Identification of novel wool keratin intermediate filament genes in sheep skin

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ABSTRACT

Keratin intermediate filament (KIF) genes encode key proteins for hair and wool formation. A total of 17 expressed human KIF genes involved in hair formation are annotated. However, to date, only eight wool KIF genes have been reported. In this study, six new cDNA sequences (*KRT32*, *KRT33B*, *KRT34*, *KRT39*, *KRT40* and *KRT82*) representing previously unreported wool KIFs were identified using a contiguous ovine sequence library constructed primarily from ESTs. The expression of three other KIF genes (*KRT36*, *KRT84* and *KRT87*) was confirmed by PCR using sheep skin total RNA. The analogue of human *KRT37* (type I) was unable to be identified, while *KRT87* (type II) was present in sheep but not in humans. Therefore 10 type I and seven type II KIF family members have been identified in sheep in comparison to 11 and six KIFs in the human. These 17 KIF genes are likely to represent the complete or near-complete set involved in wool formation. The annotation of these genes will facilitate investigation into their patterns of expression in wool follicles and their roles in the determination of fibre attributes.

Key words: keratin intermediate filament; wool; gene expression.

INTRODUCTION

The genes encoding keratin intermediate filaments (KIF) and their linked matrix proteins (keratin-associated proteins, KAP) are expressed in a highly regulated manner during hair and wool follicle growth. This is reflected by the ordered temporal and spatial distribution of their mRNA and translated proteins in growing hair and wool follicles (Langbein *et al.*, 1999; Langbein *et al.*, 2001; Langbein & Schweizer, 2005; Yu *et al.*, 2009). As keratins comprise over 90% of proteins found in wool, they are thought to play a major role in determining fibre attributes (Powell & Rogers, 1997). This hypothesis is supported by recent observations on the relationship between gene expression patterns and fibre conformation in both humans and sheep (Plowman *et al.*, 2006; Thibaut *et al.*, 2005; Thibaut *et al.*, 2007; Yu *et al.*, 2009). The study of sheep breeds producing diverse wool types has also shown correlation between relative levels of keratin gene expression and wool traits (Yu *et al.*, 2007; Yu *et al.*, 2008a). However, the limited number of reported ovine KIF and KAP genes restricts a more comprehensive analysis of underlying structure-function relationships (Powell & Rogers, 1997).

A total of 17 active hair KIF genes, representing the complete set of KIFs involved in human hair formation, have been reported. These KIFs can be divided into smaller, acidic type I and larger, basic-neutral type II members. The type I and type II families comprise 11 (*KRT31-32, 33A, 33B, 34-40*) and six (*KRT81-86*) KIFs respectively (Langbein & Schweizer, 2005; Langbein *et al.*, 2006; Langbein *et al.*, 2007). Members of each human KIF family have been classed into seven groups based on their sequence homologies (Langbein et al., 2007; Langbein et al., 2006). All KIF proteins have three large domains with different functions. The head and tail domains at the N- and C-termini are more variable, while the central rod domains have a heptad repeat of amino acids that are essential for filament assembly (Parry & Steinert, 1992; Parry *et al.*, 2007). High levels of sequence conservation occur between the members of type I and type II families, which are preserved between corresponding proteins across mammalian species, particularly in the central rod domain (Parry & Steinert, 1992; Parry *et al.*, 2007).

Although KIFs were first intensively studied in wool, only six ovine KIFs have been reported (Dowling *et al.*, 1986; Powell *et al.*, 1992; Powell & Beltrame, 1994; Powell & Rogers, 1997; Sparrow *et al.*, 1989; Sparrow *et al.*, 1992; Wilson *et al.*, 1988). Two additional members were recognised recently based on the sequence conservation using an ovine cDNA contiguous library constructed from sheep skin ESTs and other published ovine sequences (Yu *et al.*, 2009). The aim of this work was to reveal further ovine KIF genes based on the high degree of between-species sequence homology (Powell & Rogers, 1997).

MATERIALS AND METHODS

Identification of wool KIFs from an ovine cDNA library

cDNA libraries were constructed from the skin of Romney, Merino and New Zealand Wiltshire sheep breeds and sequenced (MWG Biotech AG, Munich, Germany) (Yu *et al.*, 2009; Yu *et al.*, 2008b). Contiguous sequences (contigs) were assembled from the expressed sequence tags (ESTs) and other published ovine sequences (Keane *et al.*, 2007; Yu *et al.*, 2009).

In order to identify contigs or singleton ESTs representing novel wool KIF genes, GenBank cDNA and protein sequences of non-ovine species, particularly cattle and human, were used to BLAST the contiguous ovine cDNA library (CS39). Ovine cDNA sequences with high homology (E value <e - 100) were subsequently used for whole length sequence alignment. cDNA sequences which displayed the maximal homologies to KIFs from human, cattle or other species were then translated into protein sequences for further alignment and homology assessment. Discrepancies and ambiguous matches between potential orthologues of different species were then resolved by careful examination of ovine contig assemblies, where available, and conservation between species. Once the identity of a corrected cDNA sequence was determined, the sequence and the encoded protein were named according to the current nomenclature for mammalian KIFs (Schweizer *et al.*, 2006).

Identification of novel wool KIFs in sheep skin by PCR

As no ovine cDNAs with adequate similarity to *KRT36*, *KRT37* or *KRT84* were found, primer sets for each of these genes was designed from cattle (*KRT36* and *KRT84*) and human (*KRT37*) orthologues (Table 1). PCR primers were also designed from the cDNA of a previously reported wool KIF-like gene (Powell *et al.*, 1993). Total skin RNA extraction and reverse transcription of mRNA were conducted as previously described (Yu *et al.*, 2009). The PCR amplification was conducted using Platinum Taq (Invitrogen, Carlsbad, California, USA) under standard conditions. PCR products were separated by electrophoresis in 2% agarose in TAE and visualised by incorporation of SYBR(R) Safe (Invitrogen) in agarose gel.

Grouping of type I and type II wool KIFs

The cDNA sequences were translated into proteins using the appropriate coding frame in VectorNTI (Invitrogen). The deduced protein sequences for KIFs in the same family were aligned to determine the degree of conservation or diversity within each group.

TABLE 1: Primer sequences and product sizes for missing wool KIF genes.

Gene	Forward primer	Reverse primer	Product size (bp)
KRT36	GAGCTGCTTGCCTGGTTCCTTC	CGTACCACTCGCGGATGCG	209
KRT84	ACGAGGAGATGCGAGTGACAGC	TGCCTTCAGCCTCTGGATCAGC	101
KRT87	CTATGACCTGTGGATCGGCTTGC	TGACTGACACGGAGGTGATGCAG	303

TABLE 2: Identification of eight novel wool KIF cDNA. Genes in bold are identified by PCR using sheep skin RNA.

cDNA			Encoded protein			
Gene name	Size (bp)	Sequence source	Protein name	Size (aa)	Identity (%) to orthologue	
					Bovine	Human
KRT32	714 + 761[1]	Two ESTs	K32	214 + 154	92.2 + 92.1[3]	74.4 + 74.5
KRT33B	1560	Contig	K33b	405	92.1	85.7
KRT34	1588	Contig	K34	407	95.3	82.2
KRT36	209	PCR	K36	69	97.1	85.5
KRT39	767	EST	K39	253	93.3	78.3
KRT40	898	contig	K40	299	96.3	80.9
KRT82	579	EST	K82	193	97.9	85.5
KRT84	154	PCR	K84	51	98.0	90.2
KRT87	1893[2]	EST/PCR	K87	480	NA	NA

[1]Presence of two non-overlapping ESTs.
[2]Accession number X72379.1.
[3] Numbers represent the percentages of shared residues.

RESULTS

Identification of novel wool KIFs

Ovine cDNA sequences with high homologies to 14 bovine and human KIF genes were identified. Eight of these corresponded to known wool KIFs while the remainder represented previously unreported genes. Ovine *KRT32* was represented by two non-overlapping ESTs, while *KRT39* and *KRT82* were each represented by a singleton (Table 2). The three ovine contiguous sequences which corresponded to *KRT33B*, *KRT34* and *KRT40* were larger in size, but only the *KRT33B* contig encoded a full length protein (Tables 2 and 3). The amino acid sequence for K32 was missing 33 residues in its central rod domain. It shared 92% and 74% amino acid identity in the corresponding

TABLE 3: Summary of wool KIF genes identified. The newly identified wool KIF genes are in bold. * Number represents the total or estimated number of residues for each protein. Numbers following a letter indicate the number of missing residues from the central (M) region, or N- and C-terminus.

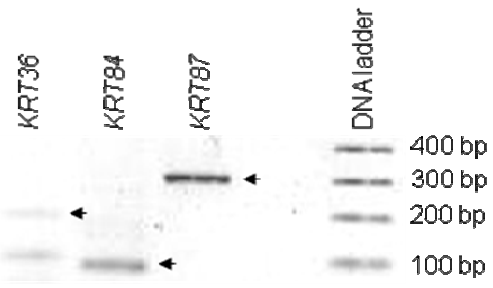
KIF type	Sheep / Human		Sheep		
	Revised names[1]		Previous names[2] and status		Current protein sequence status
	Gene	Protein	Gene	Protein	
I	*KRT31*	K31	*KRT1.1*[3]	K1.1 (8c1)	413*, Complete
	KRT32	**K32**	NA	NA	401, M33
	KRT33A	K33a	*KRT1.2*[4]	K1.2 (Partial)	404, Complete
	KRT33B	**K33b**	NA	NA	405, Complete
	KRT34	**K34**	NA	NA	433, N26
	KRT35	K35	NA[5]	NA	455, Complete
	KRT36	**K36**	NA	NA	455, N60, C326
	KRT37	K37	NA	NA	NA
	KRT38	K38	NA[5]	NA	453, Complete
	KRT39	**K39**	NA	NA	533, N280
	KRT40	**K40**	NA	NA	431, C132
II	*KRT81*	K81	*KRT2.9*[2]	K2.9	508, Complete
	KRT82	**K82**	NA	NA	510, N232, C85
	KRT83	K83	*KRT2.10*[6]	K2.10 (7c)	493, Complete
	KRT84	**K84**	NA	NA	593, N335, C207
	KRT85	K85	*KRT2.12*[7]	K2.12 (Component 5)	504, Complete
	KRT86	K86	*KRT2.11*[8]	K2.11 (Partial)	503, Complete
	KRT87	**K87**	*KRT2.13*	K2.13[9]	480, Complete

[1](Schweizer *et al.*, 2006); [2](Powell *et al.*, 1992); [3](Dowling *et al.*, 1986); [4](Wilson *et al.*, 1988); [5](Yu *et al.*, 2008b); [6](Sparrow *et al.*, 1989); [7](Sparrow *et al.*, 1992); [8](Powell *et al.*, 1992; Powell & Beltrame, 1994); [9](Powell *et al.*, 1993).

TABLE 4: Sub-groups of type I and type II wool KIFs.

Type	Group A	Group B	Group C	Group D	Group E
I	K31, K33A, K33B, K34	K32, K35, K36	K38	K39	K40
Type	Group F	Group G	Group H	Group I	
II	K81, K83, K86	K85, K87	K82	K84	

FIGURE 1: Detection of *KRT36*, *KRT84* and *KRT87* expression in sheep skin by PCR. Arrows point to cDNA bands of expected size.



segments with its bovine and human orthologues (Table 2). K33b was highly conserved across the three species, despite some degeneration in both N- and C-termini. It had 92.1% and 85.7% identical amino acids compared with cattle and sheep and human respectively (Table 2). Sheep K34 exhibited even higher overall homology to the bovine orthologue (Table 2), although 34 residues from the head domain were missing. The absence of 5' cDNA sequence from *KRT39* was reflected in approximately 280 amino acids absent from the N-terminus. Ovine K40 was also truncated; lacking 132 residues at the C-terminal (Tables 2 and 3). The encoded protein for *KRT82* derived from a single cDNA showed high homology to a region in the central rod domain of bovine and human K82 (Tables 2 and 3).

Another EST was found to have identical sequence to the previously reported *KRT2-13* (Powell *et al.*, 1993). Using a primer set derived from a region of *KRT2-13* upstream to the EST, PCR amplification resulted in a DNA fragment of the expected size and sequence (Figure 1). The much higher sequence homology of the encoded protein to type II wool KIFs (Z-D. Yu, Unpublished data) clearly indicates a new type II family member. This gene and the encoded protein were renamed as *KRT87* and K87 respectively (Tables 2 and 3).

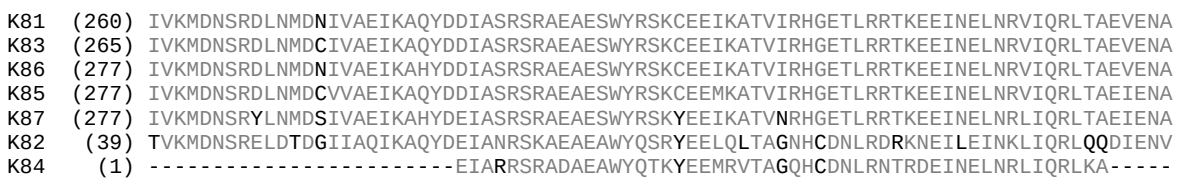
No cDNA sequences corresponding to human *KRT36*, *KRT37* and *KRT84* were identified. Using primers designed from bovine orthologues, products of expected sizes for *KRT36* and *KRT84* were amplified from sheep skin total RNA (Figure 1). Their high level of conservation with cattle and human analogues indicated that they represent ovine *KRT36* and *KRT84* (Tables 2 and 3). In contrast, two sets of PCR primers designed from the human *KRT37* failed to verify expression of an ovine orthologue in skin.

FIGURE 2: Alignments of full or partial amino acid sequences for type I Group A (a), type II Group F (b) and Groups F-I (c) wool KIFs. Arrows indicate the start and finish of central rod domain for (a) and (b). Only part of the central rod domain is shown in (c). Residues in grey are identical or similar, while those in black are distinctive. Short bars indicate missing residues or gaps created for appropriate alignment. Numbers in brackets are positions of each sequence at the start of a line.

(a) Group A

(b) Group F

(c) Groups F-I



Therefore, cDNA representing nine novel wool KIFs were found in this study (Table 3). Six were type I members (K32, K33b, K34, K36, K39 and K40), whereas three (K82, K84 and K87) belonged to the type II family. As a result, the annotated type I and type II wool KIFs have increased to 10 and seven respectively.

Subclasses of wool KIFs

Phylogenetic analysis on the available sequence information for hair and wool KIFs from human, sheep and cattle indicates that the 10 wool KIFs in the type I family fall into five groups. K31, K33a, K33b and K34 (Group A) are highly homologous in all regions apart from the C-terminus. If the extra 16 amino acids in the N-terminus of K34 are excluded, the homologous and identical residues for this group are 98% and 82% respectively (Figure 2a). In comparison, the available protein sequences in Group B (K32, K35 and K36) are conserved in the central rod domain, but are more heterogeneous in the head domain (not shown). In comparison, the diversity K38, K39 and K40 in the central rod domain indicates that they belong to three separate groups (Groups C-E) each comprising a single member (Table 4).

The seven type II wool KIFs form four groups. Group F members (K81, K83 and K86) are highly homologous despite of some degeneration in their head and tail domains (92% and 80% similar and identical residues respectively) (Figure 2b). Group G members, K87 and K85, are divergent to group F members in both head and tail domains, but are highly homologous in their central rod domain. Within the region where sequence was available for comparison from all members, K85 differs only by a methionine substitution for isoleucine, while K87 has three residue substitutions (Figure 2c). Two of the K87 substitutions are conserved in K82 and K84. However, these two KIFs differ distinctly not only from other type II members, but also from each other, thus forming the last two groups (H and I) each with a single member (Figure 2c).

DISCUSSION

Annotation of the complete human genome revealed a much larger set of KIF genes than previously anticipated (Schweizer *et al.*, 2006; Langbein *et al.*, 2007). Of the 54 active KIF genes, 26 are expressed in hair follicles. Of these, nine epithelial KIF genes only contribute to the formation of hair follicle inner root sheath, leaving 11 Type I (*KRT31-40*) and six type II (*KRT81-86*) KIF genes encoding the bulk of hair protein constituents (Langbein & Schweizer, 2005).

The sheep contiguous cDNA library used in this study has proved to be a valuable source for finding unknown KIF and KAP genes. The highly conserved sequence homology amongst the mammalian hair KIF genes and their protein products (Parry *et al.*, 1987; Powell & Rogers, 1997; Parry *et al.*, 2007) allowed the identification of six cDNA representing new wool KIF genes. The finding of two additional KIF cDNA by PCR using sheep skin total RNA which were not represented in the library suggests a low level of expression. The presence of a previously reported hair-like gene (Powell *et al.*, 1993), now renamed as *KRT87* according to the new nomenclature system (Schweizer *et al.*, 2006), was confirmed by the presence of an EST in the cDNA library, by PCR from sheep skin and finally by localising its mRNA in wool follicles by *in situ* hybridisation (data not presented). Although species-specific type I KIF genes involved in hair formation have been identified in chimpanzee and gorilla (*KRT41*) as well as mouse and rat (*KRT42*) (Rogers *et al.*, 1998; Hesse *et al.*, 2004; Schweizer *et al.*, 2006), *KRT87* is the first species-specific type II KIF gene reported to date. Although it was originally thought that a type I KIF pairs with a specific type II member during initiation of filament formation (Powell and Rogers, 1997), the imbalanced numbers of the two types of KIF genes in sheep, as well as their unmatched patterns of expression in growing wool follicles (Z-d. Yu, Unpublished data), supports promiscuous association between type I and type II KIFs during keratin formation (Langbein *et al.*, 2001; Parry *et al.*, 2007).

Despite the suggestion that one of the six human type II KIF genes, *KRT84*, is not involved in hair formation (Rogers *et al.*, 2000; Langbein *et al.*, 2001), we have amplified and cloned *KRT84* cDNA from mid-side sheep skin, and also observed its expression in wool follicles a preliminary RNA localisation study (Z-d. Yu, Unpublished data).

Although all 11 human type I KIF genes are utilised in hair growth, the expression of *KRT37* is restricted to the medulla of all male and female sexual hair, but not detectable in the terminal scalp hair (Jave-Suarez *et al.*, 2004). Its expression is also observed in vellus hair which becomes miniaturised, at least partially, in response to androgens, particularly testosterone (Langbein *et al.*, 1999). While *KRT37* expression is regulated by androgens in the human, this does not appear to be the case for other KIF genes, including the highly homogenous *KRT38* (Jave-Suarez *et al.*, 2004). The presence of active *KRT37* in primate, but not mouse and rat genomes (Hesse *et al.*, 2004), strongly suggests this gene has gone through some relatively recent changes. Consistent with this, a gene highly similar to *KRT38* in both bovine and ovine genomes appear to be pseudogenes which are not expressed (Z-d.Yu, Unpublished data).

While the total number of annotated wool KIFs is the same as in the human, the absence of *KRT37* and the presence *KRT87* in sheep alters the groupings of both type I and type II KIFs. To avoid the confusion of having the same group names for type I and type II KIFs (Rogers *et al.*, 1998; Rogers *et al.*, 2000; Langbein *et al.*, 2001; Hesse *et al.*, 2004; Schweizer *et al.*, 2006), we used an alphabetical system covering both types of KIFs. Group A members of type I wool KIFs (K31, K33a, K33b and K34) are highly conserved as in the human (Langbein *et al.*, 2007). Similarly, Group B members (K32, K35 and K36) are highly homologous in the central rod domain, but diverse in head and tail domains. Groups C-E each has only one member with even lower levels of sequence conservation.

The presence of K87 in sheep has made it more appropriate to classify type II KIFs into four groups (Groups F-I), rather than two groups as in the human. Group F members (K81, K83 and K86) are highly homologous (Rogers *et al.*, 2000). However, the distinct difference in the central rod domains of K32 and K34 suggest two different groups (groups G and H). In the human, K85 forms a diverse group with K32 and K34 (Rogers *et al.*, 2000). In the sheep, K85 and the specifies-specific K87 are both more homologous to Group F members, and form the last type II group (Group I). Although these assignments in sheep reflect the available sequence conservation, further analysis with complete sequences is required for verification. With current progress in sequencing the sheep genome the full sequences for all KIF genes and their genomic structures are likely to be available in the next few years.

The identification of a complete or nearly complete set of KIFs genes involved in wool formation has provided information that will facilitate verification of their proteins by independent approaches, such as 2-D gel and mass spectrometry. Gene-specific probes designed from the cDNA sequences have allowed the detection and comparison of their expression in wool follicles as conducted previously (Yu *et al.*, 2009). In the human, three genes (*KRT32*, *KRT39* and *KRT82*) are solely expressed in the fibre cuticle cells, while *KRT35*, *KRT39* and *KRT85* are expressed in both fibre cuticle and cortical cells. In comparison, the remaining 10 genes are predominantly expressed in the developing hair cortex (Langbein *et al.*, 2006; Rogers *et al.*, 2006; Langbein *et al.*, 2007; Langbein *et al.*, 2009). Preliminary results from our *in situ* hybridisation have indicated many similarities, but also differences, in the expression of ovine orthologues in the wool follicle. The annotation of these wool KIF genes will facilitate a more comprehensive quantitative examination on the relationships between KIF gene expression and wool traits (Yu *et al.*, 2007) , with the ultimate aim to understand their roles in imparting wool characteristics.

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