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Peer-reviewed

Citation for published item:

Porter, R.M. and Hutcheson, A.M. and Rugg, E.L. and Quinlan, R.A. and Lane, E.B. (1998) 'cDNA cloning, expression, and assembly characteristics of mouse keratin 16.', *Journal of biological chemistry.*, 273 (48). pp. 32265-32272.

Further information on publisher's website:

<https://doi.org/10.1074/jbc.273.48.32265>

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cDNA Cloning, Expression, and Assembly Characteristics of Mouse Keratin 16*

(Received for publication, August 25, 1998)

Rebecca M. Porter^{‡§}, Alene M. Hutcheson[¶], Elizabeth L. Rugg[‡], Roy A. Quinlan[¶],
and E. Birgitte Lane[‡]

From the [‡]Cancer Research Campaign Cell Structure Research Group, Department of Anatomy and Physiology and the [¶]Department of Biochemistry, Medical Sciences Institute/Wellcome Trust Building Complex, University of Dundee, Dow Street, Dundee DD1 5EH, United Kingdom

There has been speculation as to the existence of the mouse equivalent of human type I keratin 16 (K16). The function of this keratin is particularly intriguing because, in normal epidermis, it is usually confined to hair follicles and only becomes expressed in the suprabasal intrafollicular regions when the epidermis is traumatized. Previous studies suggested that K16 is highly expressed in the skin of mice carrying a truncated K10 gene. We therefore used the skin of heterozygous and homozygous mice to create a cDNA library, and we report here the successful cloning and sequencing of mouse K16. Recent *in vitro* studies suggested that filaments formed by human K16 are shorter than those formed by other type I keratins. One hypothesis put forward was that a proline residue in the 1B subdomain of the helical domain was responsible. The data presented here demonstrate that this proline is not conserved between mouse and human, casting doubt on the proposed function of this proline residue in filament assembly. *In vitro* assembly studies showed that mouse K16 produced long filaments *in vitro*. Also, in contrast to previous observations, transfection studies of PtK2 cells showed that mouse K16 (without the proline) and also human K16 (with the proline) can incorporate into the endogenous K8/K18 network without detrimental effect. In addition, K16 from both species can form filaments *de novo* when transfected with human K5 into immortalized human lens epithelial cells, which do not express keratins. These results suggest that reduced assembly capabilities due to unusual sequence characteristics in helix 1B are not the key to the unique function of K16. Rather, these data implicate the tail domain of K16 as the more likely protein domain that determines the unique functions.

Keratins are a family of proteins that form the intermediate filament cytoskeleton in epithelial cells. They are divided into two groups, the type I acidic keratins and the type II basic keratins (1). The first step in keratin intermediate filament (KIF)¹ assembly is the formation of heterodimers between a

type I and a type II keratin (2, 3). Although any type I keratin can bind with any type II keratin, only certain pairs of keratins exist *in vivo* (4). These pairs are expressed in a tissue-type and developmentally regulated manner (5). However, for some keratins, such as K17, a specific/unique partner has not been found.

K16 and its partner, K6, are expressed in various stratified epithelia including those of the oral cavity, esophagus, genital tract, and epidermis (6). In the epidermis, they are normally confined to the hair follicles, sweat and sebaceous glands, although they are also expressed in the suprabasal layer of palmoplantar epidermis (6, 7). The expression of these two keratins is of particular interest since, together with K17, they are expressed in hyperproliferative situations such as benign and malignant tumors, keratinocytes in culture, hyperproliferative skin diseases such as psoriasis, and wound healing (8–11). K6, K16, and K17 are therefore sometimes referred to as the “hyperproliferation-associated” keratins. Often associated with the expression of these keratins is the down-regulation of the differentiation-specific keratins 1 and 10, which normally predominate in the suprabasal epidermis (12). *In vitro* studies suggest that K16 has unique properties that could affect the structural organization of KIFs (13). Specifically, K16 appears to form shorter filaments than K14 with the type II keratins K5 and K6a. If this property of making shorter filaments is important for the modulation of keratinocyte behavior via alterations in the cytoskeleton during wound healing and in hyperproliferating keratinocytes, then the K16 sequence would be expected to be highly conserved among mammalian species.

Mutations in keratin genes, including K16, have now been identified as the underlying cause of several inherited epithelial disorders (14). Mutations in human K16 (hK16) result in pachyonychia congenita type I, which is characterized by nail dystrophy, focal palmoplantar keratoderma, and oral keratoses reflecting the expression pattern of this keratin (15). Despite the apparently important functions of K16 in humans, there has been some speculation as to the existence of a K16 gene in mice (16). Using a mouse model for the inherited skin disease bullous congenital ichthyosiform erythroderma, our previous studies suggested that K16 is highly expressed in the acanthotic epidermis of these mice (17). Although there is some evidence to suggest that the hyperproliferation-associated keratins are controlled, at least in part, post-transcriptionally (18), we observed a significant increase in the putative mRNA levels of K16 in K10 mutant mice. It therefore seemed logical to assume that a cDNA library generated from the skin of these mutant mice would facilitate the isolation of the gene for mouse K16 (mK16). Here we describe the cloning and sequencing of a

* This work was supported by Cancer Research Campaign Grant SP2060/01 (to E. B. L.) and Wellcome Trust Grant 46746 (to R. A. Q.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF053235.

§ To whom correspondence should be addressed. Tel.: 44-1382-344805; Fax: 44-1382-224117; E-mail: R.M.PORTER@dundee.ac.uk.

¹ The abbreviations used are: KIF, keratin intermediate filament; K, keratin; hK16, human keratin 16; mK16 mouse keratin 16; RACE,

rapid amplification of cDNA ends; PAGE, polyacrylamide gel electrophoresis.

complete cDNA for mK16 isolated from such a library made from the skin of K10 mutant mice. Using the mK16 cDNA, *in vitro* assembly studies and transfection studies were carried out to assess previous hypotheses relating specific K16 sequences to function.

MATERIALS AND METHODS

Cloning and Sequence Analysis of mK16 cDNA—Skin was removed from three homozygous, two heterozygous, and one wild-type neonatal C57Bl6 mouse as described previously (17). mRNA was extracted using a Quickprep micro mRNA purification kit (Amersham Pharmacia Biotech). cDNA synthesis and library construction were carried out using a Zap Express cDNA synthesis kit (Stratagene, Cambridge, United Kingdom). The library was screened with a probe prepared by polymerase chain reaction of hK16 cDNA using the forward primer CTG-GCTTTGGTGGTGGTTT and the reverse primer AGGCAGCTCAGT-TCTAGGAGC (40 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and synthesis at 72 °C for 1 min). Random-prime DNA labeling of the probe with digoxigenin was carried out using a DIG-high prime labeling and detection kit (Boehringer Mannheim). The probe was diluted to 10 ng/ml in standard buffer and 50% formamide and hybridized at 37 °C overnight. The filters were washed two times in 2× SSC (20× SSC = 3 M sodium chloride, 300 mM sodium citrate), 0.1% SDS at room temperature (5 min) and two times in 0.5× SSC, 0.1% SDS at 55 °C (15 min). Positive colonies were detected by a colorimetric method using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, Poole, UK) as described in the manufacturer's instructions. The positive clones were excised as pBK-CMV phagemids and sequenced with T3 or M13 primers using an automated sequencer (ABI 377). Mouse cDNA, excised by an *EcoRI/XhoI* digestion from a pBK-CMV phagemid and labeled as described above, was used as a probe to screen for longer length clones.

To obtain the sequence of the 5'-end, the rapid amplification of cDNA ends (RACE) protocol was followed using a RACE kit (Boehringer Mannheim). The following primers were used for amplification: SP1, GGATCTTCACTCCAGGTCTC; SP2, CCACAAAATCCACTACCAAGTCC; and SP3, CTGCTGAAGCTCCACCATAG. Sequencing of the RACE product was achieved using the SP3 primer. Complete sequence analysis of mK16 cDNA was obtained using the forward primers F1 (GACCATTGAGGACCTGAAGAG), F2 (TGTATGCAGCTGTCCCAGATC), F3 (CCTCAGCATGGCCACCTGCAG), and F4 (CTGAGGAGCTG-AACAAGAGG) and the reverse primers R1 (ATCTGGGACAGCTGC-ATACAG), R2 (AGCCTGGCATTGTCAATCTGC), R3 (GGCAGTTGTG-GGTGAGGAGGC), and SP1 (see above). We were also able to sequence the mK14 clone 5.1 using the mK16 primers R1 and F1 and the plasmid-specific primers T3 and M13 (–20).

Immunofluorescence—A polyclonal antibody to mK16 (RPMK16) was obtained by immunizing rabbits with the C-terminal peptide GSTS-FSQQSQQSRD of mK16 linked to keyhole limpet hemocyanin. Frozen sections were fixed in methanol/acetone (1:1) at –20 °C for 5 min and incubated with RPMK16 diluted 1:500 in phosphate-buffered saline (pH 7.5) for 1 h at room temperature. The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-rabbit IgGs (Sigma). Slides were mounted in 10% Mowiol, 2.5% diazabicyclo[2.2.2]octane (Sigma).

Transfection of mK16 cDNA into PtK2 Cells—Complete mK16 was created by reverse transcription-polymerase chain reaction from RNA extracted from K10 heterozygous mouse skins using *Taq* polymerase, forward primer F3, and reverse primer R3 and cloned into pGEM (Promega). Five clones were sequenced in both directions, and one clone with a single silent mutation was selected for subcloning of the mK16 cDNA into the *EcoRI* site of the expression vector pcDNA3 (Invitrogen).

Transfection was carried out on subconfluent PtK2 cells grown on 13-mm² glass coverslips using the calcium phosphate precipitation method. cDNAs of hK14 and hK16 cloned into pcDNA3 were used as controls. A mutant hK16 in pCR3 was also transfected. This cDNA was obtained from a patient with pachyonychia congenita and contains an L130P mutation. At 24, 48, and 72 h after transfection, the cells were fixed for 1 min at –20 °C in methanol/acetone (1:1). RPMK16 was diluted in phosphate-buffered saline (1:500), and LL025 (K16), LL001 (K14), LE65 (K18) were applied undiluted. All primary incubations were for 1 h. After washing coverslips in H₂O, Cy3-conjugated goat anti-rabbit IgGs (heavy and light chains; Jackson ImmunoResearch Laboratories, Inc., Stratech, UK), fluorescein isothiocyanate-conjugated sheep anti-mouse antibodies (Sigma), or goat anti-rabbit antibodies (Sigma) were diluted 1:50 in phosphate-buffered saline and incubated for 30 min. Coverslips were mounted in Citifluor (Agar, Stansted, UK) and sealed with nail varnish.

After removing coverslips from the culture dishes, the remaining cells were scraped from the dish and solubilized in SDS sample buffer (100 μl). One-dimensional SDS-PAGE and immunoblotting were carried out as described previously (19). Primary antibodies were diluted as follows: LE65 and LL025 (1:5) and RPMK16 (1:5000) in Tris-buffered saline, 0.1% Tween 20. Secondary antibodies were anti-mouse or anti-rabbit immunoglobulins conjugated to alkaline phosphatase diluted 1:1000 (Dako, Bucks, UK). Detection was with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Transfection of Eye Lens Cell Line H36CEI—H36CEI cells are derived from human epithelial cells of the eye lens (20). Subconfluent cells grown on 13-mm² glass coverslips were individually transfected in a well of a 24-well plate with 0.6 μg of mK16, hK16, hK5, or hK14 cDNA in pcDNA3. Double transfections were with 0.3 μg of hK5 cDNA and 0.3 μg of each type I keratin cDNA. Transfection was achieved by linking the plasmid DNA to inactivated adenovirus particles (5 × 10⁸) using 10 mM polyethyleneimine as described previously (21). Staining was carried out as described for PtK2 cells. Polyclonal antibody BL-18 to K5 was diluted 1:500 in phosphate-buffered saline.

Keratin Expression in Bacteria and Purification—hK5, mK16, and hK16 cDNAs were generated with an *NdeI* restriction site introduced at the beginning by reverse transcription-polymerase chain reaction using a 5'-primer with a 3-base pair extension of CAT. After sequencing to check that no errors had been incorporated, the cDNAs were cloned into pT7-7 (mK16) (22) or pET-23b (hK16 and hK5) (Novagen) vectors. These plasmids were individually transformed into *Escherichia coli* strain BL21(DE3) pLysS and grown to A₆₀₀ ~ 0.5 before inducing protein expression with isopropyl-β-D-thiogalactopyranoside (0.5 mM) for 4 h. Inclusion body preparation was carried out as described previously (23). Keratins were purified by ion-exchange chromatography using a Merck-Hitachi Biochromatography system with a 10 × 150-mm Fractogel-EMD DEAE-650S column (Merck) equilibrated in 8 M urea, 20 mM Tris-HCl (pH 8), 2 mM EDTA, 1 mM dithiothreitol. Fractions of 2 ml were eluted with a linear salt gradient of 0–0.4 M NaCl over 40 min at 1 ml/min and analyzed by SDS-PAGE.

In Vitro Filament Assembly and Electron Microscopy—Recombinant type I and II keratins were mixed in a 1:1 molar ratio at a concentration of 0.2 mg/ml. Assembly was achieved either by a rapid dialysis procedure against 2.5 mM Tris-HCl (pH 7.5), 25 mM 2-mercaptoethanol at 4 °C for 16–20 h (13, 24) or by a staged dialysis procedure essentially as described (25). Briefly, samples in 8 M urea-containing buffer were dialyzed in 4 M urea-containing buffer and then in 10 mM Tris-HCl (pH 8.0), 25 mM 2-mercaptoethanol. An additional dialysis against Tris-HCl (pH 7.5) was included for direct comparison with the rapid dialysis procedure. The assembly was completed in 10 mM Tris-HCl (pH 7.0), 50 mM NaCl, 1 mM MgCl₂, 25 mM 2-mercaptoethanol. Assembly was monitored at all stages by electron microscopy. Proteins were diluted to 1:10 and negatively stained using 1% (w/v) uranyl acetate. Grids were examined in a Jeol 1200EX transmission electron microscope using an accelerating voltage of 80 kV.

Sedimentation Assay—The efficiency of filament formation was assessed by sedimentation assay (25, 26). Briefly, the assembly mixture was layered on top of 0.85 M sucrose in the final assembly buffer and centrifuged for 30 min at 80,000 × g at 20 °C in a Beckman TLS-55 rotor using a TL100 bench-top ultracentrifuge. The final pellet was dissolved directly in SDS-PAGE sample buffer, and the remaining protein in the supernatant was precipitated (27) before also being resuspended in sample buffer. Both protein samples were dissolved in volumes directly proportional to the original sample volume for direct comparison by SDS-PAGE.

RESULTS

cDNA Cloning and Sequencing of mK16—We have generated a Zap Express mouse cDNA library from the skin of mice created as a model for the skin disease bullous congenital ichthyosiform erythroderma. Using the hK16 probe, two mK16 clones (4.2 and 8.1) and one K14 clone (5.1) were isolated as pBK phagemids. None of the clones contained full-length keratin cDNAs, so the library was rescreened with mK16 cDNA from the longer of the two clones. Two additional clones containing K16 were obtained (7.31 and 2.11), the largest of which was 50 base pairs short of the 5'-end (clone 7.31). The shared sequence of all four clones was identical. We obtained the 5'-sequence including the start codon and 10 base pairs of untranslated sequence using RACE. The complete cDNA se-

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0   CAGTTCCTCAGCATGGCCACCTGCAGCCGCCAGTTCACCTCCTCCAGCTCCATGAAGGGCTCCTGTGGCATCGGTGGTGGCTCTAGCCGCATG
    M A T C S R Q F T S S S S M K G S C G I G G G S S R M
94  TCCTCCATCCTGGCTGGAGGATCCTGCCGGGCTCCCAGCACCTGCGGGGGCATGTACCTCCTCTCGCTTCTCCTCTGGGGGAGTCTGT
    S S I L A G G S C R A P S T C G G M S V T S S R F S S G G V C
187 GGGATTGGAGTGGCTATGGTGGGAGCTTCCAGCAGCAGCAGTTTTGGTGGAGGACTTGGTAGTGGATTTGGTGGTTCGATTTGATGGATTTGGT
    G I G G G Y G G S F S S S S F G G G L G S G F G G R F D G F G
279 GGTGTTTTGGTGGCTGGTCTTGGTGGTGGTCTTGGCGGTGGTATTGGTGGTGGTCTCCTGGTGGGCAGTGAGAAAAGTGACCATGCAGAACCTC
    G G F G A G L G G G L G G G I G D G L L V G S E K V T M Q N L
373 AATGACCGCCTGGCCACCTACCTGGACAAGGTGCGTGCCTGGAAGAGGCCAACAGAGACCTGGAGGTGAAGATCCGGGACTGGTACCAGAGG
    N D R L A T Y L D K V R A L E E A N R D L E V K I R D W Y Q R
466 CAGCGGCCACTGAGATCAAAGACTACAGCCCTACTTCAAGACCATTGAGGACCTGAAGAGCAAGATCATTATTGCCACCCAGGAGAATGCA
    Q R P T E I K D Y S P Y F K T I E D L K S K I I I A T Q E N A
559 CAGTCACTTTGCAGATTGACAATGCCAGGCTGGCAGCTGATGACTTCAAGACCAAGTACGAGAATGAGCTGTTCTTGGCGGAGTCCGTGGAG
    Q F T L Q I D N A R L A A D D F R T K Y E N E L F L R Q S V E
652 GGTGACATCAATGGCCTGCGCAAGGTGCTAGATGAGCTGACCTGTCCAGAGCTGACCTGGAATGCAGATTGAAAACCTCAGAGAAGAGCTG
    G D I N G L R K V L D E L T L S R A D L E M Q I E N L R E E L
745 GCCTTCCTGAAGAAGAACCATGAGGAGGAGATGCTTGCCTTGGAGGGTTCAGACTGGTGGGACGTCATGTGGAGATGGAGCAGCCCCGGT
    A F L K K N H E E E M L A L R G Q T G G D V N V E M D A A F G
838 GTGGACCTCAGCCGATTCTGAATGAGATGAGGGACCAGTATGAGCAGATGGCAGAGAAGAACCAGAGATGTGGAGGCTTGGTTCTCCTGAGA
    V D L S R I L N E M R D Q Y E Q M A E K N R R D V E A W F L R
931 AAGACTGAGGAGCTGAACAAAGAGGTGGCCTCTAACAGTGATCTAATCCAGAGCAACCGCAGCGAGGTGGCTGAGCTCCGCGAGGTGTTCCAG
    K T E E L N K E V A S N S D L I Q S N R S E V A E L R R V F Q
1024 GGCTGGAGATGAACTGCAGTCCCAGCTCAGCATGAAAGCATCCTTGGAGAACAGCCTAGAAGAGACCAAAGCGAGATACTGTATGCAGCTG
    G L E I E L Q S Q L S M K A S L E N S L E E T K G R Y C M Q L
1117 TCCAGATCCAGGGTTTGGATCAGCAGTGTGGAGGAGCAGCTGGCTCAGCTTCGCTGCGAGATGGAGCAGCAGAGCCAGGAGTACAACATCTTG
    S Q I Q G L I S S V E E Q L A Q L R C E M E Q Q S Q E Y N I L
1210 TTGGATGTGAAGACAAGGCTGGAGCAGGAGATCGCCACCTACCGCCGTCTGCTGGATGGCGAGAATATCCACTCCTCCTCACAGCACTCCTCT
    L D V K T R L E Q E I A T Y R R L L D G E N I H S S S Q H S S
1303 GGACAGTCTATTCTTCTCGAGAAGTCTTCTCCTCATCCTCCCGCCAGCCCCGGTCCATCCTCAAGGAGCAAGGTTCAACCAGCTTCAGCCAG
    G Q S Y S S R E V F S S S S R Q P R S I L K E Q G S T S F S Q
1396 AGCCAAAGTCAGAGTTCAGGGACTAATGTTTTGCCTAGAGCCTCCTCACCCACAACCTGCCTCTCAAGCTGAGGGCTTGGGGCAGGACCTGT
    S Q S Q S S R D
1489 TTTCTTTGGCATTCCCCATCTGTCTCCCTACCCTCTCATGGTGGTAGGCTAATAAAGCTTTTTGGTTGATGCAAAAAAAAAAAAAAAAAA

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FIG. 1. Nucleotide sequence and deduced amino acid sequence of mouse keratin 16 cDNA. The sequence obtained from the largest clone (7.31) and the RACE product includes 10 base pairs upstream of the initial codon coding for methionine and 157 base pairs of 3'-untranslated sequence.

quence obtained from sequence analysis of the clones and the RACE product is shown in Fig. 1. When scanned against the data base, the sequence is most highly related to hK16 (GenBank™ accession number S79867). After the TAA stop codon, there is a 157-base pair untranslated region including a poly(A) addition signal (AATAAA) and an 18-residue poly(A) tail.

The K14 clone 5.1 was identical in sequence to the mK14 clone pkSCC52 reported previously (GenBank™ accession number J02644) (28). Our sequence analysis extends the mK14 sequence by an additional 17 amino acids upstream of QNLN-DRLATY. The additional amino acids are identical to mK16.

Comparison of mK16 Amino Acid Sequence with Other Keratin Sequences—The sequence codes for a protein of 469 amino acids with a calculated M_r of 51,514. All keratins have structural similarities, including an α -helical rod domain interrupted by short non-helical linker regions and non-helical head and tail domains. The rod domain is bounded by the highly conserved helix initiation and helix termination motifs and consists of four segments (1A, 1B, 2A, and 2B) of conserved length (29). The sequence of mK16 is 85% homologous to that of hK16 (Fig. 2), with the central α -helical rod domain being most highly conserved (89% identical over 308 amino acids starting at KVTMQNLNDRLATY and finishing at TYRRLDGE). The

helix initiation motif differs from that of hK16 in that it contains a threonine rather than a serine. Although mutations in this motif can cause very severe disorders, this particular change is apparently tolerated and is also observed in mK14 (28). The helix termination motif also differs from that of hK16 by one amino acid residue; in this case, an aspartic acid residue replaces a glutamic acid residue. This glutamic acid is highly conserved in epithelial keratins; however, an aspartate is seen at this position in type I hard α -keratins found in hair and in CP49/phakinin, an intermediate filament protein of the eye lens (29–32). A significant difference in the α -helical region is the lack of the proline residue in the 1B domain that has been postulated to be responsible for the formation of rather shorter keratin filaments by hK16 (13). The head domain of mK16 shares 71% homology with hK16, over the first 113 amino acids of mK16. This region of hK16 has sequence that is identical to that of hK14 and hK17 (see Troyanovsky *et al.* (44) for an alignment). Of these shared residues, 88% are conserved in the mouse sequence. In the tail domain, 70% of mK16 amino acids are present in hK16. Of particular note, 13 of the last 19 residues of hK16 are conserved. This region bears no homology to hK14 or hK17 and is likely to make K16 unique.

Mouse-specific K16 Polyclonal Antibody—The C terminus of

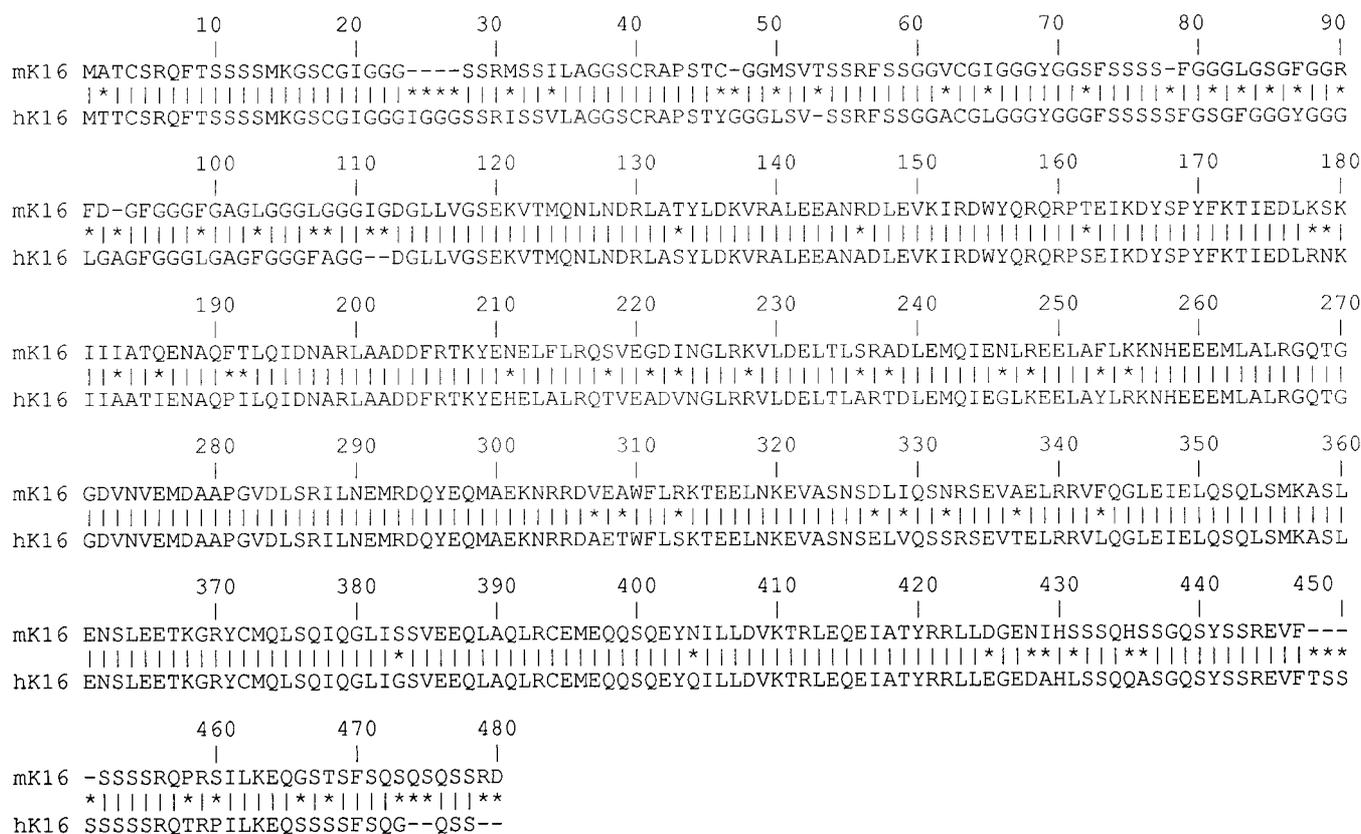


Fig. 2. Comparison of amino acid sequences of mK16 and hK16. The amino acid sequences are optimally aligned. Dashes were introduced to obtain optimal alignment of residues. Lines were drawn to indicate identical residues, and asterisks denote non-identical residues.

mK16 is longer than that of hK16 and contains two additional charged residues. Therefore, it is perhaps of no surprise that a polyclonal antibody (RPmK16) raised against the last 15 amino acid residues has proved to be specific for mK16 and does not recognize hK16. We have tested the antibody on human skin specimens including scalp, axilla, thigh, face, breast, lip, palate, and finger and observed no staining of hair follicles or suprabasal epidermis, as is readily seen with the monoclonal antibody to hK16 (LL025) (33). Similarly, the human keratinocyte cell line HaCaT, which constitutively expresses K16, did not stain with RPmK16 (34). Mouse epidermis, however, was recognized by this antibody. Hair follicles and whisker follicles were positive in normal mouse epidermis (data not shown). Also, positive staining of the suprabasal layer of the acanthotic epidermis of K10 mutant mice was observed (data not shown) and was similar to the results obtained with LL025 (35).

Transfection of PtK2 Cells with mK16 cDNA—Previous studies have reported that forced expression of hK16 in PtK2 cells causes the endogenous K8/K18 network to retract from the cytoplasmic periphery (13, 24). This reorganization was described as increasing with time, and at 72 h post-transfection, >50% of the transfected cells showed a juxtanuclear location of the keratin filaments (13). We therefore chose this time point to examine the effect of transfected mK16 in PtK2 cells. In 95% of the transfected cells, mK16 was incorporated into the existing K8/K18 network, forming normal filaments at 72 h post-transfection (Fig. 3a). In the remainder of the cells, small regular circular structures were observed (Fig. 3b), as described in other transfection experiments of PtK2 cells with normal and mutant human keratins (36–38). No perinuclear keratin filaments were observed comparable to those described by Paladini *et al.* (13). At 24 h post-transfection, mK16 had already incorporated into the endogenous K8/K18 filament matrix as shown by double immunofluorescence microscopy (Fig. 3, c and d).

For comparison, we also transfected PtK2 cells with plasmids containing either hK16 (containing the proline in coil 1B) or hK14 cDNA. Similar well extended filaments were observed as well as some small spherical aggregates found in 5% of the transfected cells (Fig. 3e). Retracted filaments (13, 24) were only observed in transfections with K16 cDNA carrying a point mutation (L130P) obtained from a patient with pachonychia congenita (Fig. 3f).

Both the monoclonal antibody to hK16 (LL025) and the polyclonal antibody RPmK16 were used to stain transfected cells (33). Whereas LL025 stained cells transfected with either mK16 or hK16, RPmK16 only stained cells transfected with mK16. Analysis of transfected cells by immunoblotting showed hK16 and mK16 to have similar relative mobilities on SDS-polyacrylamide gels and confirmed the species specificity of RPmK16 (Fig. 4).

Transfection of mK16 into H36CEI Cells—To determine if mK16 could form filaments in the absence of an established keratin network, we carried out cotransfections of mK16 with hK5 into a human lens epithelial cell line (H36CEI). This cell line has vimentin (but not keratin) intermediate filaments (20). At 24 h post-transfection, >50% of the transfected cells showed extensive networks of keratin filaments when stained with BL-18 (Fig. 5a). The filaments formed were comparable to those obtained in double transfections of hK14 or hK16 with hK5 in a similar percentage of transfected cells (Fig. 5, b and c). The remainder of the transfected cells either had not formed filaments or contained very short filaments. Sometimes large clumps of keratins were seen in the cells. These irregular filaments were seen in K5/K14-transfected cells as well as in K5/K16-transfected cells. No filaments were formed in single transfections.

In Vitro Assembly of mK16—The assembly properties of mK16 were of particular interest due to the report that hK16,

FIG. 3. Staining of transfected PtK2 cells. PtK2 cells transfected with mK16 and stained with RpmK16 72 h post-transfection showed excellent formation of mK16-containing filaments in 95% of the transfected cells (a) and spherical bodies in the remaining 5% (b). PtK2 cells transfected with mK16 at 24 h post-transfection and co-stained with RpmK16/fluorescein isothiocyanate for mK16 (c) and with LE65/Cy3 for KIFs (d) clearly showed incorporation into the endogenous keratin intermediate filament network. PtK2 cells transfected with hK16 and stained with LL025 also showed normal keratin intermediate filaments (e). Abnormal filaments were observed in cells transfected with hK16 with a L130P mutation (f). LE65 staining is shown so that normal PtK2 keratin filaments can be compared with the abnormal retracted filaments in the cell in the center of the picture.

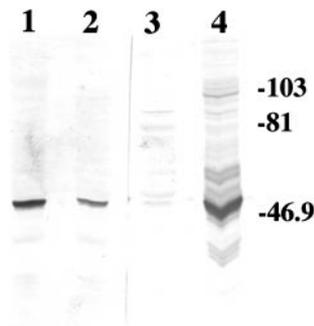
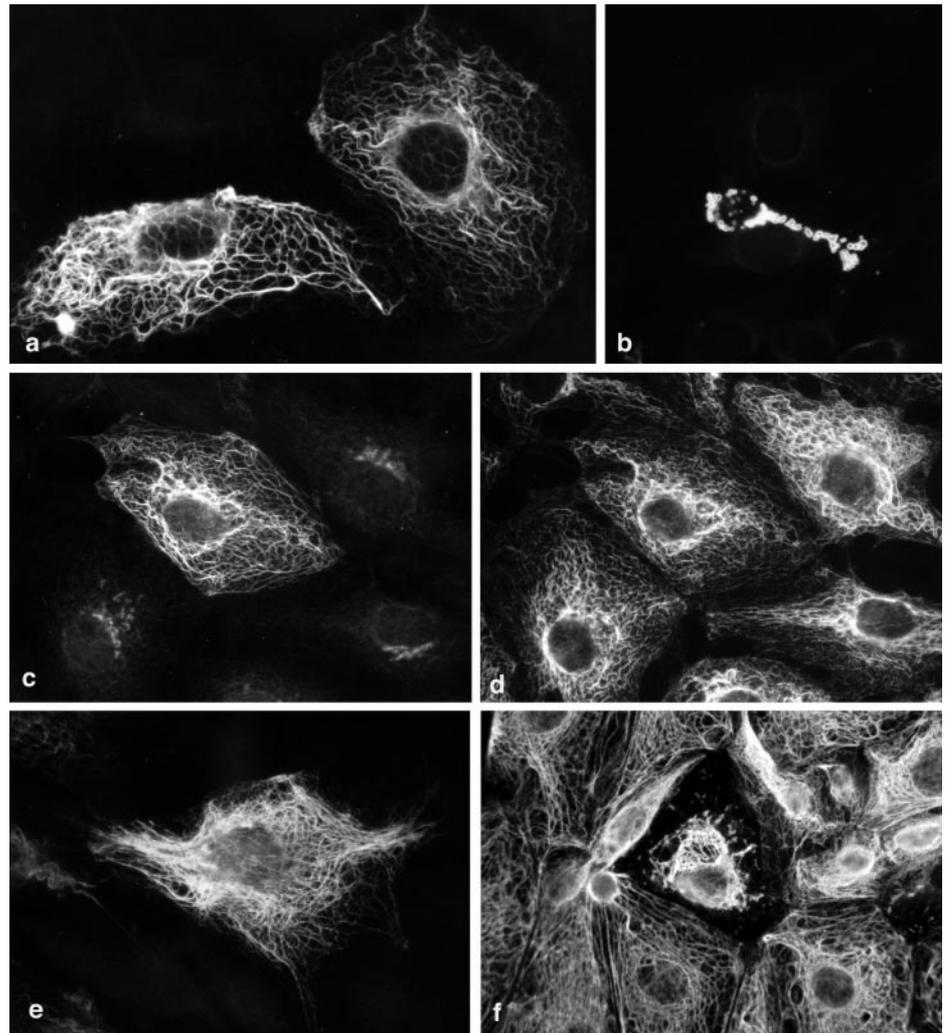


FIG. 4. Western analysis of transfected cells. Lanes 1 and 3, hK16-transfected cell lysate stained with LL025 and RpmK16, respectively; lanes 2 and 4, mK16-transfected cell lysate stained with LL025 and RpmK16, respectively. Molecular masses are indicated on the right in kilodaltons.

under certain conditions, forms shorter filaments with hK5/hK6 than does hK14 (13). Keratins from human and mouse were expressed in bacteria and purified by ion-exchange chromatography for *in vitro* assembly studies. Two different methods of assembly were performed; one used a rapid dialysis procedure, and the other employed a staged dialysis procedure to remove the urea from the protein solution. In both cases, the type I keratins were mixed in a 1:1 molar ratio with hK5. The rapid dialysis procedure was achieved by overnight dialysis into 2.5 mM Tris (pH 7.5) from 8 M urea-containing buffers. These conditions produced shorter filaments for a K5/K16 pair-

ing than for K5/K14 (13, 24). Under these assembly conditions, hK16 with hK5 formed numerous very short filaments (Fig. 6a), similar to those observed previously (13). In contrast, mK16 and K5 formed an abundance of much longer filaments (Fig. 6c). Using the staged dialysis procedure, both mK16 and hK16 formed long filaments at pH 7.5 (Fig. 6, b and d).

To assess the efficiency of the *in vitro* assembly experiment, a sedimentation assay was performed. In agreement with the electron microscopy results, the K5/hK16 filaments in the rapid dialysis procedure were not efficiently sedimented due to their short length (data not shown), unlike the K5/mK16 filaments, which sedimented under the conditions of the assay (Fig. 7). Addition of 50 mM salt to the assembly mixture promoted complete assembly of both keratins. The staged dialysis of keratins also led to complete assembly of keratin filaments (data not shown).

DISCUSSION

This study demonstrates unequivocally the existence of a mouse gene for K16. The amino acid sequence of mK16 is 85% homologous to that of hK16. hK16 possesses a proline in the helix 1B segment (13). This is not a unique situation, as another intermediate filament (CP49ins) possesses a proline in a similar position (32). Proline residues are not usually seen in helical proteins, and they would be expected to disrupt the regular helicity of the structure (39, 40). This proline, it was reasoned, could be responsible for the shorter filaments formed when K16 rather than K14 was assembled *in vitro* or in fibroblasts with either K5 or K6b. In other positions in the α -helix,

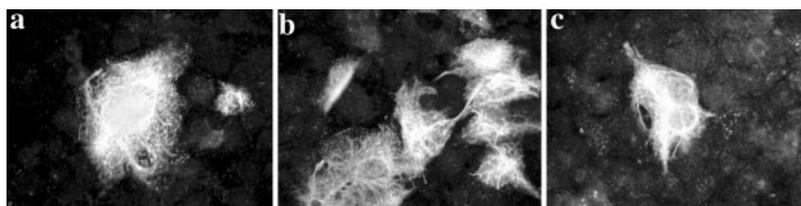


FIG. 5. **Transfection of keratins into lens cell line H36CEI.** H36CEI cells were transfected with hK5 and hK14 (a), hK5 and hK16 (b), and hK5 and mK16 (c). All cells were stained with BL-18/Cy3 and the corresponding monoclonal antibody to the type I keratin/fluorescein isothiocyanate. BL-18/Cy3 staining is shown in each case.

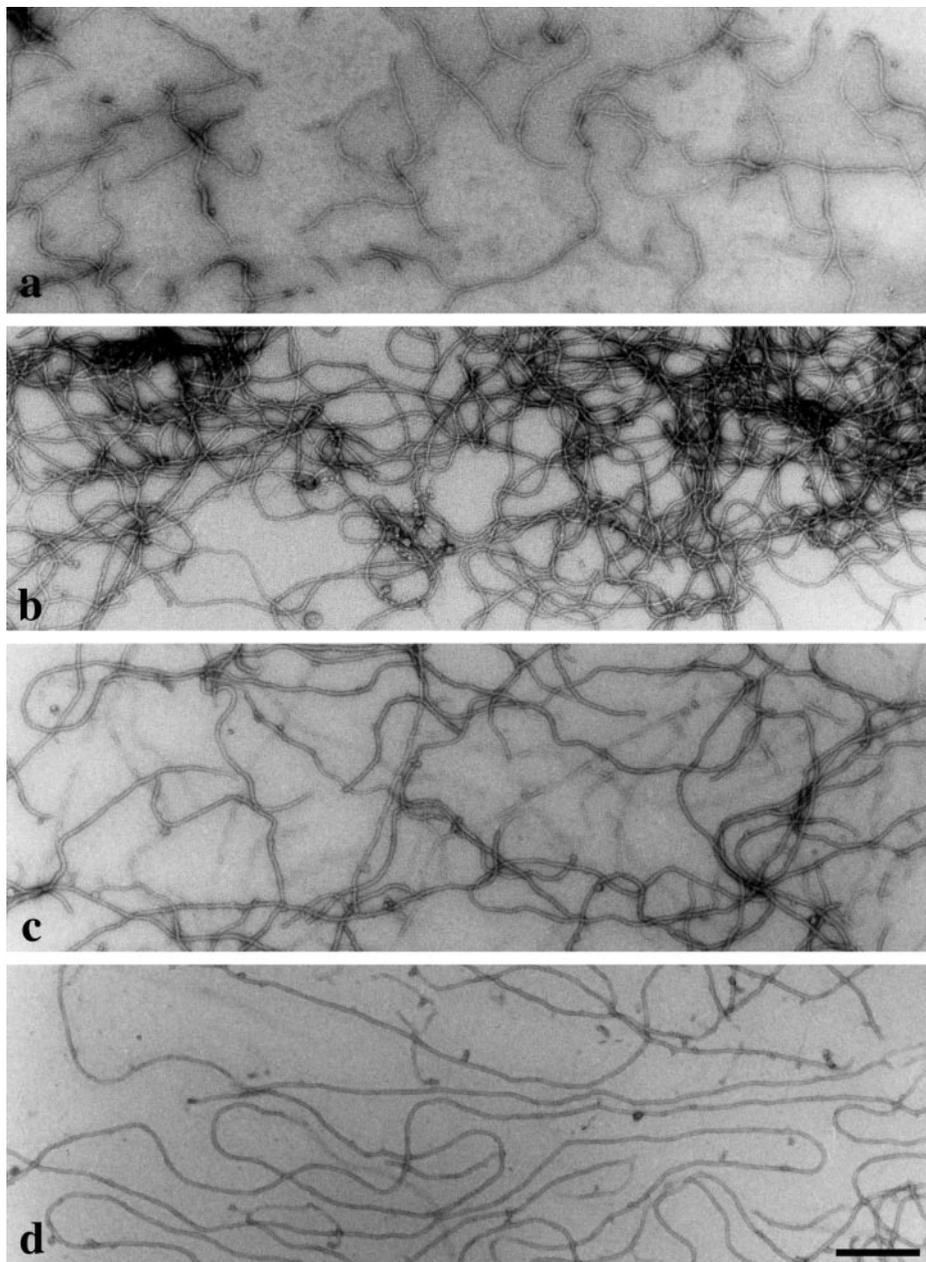


FIG. 6. **Electron micrographs of K5/K16 filaments assembled *in vitro*.** K5/hK16 formed short filaments (a) when assembled by the rapid dialysis procedure and long filaments (b) using the staged dialysis procedure. K5/mK16 formed long filaments using the rapid dialysis procedure (c) and the staged dialysis procedure (d). Scale bar = 250 nm.

a proline can be detrimental to KIF assembly, and in many epidermal keratins including K16, such a proline can lead to skin disease (41). In one study, a proline residue was introduced experimentally into the α -helical domain at a number of different points; however, an effect on filament assembly was observed only when it was introduced at the ends of the helical region (42). To support the possibility that the proline could be responsible for the unique properties of K16, Wawersik *et al.* (24) replaced this residue with valine, the corresponding amino

acid in hK14, and showed that hK16 could be made to form more stable tetramers. The mouse sequence for K16 lacks this proline residue, which is replaced by a phenylalanine (see Phe-191 in Fig. 2). As this proline is not conserved between mouse and human, it is unlikely to play a key role in the function of K16.

There are several published sequences for hK16 with and without the proline residue (43–46), and it is possible that several different K16 genes exist, as observed with K6 (47).

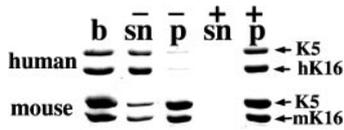


FIG. 7. **Sedimentation assay of K5/K16 assembly.** The rapid dialysis procedure was used to assemble *in vitro* K5/hK16 or K5/mK16 before (–) and after (+) addition of 50 mM NaCl. Keratins in the supernatant (sn), in the pellet (p), and before (b) sedimentation were subjected to SDS-PAGE. Note that in the absence of salt, most of the K5/hK16 protein was in the supernatant, in agreement with the electron microscopy data showing that this keratin combination formed short incomplete filaments using the rapid dialysis procedure.

However, no other evidence suggesting more than one hK16 gene exists at present. In our experience, the human sequence containing the proline (GenBank™ accession number S79867) is the correct one. All four mK16 clones obtained from the expression library in this study were of identical sequence, so we would expect that the sequence reported here (without the proline in coil 1B) is the predominant form of mK16, if not the only form.

Our data do indicate some differences in the *in vitro* assembly properties of hK16 and mK16. For instance, mK16 forms long filaments using the rapid dialysis procedure, whereas hK16 produces only short filaments with an identical type II partner. Long filaments can be produced *in vitro* with hK16 or mK16 with hK5 by the staged dialysis procedure. The results obtained using the two methods suggest that hK16 and mK16 have different assembly properties, which may be due to sequence differences between the two keratins. The proline residue is likely to be at least partly responsible for these differences since it has been shown by mutation analysis to affect tetramer stability (24). The reduced tetramer stability of hK16 may mean that this keratin requires more time to reach an energetically favorable state, as provided by the staged dialysis procedure. The weaker interactions at the tetramer stage do not, however, affect the end point of assembly *in vitro* where both hK16 and mK16 form long filaments. There are also no apparent differences when filaments assemble in transiently transfected cells. The fact that the mouse protein does not share the *in vitro* filament assembly properties of the human protein suggests that the assembly pathway is secondary to the unique function of this keratin.

In contrast to previous studies (13, 24), we observed no irregularity of the KIF cytoskeleton in K16 transfection studies. In PtK2 cells, we see that both mouse and human K16 are capable of incorporating into the existing K8/K18 keratin filaments. We only see irregular filament formation when the transfected keratin gene carries a pathogenic point mutation as found in patients with keratin disorders. It is possible that the different observations can be accounted for by varying levels of expression of the K16 constructs. However, we used the same method of transfection with an identical expression vector as Paladini *et al.* (13), and we would therefore expect similar transfection efficiencies. Also, both mK16 and hK16 form filaments similar to those formed by hK14 and hK5 in the lens epithelial cell line H36CEI. One possible explanation for these differences from previously published data (13) is a difference in K16 sequence. The original clone of hK16 sequenced by Paladini *et al.* (43) begins with the sequence MAT rather than MTT, which is an error caused by a cloning artifact. Other than this very minor difference, our hK16 is identical in sequence. This variation is unlikely to explain the different observations, as the mouse sequence also begins with MAT not MTT, but at present, it is the only apparent difference to explain the two sets of results.

Subtle differences in the α -helical domain may lead to dif-

ferent filament-forming abilities and cytoskeletal rigidity. However, the sequence of mK16 shows a high degree of amino acid sequence conservation to hK14, hK17, and hK16 in the α -helix (44). There is also a high degree of sequence homology (83%) between mK14 and mK16 in the α -helical rod domain (28). The most unique amino acid sequences of individual keratins are found in the head and tail domains. Common features are observed between keratins with similar expression patterns. For example, the end domains of both type I and II keratins of simple epithelial keratins are rich in serine residues, whereas those of complex epithelia are rich in glycine residues, and hair keratins are rich in cysteine (48). It seems therefore likely that these domains should at least be partly responsible for the individuality of the keratin molecule. So far, too few disease-causing mutations have been identified in the head and tail domains to give clues as to the function of the head and tail domains (reviewed in Ref. 14). The most unique region of K16 is found in the C terminus since it shows 70% homology (over 49 residues) to hK16, but only 12% homology to the C terminus of mK14. In contrast, the N terminus of mK16 is 72% homologous to that of hK14.

In conclusion, it would appear there is little unusual about the assembly properties of K16 that are likely to be functionally significant for the following reasons. 1) Our transfection studies show that K16 can not only integrate into an existing keratin network, but can also form filaments *de novo* in vivo. 2) The decreased assembly capability of hK16 *in vitro* only occurs under certain conditions and is not apparently shared by mK16. 3) Both hK16 and mK16 are equally capable of forming long filaments *in vitro* with K5. The restricted distribution of K16 in normal tissue and its altered expression pattern in hyperproliferative states such as wound healing and tumorigenesis suggest a specific and specialized role for this keratin. The existence of a mK16 strengthens the evidence for an essential role for this protein that is not provided by other keratins. The unique sequence of the tail domain of K16 is highly conserved between mouse and human and is a favored candidate to provide clues to the function of this protein. Mutation analysis by genetic manipulation could be useful to resolve this issue.

Acknowledgments—We thank Dr. D. W. Melton for providing the mice for this project. We are grateful to N. J. Wilson and G. J. Magee for sequencing the keratin cDNAs and to P. H. Ogden and Dr. A. R. Prescott for help with transfections.

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cDNA Cloning, Expression, and Assembly Characteristics of Mouse Keratin 16
Rebecca M. Porter, Alene M. Hutcheson, Elizabeth L. Rugg, Roy A. Quinlan and E.
Birgitte Lane

J. Biol. Chem. 1998, 273:32265-32272.

doi: 10.1074/jbc.273.48.32265

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