis was conducted in the first dimension. To the in vitro translation products 4 μl of 10 mM Trisphosphate pH 7.0, 4 μl 80% glycerol and 1 μl 1% bromophenol-blue (BPB) were added before loading onto the first dimension gel. To other samples an equal volume of a buffer containing 5 mM Tris-phosphate pH 7.0, 0.1% BPP, 50% glycerol was added. A slab gel (140 × 1 mm containing 100 mm 7.5% separating gel and 30 mm 4% stacking gel) was used for the first dimension. Electrophoresis was conducted at 10 mA. Strips containing protein samples were cut out after the BPB had migrated 80 mm through the separating gel and placed in a solution containing 0.125 M Tris-HCl pH 7.0, 0.1% SDS for 15 min.

SDS PAGE in the second dimension was conducted as described by Laemmli (1970), with the following modifications. Electrophoresis was conducted in a Hoefer slab gel apparatus which could accommodate up to four gel slabs. The excised strips from the first dimension were placed on top of a 140 × 140 × 1.5 mm slab gel and held in place with 1% agarose. A 10% separating gel was used. Prestained molecular weight markers (low molecular weight range; Bethesda Research Laboratories, Gaithersburg, MD) were loaded onto second dimension gels in a well formed in the stacking gel beside the first dimension strips. Electrophoresis was conducted at 30 mA until the tracking dye had migrated 80 mm through the separating gel. After electrophoresis the gels were soaked with agitation in 25% ethanol, 5% acetic acid for 30 min at room temperature and were transferred to 1 M sodium saline/coclylate solution for 30 min with agitation at room temperature. The gels were removed from this solution and dried onto paper (3 MM; Whatman Inc., Clifton, NJ) using a Hoefer gel-drying apparatus.

Samples fractionated in the same experiment on different gel slabs could be directly compared by alignment of the molecular weight markers.

**Southern Blot Analysis**

Restriction fragments to be used as probes were radiolabeled to high specific activity by the method of Feinberg and Vogelstein (1983) using a Bresatec kit (South Australia). Restriction enzyme cleaved plasmid and genomic DNA fragments were electrophoresed on 1% agarose and transferred to Zeta-Probe (Bio-Rad Laboratories, Richmond, CA) in 0.4 M NaOH as described by Reed and Mann (1985). For hybridization, filter bound DNA was prehybridized for at least 2 h at 41°C in 47% formamide, 3 × SSPE, 1% SDS, 0.5% Biotto, and 0.5 mg/ml autoclaved salmon sperm DNA. Labeled probe was then added to the same solution and hybridization carried out overnight. After hybridization, filters were washed in two changes of 2 × SSPE, 0.1% SDS at room temperature and then twice at 65°C for 1 h (low stringency). High stringency washing was conducted in 0.2 × SSPE, 0.5% SDS at 65°C, with occasional agitation, for 1 h.
Total RNA was isolated from follicles of Border Leicester-Merino cross wethers as described by Kuczek and Rogers (1985). Glyoxylated RNA (10 μg) was fractionated through 1.5% agarose as described by Thomas (1983) and transferred to Zeta-Probe (Bio-Rad Laboratories) by a modification of the method of Southern (1977). RNA was transferred without pretreatment of the gel using 10× SSC as the transfer buffer. After transfer, the Zeta-Probe membrane was washed in 10 mM NaOH for 5 min and neutralized in 2× SSPE. Hybridizations and washings were conducted under the same conditions as described for Southern blot analysis.

**Tissue In Situ Hybridization**

In situ hybridizations on fixed and sectioned skin biopsies of male Caucasian human head and beard hair follicles, Border Leicester-Merino sheep wool follicles and Lincoln sheep wool follicles were performed as described by Powell and Rogers (1990) with 35S-labeled cRNA probes prepared as described above. Final wash stringencies were 2× SSPE at 50°C for 30 min for coding region probes and 0.1× SSPE at 50°C for 30 min for 3′-noncoding probes.

DNA fragments cloned for in situ hybridization were as follows. Sheep UHS keratin coding region template: a 1,020-bp BAL31-Eco RI deletion fragment (base 697-base 1,716 in Fig. 3 a) covering the 5′-noncoding region, coding region and most of the 3′-noncoding region of the gene. Sheep UHS keratin 3′-noncoding template: a 531-bp Bgl II-Bam HI fragment from the 3′-noncoding region (base 1,294-base 1,825 in Fig. 3 a). UHS keratin cDNA clone K4 3′-noncoding template: a 307-bp Mbo I fragment from the 3′-noncoding region. Human UHS keratin coding region template: an 800-bp BAL31-deletion fragment (base 162-base 961 in Fig. 2 a) covering the 5′-noncoding region, coding region and 216-bp of the 3′-noncoding region.

**Results**

**Isolation and Sequence of Human and Sheep UHS Keratin Genes**

Using a partial wool follicle cDNA clone (K4) encoding a presumptive sheep UHS keratin protein (Powell and Rogers, 1986) several unsuccessful attempts were initially made to isolate a sheep UHS keratin gene from various λ libraries. In contrast, when a human cosmid library was screened under low stringency conditions with the same probe >30 positive colonies were detected in the first round screening. After rescreening twice at low density 16 positive colonies were purified and one was analyzed in detail.

Southern transfer analysis indicated the presence of two Eco RI fragments (1 and 17.4 kb) in the clone which hybridized to the K4 probe (Fig. 1 a). With several restriction enzymes tested at least two restriction fragments hybridized strongly to this probe, suggesting the presence of more than one gene in this cosmid. The hybridization-positive 1-kb Eco RI fragment was subcloned and completely sequenced (Fig. 2 a). A presumptive UHS keratin gene was located within this sequence, encoding a protein of 168 residues, of which 36 mol% were cysteine. The cysteine residues occur uniformly throughout the predicted protein which is predominantly composed of three types of peptide repeats: a cysteine-rich decapeptide, a glycine-rich decapetide, and a serine-rich nonapeptide (Fig. 2 b). Upstream from the open reading frame were noted sequences similar or identical to the Kozak sequence (Kozak, 1981, 1984) which is contiguous with the methionine initiation codon, the TATA box which is common to most eukaryotic promoters (Breathnach and Chambon, 1981) and to a lesser extent the CCAAT box (Benoist et al., 1980; Efstradiadis et al., 1980; Johnson and McKnight, 1989).

Following the successful isolation of a human UHS keratin gene another sheep λ library was then screened using as a probe the 1-kb Eco RI fragment which contained the complete human gene. One positive phage was obtained from the...
Figure 2. Sequence of the human UHS keratin gene. (A) The DNA sequence of the 1.0-kb Eco RI fragment containing the human UHS keratin gene is shown. The derived protein sequence of the gene appears above the DNA sequence. The predicted protein of 168 amino acids contains 36 mol% cysteine. The CCAAT and TATA box homologies in the 5'-flanking region are boxed and highlighted by reverse text and extended sequence homology flanking the two putative CCAAT boxes is underlined. Sequences homologous to the Kozak consensus sequence contiguous with the presumptive methionine initiation codon (Kozak, 1981, 1984) are also underlined. Two possible locations of the mRNA cap site are shown by open triangles. NO AATAAA motif indicative of the polyadenylation signal (Proudfoot and Brownlee, 1976) is present in the sequenced DNA downstream of the coding region of the gene and it is likely to be located outside of this Eco RI fragment. The three repeats found in the coding region of the gene are identified as follows: cysteine-rich decapeptide repeats (n); serine-rich nonapeptide repeats (m); glycine-rich decapeptide repeats (k). The arrows 74 bp upstream (base 162) from the methionine initiation codon and 216 bp downstream (base 961) of the termination codon define the BAL 31 fragment which was end-filled and subcloned into a pGEM2 Sma I vector for in vitro transcription. (Note: A preliminary and shorter version of this sequence and its analysis has already been published; see Fig. 5 of Rogers et al. [1989].) (B) Alignment of the human UHS peptide repeats. The amino acid sequences of each group of repeats are presented in the order in which they occur from the NH₂ terminus of the predicted protein. Differences between repeats are boxed and highlighted. Note that the second amino acid of the cysteine-rich repeats is equally likely to be cysteine or serine. These sequence data are available from EMBL/GenBank/DDBJ under accession number X55293.

first round screening and purified. Southern transfer analysis indicated the presence of two Bam HI fragments (2.8 and 9 kb) that hybridized to the probe (Fig. 1 b) and they appear to represent two genes on the basis of a partial restriction map (Fig. 1 c). The strongly hybridizing 2.8-kb Bam HI fragment was subcloned and partially sequenced. The sequence of the complete presumptive transcription unit of the sheep UHS keratin gene and some of the 5' and 3' flanking sequence was determined (Fig. 3 a). The predicted protein of 181 amino acids contains 31 mol% cysteine and, like the predicted human UHS keratin protein, is composed mainly of repeating peptides, in this case a cysteine-rich 21-amino acid repeat and a glycine-rich decapeptide repeat (Fig. 3 b). The coding region of the presumptive gene is flanked by consensus promoter (Benoist et al., 1980; Efstratiadis et al., 1980; Breathnach and Chambon, 1981) and polyadenylation signal sequences (Proudfoot and Brownlee, 1976; Birnstiel et al., 1985).

Electrophoretic Identification of the In Vitro Translation Products of the UHS Keratin Genes

The translation products of both predicted UHS keratin proteins were compared electrophoretically with keratin protein isolated from both wool and human hair. The protein products of the two UHS keratin genes were synthesized by in vitro transcription of a DNA template followed by in vitro translation. Clones for in vitro transcription were con-
Figure 3. Sequence of the sheep UHS keratin gene. (A) The DNA sequence obtained from part of the 2.8-kb Bam HI fragment containing the sheep UHS keratin gene is shown. The derived protein sequence of the gene appears above the DNA sequence. The predicted protein of 181 amino acids contains 31 mol% cysteine. The sequences homologous to the CCAAT and TATA motifs in the promoter region and the AATAAA motif in the 3'-noncoding region are boxed and highlighted by reverse text. The T/G cluster (McLauchlan et al., 1985) in the 3'-flanking region is also boxed. The Kozak homology contiguous with the methionine initiation codon (1981, 1984) is underlined and possible locations of the mRNA cap site are indicated by open triangles. The two repeats found in the coding region of the gene are identified as follows: cysteine-rich 21 residue repeat (n); glycine-rich decapeptide repeats (m). The arrows 56 bp upstream (base 697) of the methionine initiation codon and 416 bp downstream (base 1,716: Eco RI site) define the BAL 31 deletion-Eco RI fragment which was end-filled and subcloned into a pGEM2 Sma I vector for in vitro transcription. The underlined BglII and Bam HI sites define the restriction fragment that was end-filled and subcloned into a pGEM2 Sma I vector for in situ hybridization and also used as a Y-non-coding probe in Southern and Northern analysis. (B) Alignment of sheep UHS keratin peptide repeats. The amino acid sequences of each group of repeats are presented in the order in which they occur from the NH2 terminus of the predicted protein. The two human glycine-rich repeats contain an extra serine residue. The two amino acid sequences of each group of repeats are presented in the order in which they occur from the NH2 terminus of the predicted protein. The two human glycine-rich repeats contain an extra serine residue. The two human glycine-rich repeats are presented below the line for comparison to the sheep repeats. Differences between repeats are boxed and highlighted. These sequence data are available from EMBL/GenBank/DDBJ under accession number X55294.
Figure 4. In vitro transcription and translation analysis of the UHS keratin genes by two-dimensional PAGE. In vitro sense transcripts of both UHS keratin genes were capped and translated in vitro in the rabbit reticulocyte lysate (Materials and Methods). The translation products were labeled using [3H]serine, because this amino acid is abundant (23 mol%) in the predicted sequence of both proteins and, unlike cysteine, has a relatively inert side group. After translation the proteins were S-carboxymethylated and separated by two-dimensional PAGE, with pH 8.9 electrophoresis in the first dimension and SDS-PAGE in the second dimension. Sheep wool and human hair protein were reduced and S-carboxymethylated using [14C]iodoacetic acid before two-dimensional PAGE. Electrophoretic separation of the three samples in each panel (A-C, D-F) was conducted in the same apparatus under identical electrophoretic conditions and included molecular weight markers so that the patterns of each were directly comparable to the others. All gels were fluorographed. (A) Wool protein. The arrow indicates the position of the human UHS keratin gene protein product. (B) Human hair protein. The arrow indicates the position of the human UHS keratin gene protein product. (C) Human UHS keratin gene protein product. (D) Wool protein. The arrows indicate the positions of the sheep UHS keratin gene and HS BIIIA gene protein products. (E) Sheep UHS keratin gene protein product. (F) Sheep UHS keratin gene and BIIIA gene protein products. The in vitro translation products of a sheep HS keratin BIIIA gene (Crocker, L., and B. C. Powell, unpublished results) and the sheep UHS keratin gene were electrophoresed together. The positions of each were determined by comparison to gels in which the in vitro translation product of each gene was electrophoresed individually.
There Are Several Related UHS Keratin Genes in the Sheep and Human Genomes

Genomic Southern blot analysis of human and sheep DNA was firstly conducted using a human UHS keratin gene coding region probe to examine the number and extent of conservation of any related genes in the human and sheep genomes (Fig. 5). Several Eco RI and Hind III restriction fragments cross-hybridized to the human probe, indicating the presence of related sequences in both genomes (Fig. 5 A). Subsequently, the filter was stripped and rehybridized with a coding region probe from the sheep UHS keratin gene and the hybridization patterns produced were identical, indicating that the human and sheep UHS keratin gene families were closely related. Subsequently, the filter was stripped and rehybridized with a coding region probe from the sheep UHS keratin gene and the hybridization patterns produced were identical, indicating that the human and sheep UHS keratin gene families were closely related.
Figure 7. In situ localization of UHS keratin gene expression in longitudinal sections of developing human hair and sheep wool follicles. Longitudinal 7-μm sections of human beard hair follicles or fine sheep wool follicles from a Border Leicester-Merino were hybridized with 35S-labeled UHS keratin antisense or sense (data not shown) RNA probes. *Bulb*, follicle bulb; *DP*, dermal papilla; *O*, outer root sheath; *I*, inner root sheath; *C*, cuticle; *Co*, cortex; *M*, medulla. The lines in *A* and *B* mark the approximate upper limit of the follicle bulb. (*A*) Brightfield view of a human beard hair hybridized with a human UHS keratin coding region probe (see Materials and Methods). The human cuticle is several cell layers thick and cuticle hybridization is readily distinguished and is indicated by the large arrow. The adjacent inner root sheath, which does not show any hybridization signal, is indicated by small arrows. This photograph is a composite of two photographs. Exposure was for 28 d. (*B*) Brightfield view of a sheep wool fiber hybridized with a sheep UHS keratin coding region probe (see Materials and Methods). The sheep wool cuticle consists of a single cell layer. Cuticle hybridization is indicated by the large arrow. Exposure was for 31 d. (*C*) Darkfield view of *B*. (*D*) Enlarged view of upper region of *B*. Note that the hybridization signal is specific to the cuticle (*large arrow*) and does not occur in the adjacent inner root sheath (*small arrow*). Bars: (*A*) 120 μm; (*B* and *C*) 50 μm; (*D*) 22 μm.
Figure 8. In situ localization of UHS keratin gene expression in transverse sections of developing human hair and sheep wool follicles. Transverse 7 μm sections of human head hair follicles or sheep wool follicles from Border Leicester–Merino (fine wool) or Lincoln sheep (coarse wool) were hybridized with 35S-labeled UHS keratin antisense or sense (data not shown) RNA probes. O, outer root sheath; I, inner root sheath; C, cuticle; Co, cortex. (A) Brightfield view of a human head hair hybridized with a human UHS keratin coding region probe (see Materials and Methods). Exposure was for 28 d. (B–D) Brightfield views of sheep Merino fine wool fibers; (F–H) darkfield views of the same fibers. (E) Brightfield view of a thick wool fiber from the Lincoln breed of sheep; (I) darkfield view of the same fiber. (B and F; E and I) Sheep UHS keratin coding region probe. (C and G) Sheep UHS keratin 3'-noncoding region probe. (D and H) Sheep UHS keratin cDNA clone K4 3'-noncoding probe. The same fibers are indicated by arrows in the complementary bright and darkfield panels. Sections shown in B–I were all exposed for 31 d. Note that not all the sheep follicles in each section show expression of the UHS keratin probes. This is a result of a combination of the narrow window of expression during follicle development (see Fig. 7 and text), the independent activity of each follicle, and the different depths at which the follicles are embedded in the skin. Bars: (A) 67 μm; (B–I) 120 μm.
Figure 9. Comparison of amino acid and nucleotide homologies between the UHS keratin genes and proteins. (A) Alignment of NH₂-terminal halves of the sheep (top) and human (bottom) UHS keratin gene coding regions commencing from the methionine initiation codon. An insertion of 21 nucleotides (7 amino acid residues; boxed) and a deletion of 3 nucleotides (1 amino acid; dash) have been allowed in the human gene to maximize the alignment. A match between respective nucleotides is indicated by a star, whereas identical amino acids are highlighted by reverse text. (B) Homology in the 5'-noncoding region between the sheep, human, and mouse (McNab et al., 1989) UHS keratin genes. Conserved nucleotides are highlighted by reverse text. Several single nucleotide insertions and deletions have been introduced into the human and mouse sequences to facilitate alignment. The methionine initiation codons for the respective mRNAs are underlined. (C) Comparison of the protein sequences predicted from the sheep UHS keratin partial eDNA clone K4 (Powell and Rogers, 1986) with the corresponding COOH-terminal end of the sheep gene and the cysteine-rich repeat from the human UHS keratin gene. Conserved amino acids are boxed and highlighted by reverse text. The cysteine-rich decapeptide repeats are highlighted by stippled boxes (●).

have been strongly conserved during mammalian evolution (e.g., compare Fig. 5 a, human UHS keratin coding region probed to sheep DNA/HindIII track, with Fig. 5 b, sheep UHS keratin coding region probed to the same track). A coding region probe from the sheep high sulfur keratin B2 family hybridized to different restriction fragments (data not shown). Fragments from the sheep gene 5'- and 3'-noncoding/flanking regions were also serially hybridized to the stripped filters (Fig. 5 b). The sheep gene 5'-noncoding/flanking region probe hybridized strongly to all the sheep DNA fragments to which the coding region probe hybridized, whereas the 3'-noncoding region probe hybridized to only two fragments (Fig. 5 b). The 5'-probe also hybridized weakly to human DNA (data not shown).

Northern Analysis of Sheep UHS Keratin Gene Expression in Wool Follicle RNA

The expression of the sheep UHS keratin gene described in this paper, and the related sheep UHS cDNA clone K4, was examined in the wool follicle (Fig. 6). All probes hybridized to an mRNA band approximately 1.5 kb in size. The signal obtained with the sheep UHS keratin gene coding probe was several fold stronger than that observed with the 3'-noncoding probe, and probably reflects the detection of several UHS keratin mRNA species with highly conserved coding regions but different 3'-noncoding regions. Indeed, the 3'-noncoding regions of the sheep UHS keratin gene and the cDNA clone K4 are different (data not shown). In this regard, we note that the K4 probe used in the Northern analysis contains, in addition to 241 bp of 3'-noncoding sequence, 69 bp of coding sequence in which there is a 26/27-bp match with the equivalent region of the related sheep UHS keratin gene. Thus, in part, the strong signal obtained with this probe after high stringency washing may reflect some cross-hybridization between conserved coding regions.

Localization of the UHS Keratin Gene Transcripts in Developing Hair Follicles

The sites of expression of the UHS keratin genes in hair and wool follicles were detected by in situ hybridization conducted on human and sheep follicle tissue. 35S-labeled cRNA probes were constructed from the conserved coding regions of the human and sheep UHS keratin genes, from the 3'-noncoding region of the sheep UHS keratin gene and from the sheep UHS keratin cDNA clone K4 (Figs. 7 and 8). With all the UHS keratin probes used, and with both sheep and hu-
Table 1. Amino Acid Composition of UHS Keratin Proteins

| Amino acid | Sheep UHS protein fraction | Human UHS gene | Sheep UHS gene | Sheep UHS cDNA 'K4' | Mouse UHS gene |
|------------|-----------------------------|----------------|----------------|---------------------|----------------|
| Ala        | 2.0                         | 1.2            | 1.7            | 2.7                 | 1.6            |
| Arg        | 6.9                         | 2.4            | 1.1            | 8.0                 | 4.8            |
| Asn        | -                           | 0.6            | 0              | 0                   | 0.5            |
| Asp        | 0.6*                        | 0.6            | 0              | 1.3                 | 2.7            |
| Cys        | 29.9                        | 35.5           | 31.0           | 37.3                | 37.6           |
| Gin        | -                           | 4.1            | 1.1            | 6.7                 | 9.1            |
| Glu        | 7.9*                        | 0              | 0              | 1                   | 1.1            |
| Gly        | 4.2                         | 16.0           | 28.0           | 5.3                 | 2.7            |
| His        | 1.3                         | 0.6            | 0              | 1.3                 | 3.2            |
| Ile        | 1.7                         | 0.6            | 0              | 1.3                 | 0.5            |
| Leu        | 1.4                         | 0              | 0              | 1.3                 | 1.1            |
| Lys        | 0.9                         | 5.0            | 5.5            | 1.3                 | 5.3            |
| Met        | 0                           | 0              | 0              | 0                   | 11.3           |
| Phe        | 0.5                         | 0              | 0              | 0                   | 1.1            |
| Pro        | 12.8                        | 6.0            | 4.4            | 6.7                 | 13.4           |
| Ser        | 12.7                        | 23.1           | 20.0           | 21.3                | 7.0            |
| Thr        | 11.1                        | 0              | 0              | 1.3                 | 0.5            |
| Trp        | ND                          | 0              | 0              | 1.3                 | 0.5            |
| Tyr        | 1.8                         | 1.8            | 0              | 0                   | 0              |
| Val        | 4.3                         | 3.0            | 6.1            | 5.3                 | 2.1            |

The amino acid contents (as residues per 100 residues) of the predicted proteins of the sheep and human UHS keratin genes described in this paper and data derived from the mouse UHS keratin gene (McNab et al., 1989) and the sheep UHS keratin cDNA clone, K4 (Powell and Rogers, 1986), are compared along with the amino acid analysis of a UHS keratin protein fraction from normal sheep wool (Gillespie and Broad, 1972; reproduced from Australian Journal of Biological Sciences, with copyright permission of CSIRO Australia). Note that the K4 amino acid analysis is tentative because the coding region is truncated in this partial keratin cDNA clone. * In the amino acid analysis of the sheep UHS keratin protein fraction the individual values for the Asn/Asp and Gin/Glu pairs were not distinguished.

human follicle tissue, hybridization was localized to the cuticle of the nascent fiber. No specific hybridization was observed with any sense probe and no hybridization was observed to the epidermis with any probe (data not shown). Hybridization to the cuticle was most obvious in the human hair follicles, where the cuticle is 8-10 cell layers thick (Figs. 7 a and 8 a), compared with the single cell layer of the wool fiber (Figs. 7, b–d and 8, b–i). Hybridization to the cuticle of the inner root sheath, which apposes the hair fiber cuticle, can be excluded since longitudinal sections of both human and sheep follicles (Fig. 7), where the inner root sheath has started to separate from the hair shaft as part of the normal sloughing process showed a strong signal along the edge of the fiber and not over the inner root sheath. When the UHS keratin probes were hybridized to longitudinal sections of wool follicles the grains were localized to the more distal regions of the fiber, well above the follicle bulb and dermal papilla (Fig. 7, a and b). In both cases these UHS keratin cuticle genes are restricted to a narrow window of expression during follicle differentiation and their mRNAs are first detected ~240 µm above the follicle bulb. The sheep UHS keratin coding region probe hybridized equally well to the fine wool follicles (~22 µm) of Border Leicester–Merino sheep (Fig. 8, b and f) and to the thicker wool follicles (~45 µm) of Lincoln sheep (Fig. 8, e and i). Specific 3'-noncoding probes from the sheep UHS keratin gene reported here and the sheep partial keratin cDNA clone K4 both hybridized to the cuticle cells of sheep follicles (Fig. 8, c and g, d and h, respectively).

Discussion

We have described the molecular characterization of two genes expressed in hair cuticle cells during hair follicle differentiation. About one-third of the amino acid residues of each predicted protein are cysteine and, accordingly, the proteins are classified as UHS keratin proteins on the criterion for this group of hair proteins of a half-cystine content >30 mol% (Gillespie and Broad, 1972). The in vitro expression data confirm that the gene products migrate to the same regions of two-dimensional polyacrylamide gels as authentic wool and hair UHS and HS keratin proteins.

Evolutionary Conservation of the Human and Sheep UHS Keratin Gene Coding Regions

The predicted sheep and human UHS keratin proteins contain 181 and 168 amino acids, respectively, and both have molecular masses of 16 kD. Both proteins are predominantly (75 mol%) composed of three amino acids, cysteine, serine, and glycine, which are found in various repeating units. The human and sheep UHS keratin proteins show striking homology, most extensive in the NH₂- and COOH-terminal regions, with the central regions of both proteins being more divergent. Comparing the first 71 amino acids of the sheep protein with the first 78 of the human UHS keratin protein, 63 are identical if a single insertion of 7 amino acids and a deletion of one amino acid in the human sequence is allowed (Fig. 9 a). A shorter region of strong homology is observed towards the COOH terminus with 13 of the last 16 residues identical (compare Figs. 2 a and 3 a). Between these regions the strict conservation breaks down and the remainder of each protein is composed of mixed repeats (compare Figs. 2 a and 3 a). For example, in the sheep protein, 21-residue cysteine-rich repeats are interspersed with glycine-rich repeats, whereas, in the divergent part of the human protein,
decapetide cysteine-rich repeats are present and are inter-
spersed with serine-rich nonapetide repeats. The cysteine-
rich decapetide repeats of the human protein are homolo-
gous (6/10 amino acids) with the first half of the sheep 21
amino acid cysteine-rich repeat.

The partial wool follicle cDNA clone K4 used to isolate
the genes described in this paper was predicted to be a UHS
keratin cDNA clone (Powell and Burke, 1986). We have
shown that it shares extensive nucleotide and amino acid se-
quence homology with the sheep and human UHS keratin
coding regions and that it is also expressed in the wool cuti-
cle. Of the last 42 amino acids of the sheep UHS keratin pro-
tein 33 are identical to the predicted keratin coding sequence
of K4, but they are displaced ~30 amino acids up from the
K4 termination codon (Fig. 9 c). In succession from the
NH$_2$-terminal end of the partial K4 sequence they form an
almost complete 21-residue cysteine-rich repeat and a gly-
cine-rich repeat. At the COOH-terminal end of the predicted
K4 protein are 4 cysteine-rich decapetide repeats that are
similar to the human repeats (Fig. 9 c) and to the first 10
amino acids of the sheep cysteine-rich 21-residue repeat
(compare with Fig. 3 b).

In contrast, the protein encoded by the mouse hair UHS
keratin gene (McNab et al., 1989) differs from the human
and sheep UHS keratin genes reported here. There are nota-
table differences in amino acid composition, one of the most
striking being the low proportion of glycine residues (Table
1). Although the predicted coding sequence of the K4 cDNA
clone has a similarly low proportion of glycine residues it is
too preliminary to make comparisons with this incomplete
cloned based on this amino acid because in the sheep and hu-
man UHS keratin proteins the distribution of glycine res-
dues is polarized towards the NH$_2$-terminal half of the pro-
teins. McNab et al. (1989) identify a tetrapetide, CCQP,
repeated 12 times in the mouse UHS keratin protein, al-
though most of these can be rearranged into several octapet-
dide repeats of the form, SSCCQPC, homologous to the
cysteine-rich decapetide repeats of the human and sheep
proteins described here (data not shown, but compare Figs.
2 b and 3 b). The mouse gene is nevertheless quite different.

**The UHS Keratin Multigene Family**

The human and sheep UHS keratin genes and the sheep UHS
keratin clone K4 are homologous and seem to belong to the
same family in different genomes. There could be at least six
highly conserved UHS keratin genes in each genome (Fig.
5). As the human and sheep UHS keratin coding region
probes hybridize to the same restriction enzyme fragments
(Fig. 5) there is a close relationship between all the sheep
and human genes. These genes may be clustered in both ge-

omes. Southern blot analysis and partial mapping data (Fig.
1, and data not shown) indicate at least two genes in each of
the two isolated clones. Additionally, human chromosomal
in situ hybridization experiments with the human UHS kerat-
in gene coding probe indicate only two chromosomal loci,
both on chromosome 11 (MacKinnon et al., 1991). The
sheep gene may have been recently duplicated or a polymor-
phism may be present in the 3'-noncoding/flanking region of
the gene because a fragment from this region detected two
Hind III bands on a genomic Southern blot (of DNA derived
from a different source to that of the cosmid clone) yet the
isolated gene itself did not contain a Hind III site.

It is likely that both genes lack introns, although formal
proof would require primer extension and S1 protection anal-
ysis. Similarly, the mouse UHS keratin gene does not appear
to contain any introns (McNab et al., 1989) and neither do
the other sheep wool keratin matrix genes that have been se-
quenced (Powell et al., 1983; Kučzek and Rogers, 1987;
Frenkel et al., 1989; Powell, B., R. Keough, L. Cracker, and
G. Rogers, manuscript in preparation). The most direct evi-
dence that the coding regions of the two UHS keratin genes
do not contain introns comes from in vitro expression of
gene constructs containing the open reading frames. The
synthesized protein products comigrate on two-dimensional
gels with the bulk of the UHS and HS keratin protein isolated
from wool and hair (Fig. 4).

**The 5'-Noncoding Regions of the UHS Keratin Genes
Are Highly Conserved**

The human and sheep UHS keratin genes have very similar
5'-noncoding regions with over 80% homology between them (Fig. 9 b). All genes in this sheep family may have simi-

larly conserved sequences because the 5'-noncoding region
probe cross-hybridized strongly to the same genomic restric-
tion fragments as a coding region probe (Fig. 5 b). The probe
also contained 5'-flanking sequence; thus the homology may
extend further upstream into the promoter regions. The 5'-
noncoding homology observed between the human and sheep
UHS keratin genes, which have been separate for ~90
Myr, suggests that these sequences may have some func-
tional significance, perhaps in the regulation of transcription
or translation. It is noteworthy then that the 18-bp sequence
present in the 5'-noncoding region of most HS and high-
glycine/tyrosine keratin genes (Powell et al., 1983; Powell
and Rogers, 1986) is lacking from the sheep UHS keratin
gene. This may indicate a difference in the mechanism of
regulation between the UHS keratin genes and the other ker-
atin genes.

**Developmental Expression of UHS Keratin Genes in
the Hair Follicle**

When the human UHS keratin gene was hybridized in situ
to human hair follicles hybridization occurred around the pe-

riphery of the nascent hair fiber. Similar observations were
made with the sheep UHS and K4 cRNA probes on wool fol-
licle tissue. Thus, these UHS keratin mRNAs are expressed
in the cuticle cells and not in the hair cortex. This is in accord
with protein analysis studies that report cysteine-rich pro-
teins in the wool cuticle (Swift, 1976; Ley and Crewther,
1980; Marshall and Ley, 1986). Histochemical studies on
the ultrastructure of the hair cuticle have described the initial
appearance of cysteine-rich granules in the cytoplasm of
differentiating cuticle cells in the upper region of the follicle
bulb with progressive accumulation of granules during folli-
cle development (Swift, 1977; Woods and Orwin, 1980).
However, the UHS cuticle keratin genes reported here are
expressed relatively late in follicle differentiation and are re-
stricted to a narrow developmental window (Fig. 7). It there-
fore seems likely that there are other UHS keratin genes
which have a different cuticle developmental pattern and are
expressed at an earlier stage of follicle differentiation.
Sulfur-enrichment of sheep wool, through infusion of cysteine or cysteine-rich proteins into the abomasum, leads to an increase in the UHS keratin protein fraction (Gillespie et al., 1964; Gillespie, 1983). Concomitantly, electron micrographs of sectioned wool fibers reveal changes in the filament/matrix arrangement of cortical cells, notably an increase in the spacing of the filaments (Gillespie et al., 1964; Rogers, 1964). This was interpreted as an increase in the amount of cortical matrix protein and together these observations suggest that UHS keratin proteins are synthesized in the cortex of sulfur-enriched wools. In this regard we note that the amino acid compositions of the predicted sheep and human UHS keratin proteins presented here differ from that of a purified UHS keratin fraction from normal sheep wool in several respects (Table I). The wool analysis more closely resembles that of the predicted mouse UHS keratin protein (Table I). Unfortunately there are no in situ hybridization data available on the location of expression of the mouse gene. Although our in situ hybridization data and the protein isolation studies (Ley and Cree, 1980; Marshall and Ley, 1986) show that the hair cuticle is a major site of expression of UHS keratin proteins, it is more likely that other UHS keratin proteins with different protein compositions and electrophoretic properties are expressed in the cortex and respond to cysteine infusion. Preliminary experiments in our laboratory suggest that there is indeed another cysteine-rich protein family which is expressed in the wool fiber cortex (Powell, B., et al., unpublished observations).

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