Transient Expression of Mouse Hair Keratins in Transfected HeLa Cells: Interactions Between "Hard" and "Soft" Keratins

Da-wen Yu, Susana Yuk-Ying Pang, Daniel M. Checkla, Irwin M. Freedberg, Tung-Tien Sun, and Arthur P. Bertolino

Epithelial Biology Unit, Departments of Dermatology and Pharmacology, Kaplan Cancer Center, New York University School of Medicine, New York, New York, U.S.A.

Although it has been shown previously that an acidic (type I) "soft" keratin can interact with many basic (type II) "soft" keratins to form 10-nm intermediate filaments, it has been unclear whether "soft" keratins are compatible with the "hard" keratins typically found in hair and nail. To address this issue and to generate more structural information about hard keratins, we have isolated and sequenced a cDNA clone that encodes a mouse hair basic keratin (b4). Our sequence data revealed new information regarding the structural conservation of hard keratins as a group, being significantly different from soft keratins. Using expression vectors containing appropriate cDNA inserts, we studied the expression of this basic (b4) as well as an acidic (a1) mouse hair keratin in HeLa cells. The expression of these alien hair keratins in the transfected cells was surveyed using a panel of monoclonal and polyclonal antibodies. Our results indicated that the basic and acidic hair keratin readily incorporated into the existing endogenous soft keratin network of HeLa cells. Overproduction of hair keratin, however, occasionally led to the formation of cytoplasmic aggregates containing both hard and soft keratins. These data suggest that although small amounts of newly synthesized hair keratins can incorporate into the "scaffolding" of the preformed soft keratin filament network, possibly through dynamic subunit exchange, overproduction of hard keratins can lead to the partial collapse of the soft keratin network. These observations, along with the deduced amino acid sequence data, support and extend the concept that hard and soft keratins, although closely related, are divergent enough to justify their being divided into two separate subgroups. *J Invest Dermatol 97:354–363, 1991*

Keratins are a group of about 30 highly insoluble proteins that form intermediate filaments in various epithelial tissues and their appendages, such as hair and nail. These proteins can be divided into two groups: the type I keratins that are, in general, relatively acidic and the type II keratins that are neutral to basic [1–6]. In vitro reconstitution experiments have provided several examples in which an isolated acidic keratin can polymerize with a number of basic keratins, forming morphologically intact 10-nm keratin filaments [7–9]. This minimum requirement of at least one acidic and one basic keratin for 10-nm filament formation has been used to explain the existence of these two keratin groups. Recent data indicate that the acidic and basic keratins interact starting with the formation of a heterodimer (consisting of an acidic and a basic monomeric keratin), two of which then form a tetramer that further polymerizes, giving rise to the final 10-nm filamentous structure [10–12].

Systematic analyses of the keratins from a large number of epithelial tissues established that each epithelium expresses a set of 2–10 keratins; the composition of these keratins varies depending on the tissue type, developmental stage and environmental factors [2,6,13]. Moreover, in the cases of stratified epithelia, major changes in keratin composition can occur when cells leave the basal cell layer and become terminally differentiated [14–21]. This type of analysis showed that specific acidic and basic keratins tend to co-express, forming what has been known as keratin "pairs" [6,22]. Because the expression of most of these keratin pairs are tissue- and differentiation-stage–dependent, they can serve as molecular markers for different pathways of epithelial differentiation [23,24]. For example, the basic K5 and acidic K14 keratins (K5/K14 pair) are synthesized by basal cells of the epidermis and almost all other stratified squamous epithelia (markers of basal keratinocytes [14–21]). The basic K1 and acidic K10 keratins (K1/K10 pair) are mainly associated with suprabasal cells of keratinized epidermis and have been referred to as markers of keratinization or skin-type differentiation [14–20,25]. Similarly, prominent groups of four basic (Hb1, Hb2, Hb3 and Hb4) and four acidic (Ha1 to 4) keratins are found in the developing and mature hair shaft [4,5,26]. These keratins are also found in nail and filiform papillae of dorsal tongue epithelium [27,28], and may be thought of as markers for an advanced stage of hair-type differentiation.

Like other intermediate filament proteins, the primary structure

Manuscript received February 1, 1991; accepted for publication March 11,1991.

This work was supported in part by NIH grants AM06507, AR35644, AR34511, and AR39749 and a grant from the Rudolf L. Baer Foundation for Skin Diseases, Inc.

Reprint requests to: Dr. Arthur P. Bertolino, Department of Dermatology, N.Y.U. Medical Center, 550 First Avenue, New York, N.Y. 10016.

Abbreviations:

cDNA: complementary deoxyribonucleic acid
DMEM: Dulbecco's modified Eagle medium
EDTA: ethylene diaminetetraacetic acid
EGTA: ethyleneglycol-bis-(beta-aminoethyl ether) N,N,N′,N′-tetra-acetic acid
IEF: isoelectric focusing
MW: molecular weight
PAGE: polyacrylamide gel electrophoresis
PMSF: phenylmethyl-sulfonyl fluoride
SDS: sodium dodecyl sulfate

0022-202X/91/$03.50

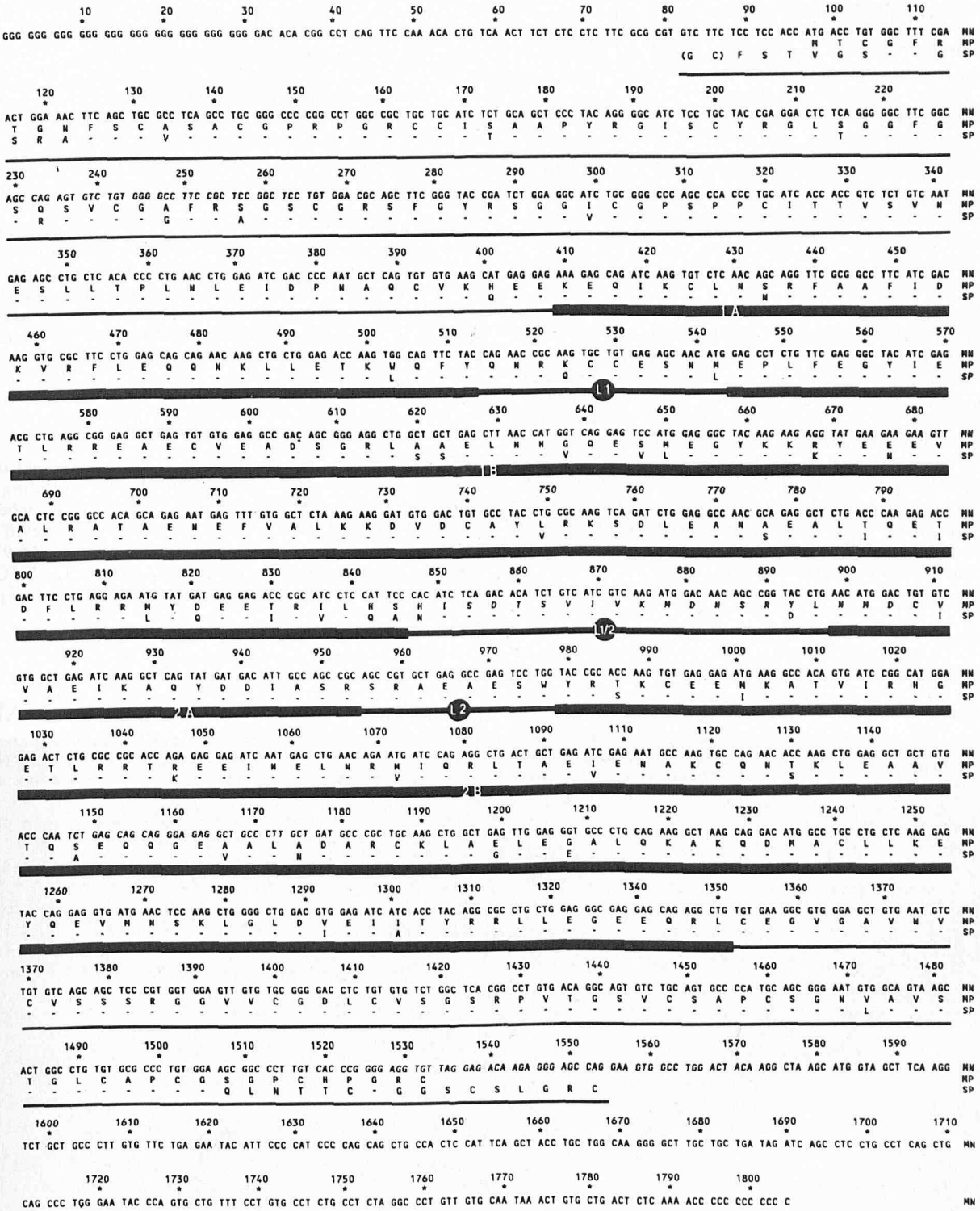

Figure 1. Nucleotide sequence of cDNA clone MHKB-2, and a comparison of its deduced amino acid sequence with a sheep wool keratin. MN denotes mouse cDNA nucleotide sequence; MP denotes the deduced mouse protein sequence; SP denotes sheep protein sequence of wool keratin 7c [47]. In the sheep sequence, amino acid residues identical to those of the mouse sequence are represented by *dashes*. Different structural domains of the molecules are depicted in the bar diagram underlying the sequences: *thin bars*, non-helical amino- and carboxy-termini, respectively; *thick bars*, helical domains (1*A*,*B*, 2*A*,*B* as marked); *intermediate bars*, linker regions (L1, L1/2, and L2 as marked).

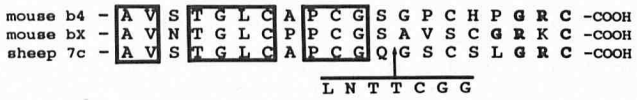

Figure 2. A comparison of the amino acid sequences of the distal carboxy-termini of several type IIa keratins. The C-terminal sequence of mouse keratin b4 is compared with those of a second mouse sequence (bX) and a sheep (7c) keratin. Identical residues are boxed and the conserved GRC terminal residues are in bold. The seven additional amino acids of sheep keratin 7c were listed separately as an insert in order to maintain register.

of the keratins can be divided into several reasonably well-defined structural domains including the non-helical N- and C-termini, and a central, largely helical rod domain (of approximately 310 amino acids) that is interrupted by three linker regions (L1, L1/2, and L2) [10]. The rod domains show high homology within each keratin type (type I and type II). Based upon limited amino acid sequence data of "hard" keratins, Conway and Parry [29] proposed to further divide keratins into type Ia and IIa ("hard" keratins) and type Ib and IIb (epidermal and other "soft" keratins). The sequence divergences between "hard" and "soft" keratins led them to hypothesize that although type Ia chains could aggregate with type IIa chains and type Ib chains could aggregate with type IIb chains, the cross-combinations would be highly improbable [29].

To provide a direct test of this hypothesis, we have isolated and characterized two mouse hair follicular cDNA clones that encode one of the four known major basic mouse hair keratins (mHb4) and one of the four major acidic hair keratins (mHa1 [26,30]). We were therefore able to force the expression of each of these hair keratins in HeLa cells which contain a preformed soft keratin network. Using a panel of antibodies as probes, we demonstrated that each hair keratin introduced into HeLa cells could readily incorporate into the existing soft keratin network. However, overproduction of hair keratin can lead to the partial collapse of the endogenous soft keratin network, resulting in the formation of non-filamentous cytoplasmic aggregates. These data indicate that, although small amounts of hard keratins can interact with soft keratins in preformed soft keratin filaments, overproduced hard keratins are incompatible with soft keratins in filament formation. These results, in conjunction with our sequence data, support and extend the concept that hard and soft keratins, although closely related, are divergent enough to warrant their being divided into two separate subgroups.

MATERIALS AND METHODS

cDNA Library Screening and Plasmid Isolation We screened our mouse hair follicle cDNA library [26] using a ^{32}P-labeled, nick-translated probe prepared from a type IIa wool keratin cDNA [31] (clone SWK3; kindly provided by Dr. Kevin Ward of C.S.I.R.O., Australia). Positive clones were grown in M-9 minimal media in the presence of tetracycline (12.5 μg/ml) at 37°C and the plasmid copy number was amplified with chloramphenicol (170

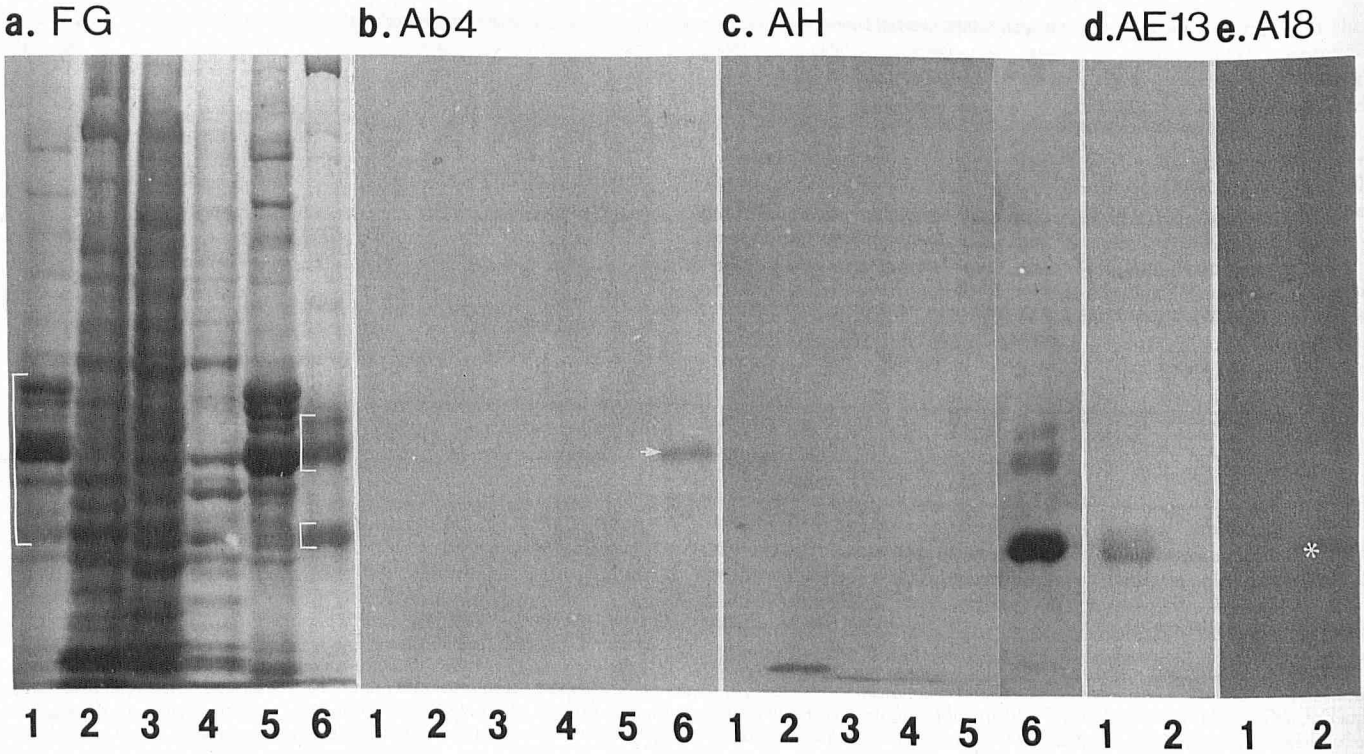

Figure 3. Antigenic specificity of the antibodies as determined by one-dimensional immunoblotting. Water-insoluble cytoskeletal proteins from different cells and tissues were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose paper, and stained with (*a*) Fast Green to reveal the protein patterns, or immunochemically with (*b*) a rabbit antiserum (Ab4) raised against a synthetic peptide corresponding to the C-terminus of the mouse basic type IIa keratin encoded by MHKB-2, (*c*) a rabbit antiserum (AH) raised against total human hair keratins, (*d*) monoclonal antibody AE13; and (*e*) monoclonal antibody A18 (Ks18.174) which recognizes K18 (*asterisk*). Samples in (*a*)–(*c*) are *lane 1*, mouse esophageal keratins; *2*, mouse liver total proteins; *3*, HeLa total proteins; *4*, HeLa keratin-enriched extract; *5*, mouse epidermal keratin extract; and *6*, mouse hair root proteins. Samples in (*d*) and (*e*) are *lane 1*, mouse hair root proteins; and *2*, HeLa keratin enriched extract. Note in (*b*) that antiserum Ab4 reacts specifically with mHb4 (*arrowhead*). Also note in (*c*) that anti-hair keratin, AH, reacts strongly with acidic keratins and weakly with basic hair keratins. [*Brackets*, keratin molecular weight range or acidic/basic keratins in (*a*).]

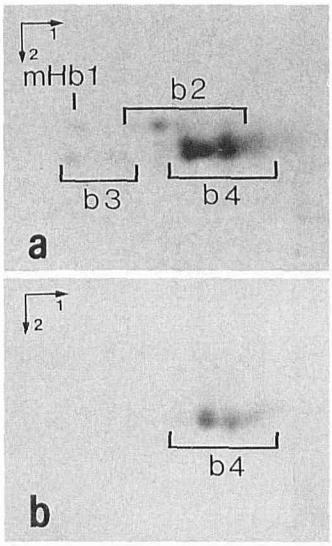

Figure 4. Antigenic specificity of the anti-peptide antibody Ab4 as determined by two-dimensional immunoblotting. (*a*) Mouse hair root proteins, labeled in vivo with ^{35}S-methionine, were resolved by two-dimensional PAGE and visualized by autoradiography (only the basic keratins are shown). (*b*) Staining of these basic hair keratins with the antibody to a synthetic peptide corresponding to the distal C-terminal sequence of mHb4. Note the selective staining of keratin b4; the other three basic hair keratins, b1, b2, and b3, were not stained.

μg/ml). Bacteria were harvested and lysed with alkali and plasmid DNA was purified by cesium chloride density centrifugation [32].

Nucleic Acid Sequencing and Analyses The five Pst I fragments of MHKB-2 cDNA [in order from the 5′-end to 3′-end: 177 bp, 1040 bp, 233 bp, 262 bp and 92 bp (96 bp including the 3′-regenerated Pst I site)] were subcloned into M13mp18 and pGEM-3Z and sequenced by the dideoxy method [33] using the Sequenase single-stranded or double-stranded sequencing systems (United States Biochemicals, Cleveland, OH). M13, T7, and SP6 primers as well as synthetic primers corresponding to segments of derived sequences were used for sequencing reactions. Occasional sequence ambiguities were resolved by sequencing in both directions and by sequencing intact MHKB-2 cDNA across restriction sites. Sequence analyses were performed using Pustell Sequence Analysis Programs (International Biotechnologies, Inc., New Haven, CT).

The isolation and characterization of the type Ia clone, MHKA-1, have been previously described [26].

Protein Preparation Mouse hair root keratins were extracted from the plucked dorsal hairs of 9-day-old animals as previously described [34]. Additional keratin-enriched extracts from HeLa cells, mouse epidermis, and mouse esophageal epithelium, as well as total protein extracts of HeLa cells and mouse liver, were also prepared [5,9].

Confluent HeLa cell cultures were rinsed twice with phosphate-buffered saline (PBS), collected from culture dishes with a rubber policeman, and homogenized in 25 mM Tris·HCl (pH 7.4), 1% Triton X-100, and 0.6 M KCl to remove water-soluble proteins [13,22]. Protease inhibitors including antipain (5 μg/ml), pepstatin (5 μg/ml), 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF were added to all buffers [15,22]. The homogenates were centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant fractions were discarded and the insoluble pellets were resuspended in the same buffer and the extraction procedure repeated three more times. The water-insoluble fractions containing the keratins were heated at 95°C for 10 min in 25 mM Tris·HCl (pH 7.4) and 2% SDS. Keratins were similarly isolated from epidermal scrappings from frozen (liquid N_2) newborn mouse skin as welll as from dispase-isolated mouse esophageal epithelium.

Total protein extracts from HeLa cells and mouse liver were obtained by heating homogenized tissues as 95°C for 10 min in 25 mM Tris·HCl (pH 7.4), 2% SDS solution. After the protein extracts were passed through a 25 gauge needle to reduce their viscosity, the insoluble materials were pelleted using a microfuge and were discarded.

Antibody Preparation An oligopeptide, representing the last 18 amino acids (STGLCAPCGSGPCHPGRC) of the carboxyl-terminal domain of keratin mHb4, was synthesized (Peninsula Laboratories, Inc., Belmont, CA) and was conjugated to bovine thyroglobulin using the EDC method [35]. The conjugate was then used to immunize rabbits [36]. Antisera (designated Ab4) collected 10 d after the final immunization were assayed for specificity by immunofluorescence staining of hair follicles in mouse skin sections.

Human hair keratins, isolated as previously described [5], were used to immunize female New Zealand white rabbits. The resultant antiserum was affinity-purified to remove antibodies that cross-reacted with the soft keratins by passing it through a Sepharose column containing immobilized human callus keratins [5] chemically conjugated according to the manufacturer's recommendations (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). This affinity-purified antiserum (designated AH) recognized both acidic and basic hard keratins but not the soft keratins.

Other antibodies used in this study have been previously described; AE13 [5] and Ks18.174 ([37]; a gift from Dr. Roland Moll, University of Mainz, Federal Republic of Germany). Cells for immunofluorescence staining were grown on 12-mm glass coverslips, and fixed and permeabilized with acetone/methanol (1:1) as described [38].

Skin from 6-day-old mice was embedded in O.C.T. medium (Miles Scientific, Napaville, IL) and 5-μm frozen sections were prepared. Cultured cells or frozen skin sections were stained with antibodies singly or doubly as described previously [38,39].

Gel Electrophoresis and Immunoblots SDS-PAGE was performed according to Laemmli [40]; two-dimensional PAGE (IEF/SDS-PAGE [41]) was performed using Servalyt ampholines, pH 3 to 10 (Serva, Westbury, NY). The resolved proteins were electrophoretically transferred to nitrocellulose sheets and were visualized by Fast Green staining [15,42]. The immunoreactivity of the transferred proteins was determined by immunochemical staining using the peroxidase-antiperoxidase (PAP) technique [43].

Expression Vectors pB2 and pA1 The type IIa keratin cDNA insert of clone MHKB-2 was excised with Hinc II yielding an approximately 2-kb fragment beginning in the 5′-untranslated region and ending in the pBR322 vector 300 bp past the cloning Pst I site. After the addition of Eco RI linkers to both ends of this segment, it was inserted into the Eco RI cloning site of an expression vector, PECE [44]. This construct (pB2) therefore contains the full-length cDNA, a small segment of pBR322, and Eco RI linkers. An analogous construct (pA1) containing the full-length cDNA (MHKA-1) of a type Ia keratin [26,30] was similarly prepared.

The plasmids were twice purified by cesium chloride density centrifugation. Transfection was done using the calcium phosphate precipitation method [45] followed by a 10% glycerol shock for 3 min [46]. Cells were fixed 48 h after transfection.

RESULTS

A cDNA Clone Encoding a Mouse Hair Basic Keratin We have previously isolated a full-length cDNA clone of an acidic type Ia mouse hair keratin [26]. In order to isolate a "complementary" basic hair keratin cDNA, we screened our mouse hair follicular cDNA library with a partial cDNA clone of sheep type IIa wool

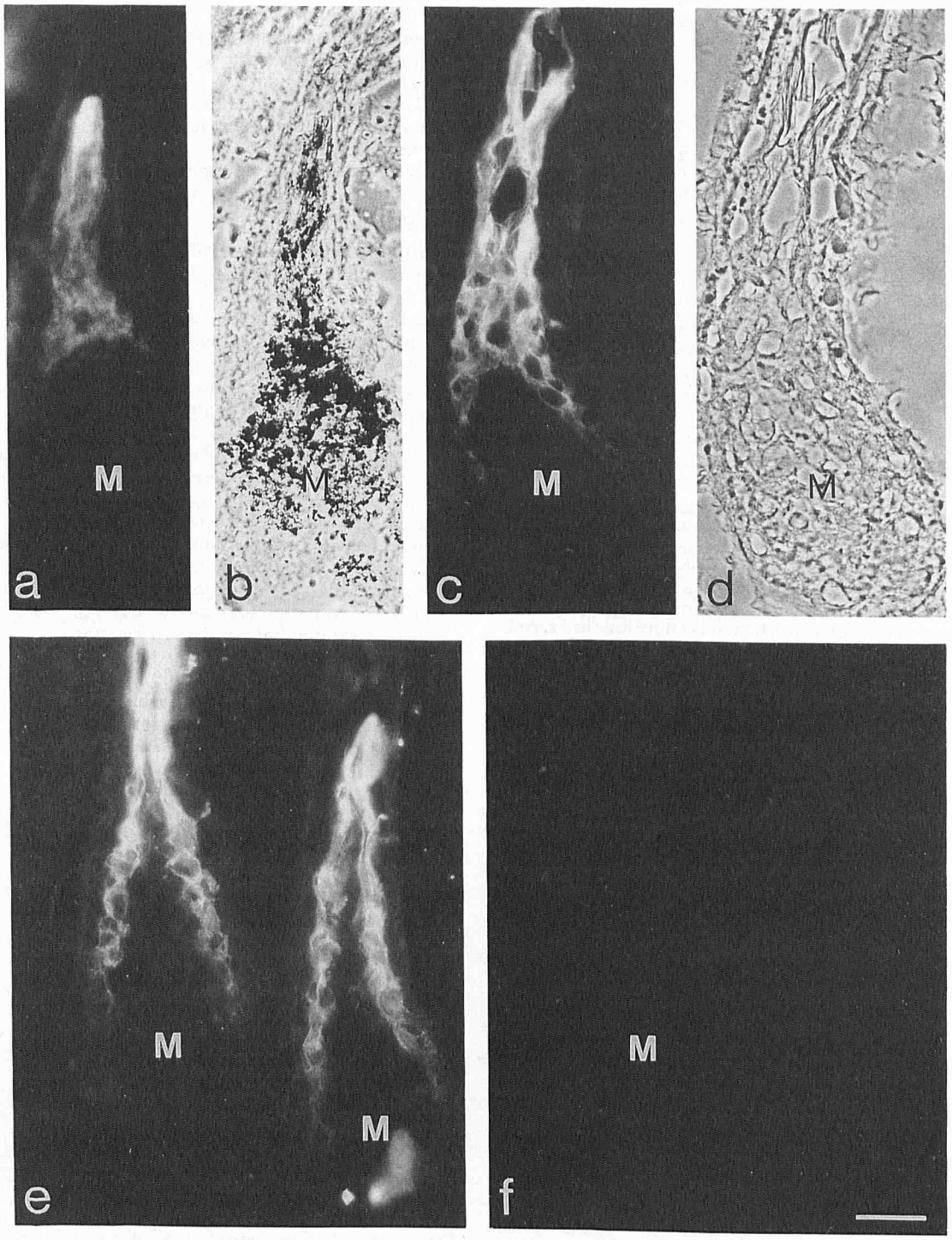

Figure 5. Immunofluorescence staining of mouse hair follicles. (*a*) Staining pattern produced by rabbit antiserum, Ab4, prepared against a synthetic peptide corresponding to the C-terminal amino acid sequence of mouse hair basic keratin, mHb4. (*b*) Corresponding phase-contrast image of the same field. (*c*) Immunofluorescence staining pattern using AE13. (*d*) Corresponding phase-contrast image of the same field in *c*. (*e*) Immunofluorescence staining pattern using affinity-purified rabbit antiserum (AH) to total human hair shaft keratins. (*f*) Immunofluorescence staining with monoclonal antibody A18 (Ks18.174) to soft keratin K18. Note the strong staining of cortical cells by Ab4, AE13, and AH, and the lack of staining by A18 (Ks18.174). All fields are the same magnification; *bar*, 30 μ. M, matrix area.

keratin and obtained a cDNA clone MHKB-2 (Fig 1). This clone contains a cDNA insert of 1804 bp—including some 5′-untranslated sequence, a presumptive ATG initiation condon (bp position 97), 1437 bp of uninterrupted reading frame, a TAG stop condon (bp 1534), a polyadenylation signal (AATAAA; bp 1769), and a small poly (A)-tail (bp 1789).

The deduced amino acid sequence of the coding region (Fig 1) corresponds to a protein with 479 amino acids, a molecular weight (MW) of 52,822, and a pI of 6.29. This sequence shows a high degree of homology to the sequences of type II keratins, with a clearly defined central helical domain flanked by non-helical N- and C-terminal sequences. A detailed comparison of this sequence

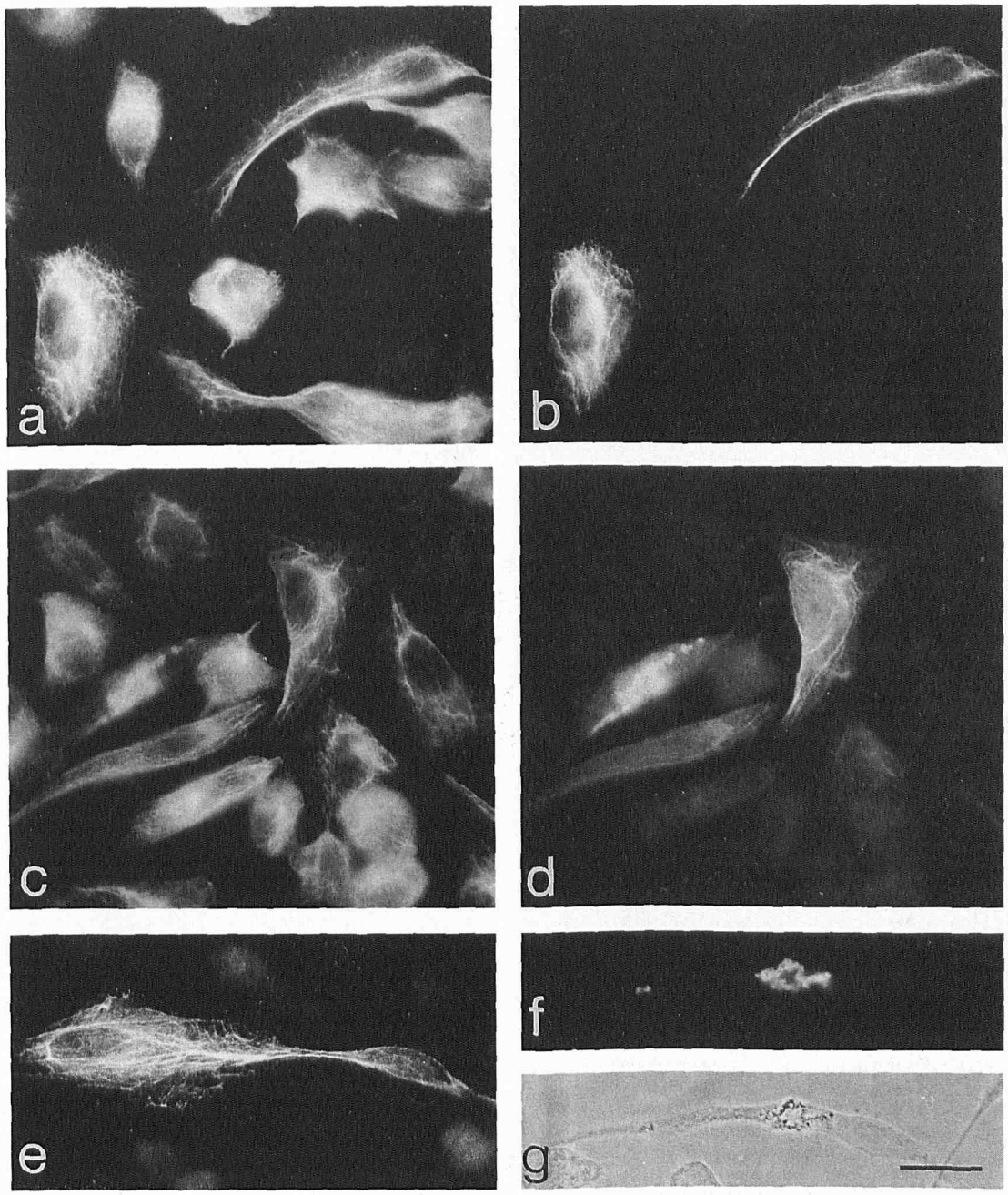

Figure 6. Expression of mouse hair keratin b4 in transfected HeLa cells. The endogenous keratin network of HeLa cells was detected with A18 (Ks18.174), which stains keratin K18, whereas the alien basic hair keratin was detected using antibodies AH and Ab4. (*a*)–(*d*) are double immunofluorescence staining patterns. (*a*) and (*c*) show the distribution of the endogenous soft keratins stained with A18, and (*b*) and (*d*) are the same two fields showing the distribution of the alien hair keratin stained with AH. Note that some of the transfected cells exhibit a filamentous keratin network containing both hard and soft keratins, suggesting that hair keratins can incorporate into the endogenous soft keratin network. Also note in (*d*) that in some transfected cells the hair keratin forms aggregates with endogenous soft keratins. (*e*) Staining of a transfected cell with rabbit antiserum (AH) to total human hair keratins. Note that the alien basic hair keratin exists in a filamentous network. (*f*) A transfected cell stained with antibody Ab4 showing aggregates of basic hair keratins. (*g*) The phase-contrast image corresponding to (*f*). Note that these keratin aggregates are phase dense and are thus easily visible. All fields are the same magnification; *bar*, 25 μ.

with basic wool keratin 7c [47] revealed over 87% identical amino acid residues, indicating that the sequences of type IIa keratins are highly conserved across species.

Specificities of the Antibodies to Hair Keratins To identify and to characterize the hair keratin encoded by this cDNA clone (MHKB-2), we synthesized an oligopeptide corresponding to the C-terminal 18 amino acid residues of the deduced sequence. Previous sequence data on various soft keratins have established that their C-terminal sequences tend to be highly divergent and characteristic of individual keratin chains [36]. These chain-specific sequences are highly conserved, however, across animal species indicating that they are probably functionally important. A comparison of the available C-terminal sequences of two mouse and one sheep type IIa hair keratins clearly shows that a similar situation exists for the hard keratins (Fig 2). Consistent with this result, we found that rabbit antisera raised against the C-terminal portion of the above mentioned, deduced protein sequence reacted specifically with one

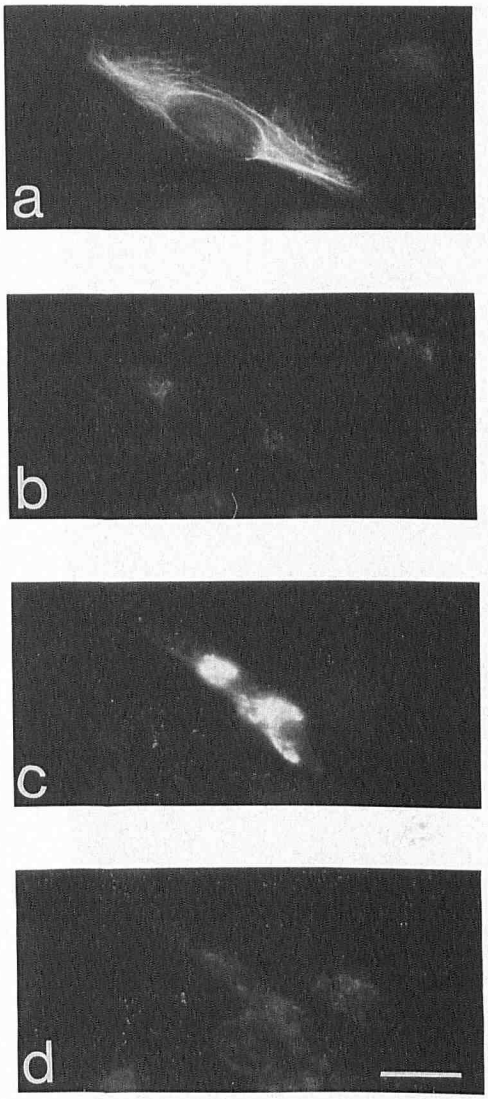

Figure 7. Transient expression of hair basic keratin b4 does not induce the expression of a hair acidic keratin in HeLa cells. (*a*) – (*d*) are double immunofluorescence staining patterns of pB2-transfected HeLa cells. (*a*) and (*c*) are stained with rabbit antiserum (AH) to total human hair keratins; this antibody reacts with both acidic and basic hair keratins. (*b*) and (*d*) are the corresponding staining patterns with monoclonal antibody AE13, which stains only acidic hair keratins. Note that acidic hair keratin cannot be detected in the keratin filaments (*b*), nor in the keratin aggregates (*d*). All fields are the same magnification; *bar*, 25 μ.

of the four basic mouse keratins, mHb4, according to both one- (Fig 3*b*) and two-dimensional immunoblotting (Fig 4). The highly specific nature of this antiserum was also supported by the observation that this antiserum selectively stains, by immunofluorescence, the distal cell layers of mouse hair cortex, without crossreacting with the outer root sheath or the epidermis (Fig 5*a* and data not shown).

We have also determined the specificities of several other antibodies. Figure 5*e* documents the reactivities of an affinity-purified rabbit antiserum prepared against total keratins of human hair shaft. This antibody (AH) reacts strongly with acidic mouse hair keratins and weakly with basic hair keratins but does not react with any soft keratins (Fig 3*c*). As we have shown earlier [5], AE13, a mouse monoclonal antibody, stains hair shafts (Fig 5*c*) and reacts with all four major acidic mouse hair keratins — without cross-reacting with any known basic keratins (Fig 3*d, lane 1*). A18 (Ks18.174), another mouse monoclonal antibody [37], does not show significant staining of the mouse hair follicle (Fig 5*f*) or react with any hard keratins (Fig 3*e, lane 1*), but it reacts with keratin K18 from HeLa cells (Fig 3*e, lane 2*).

Transient Expression of Hard Keratins in Cultured HeLa Cells Using this panel of antibodies to various hard and soft keratins, we surveyed the fate of hard keratins when they were artificially expressed in cultured HeLa cells. To perform these experiments, we inserted the cDNA of mouse hair basic keratin mHb4 and mouse hair acidic keratin mHa1 into an expression vector (PECE). In one series of experiments, we introduced the "b4" construct into HeLa cells by transfection using the calcium phosphate technique. Two days later, the transfected cells were fixed and double stained with antibody A18 (to detect the endogenous K18 keratin) and rabbit antiserum to total hair keratins (AH). We found that at least some hard keratin incorporated into cytoplasmic filament bundles that were morphologically indistinguishable from the endogenous soft keratin network. Interestingly, we also observed in some cells the formation of nonfilamentous cytoplasmic aggregates containing both mouse hair basic keratin and endogenous soft keratin (Fig 6*c,d*), suggesting that in these cases the hard keratins actually induced the partial collapse of the soft-keratin filaments. Finally, we found no cases where the expression of hair basic keratin in HeLa cells induced the expression of a hair acidic keratin (Fig 7).

Analogous results were obtained when we performed the opposite experiments, in which we introduced the cDNA of mouse hair acidic keratin "a1" (which we cloned and characterized earlier [26,30]) into HeLa cells (Fig 8). Specifically, the transiently expressed hair keratin (stained by mouse antibody AE13) was found to incorporate into the existing soft keratin network of HeLa cells. Moreover, nonfilamentous cytoplasmic aggregates of the mouse hair acidic keratin with or without endogenous soft keratin were observed.

DISCUSSION

Structural Conservation of Hair Keratin Sequences We have isolated and characterized in detail a cDNA clone encoding mHb4, a mouse hair basic keratin. The calculated pI of mHb4 corresponds extremely well to the experimental value, suggesting that the ATG (position 97) indeed represents the initiation codon and that the deduced sequence shown in Fig 1 represents the full-length sequence. Although the calculated MW (52,822) is considerably smaller than the 59-K value determined by SDS-PAGE, this difference most likely reflects anomalous electrophoretic behavior of the protein because a similar disparity in the calculated versus SDS-PAGE – determined MW was reported for a wool keratin [47].

Although the deduced amino acid sequences of numerous soft keratins are known, relatively little is known about the detailed amino acid sequences of hard keratins. Consequently, hair-keratin structure, particularly the N- and C-terminal domains, remains the least understood among all intermediate filaments (see, e.g., [29]). Using protein-chemical techniques, Sparrow et al [47] and Dowling et al [48] have determined the complete amino acid sequences of wool basic keratin 7c and wool acidic keratin 8c1. We have recently published two mouse hair acidic keratin sequences as deduced from cDNA data [26,30], and in this paper we present for the first time the deduced amino acid sequence of a mouse hair basic keratin (Fig 1). In addition, we have recently characterized and sequenced a partial cDNA clone that encodes another mouse hair basic keratin. Therefore, we have now accumulated sufficient primary sequence data enabling us to draw conclusions regarding the general structural features of hard keratins as a group.

(i) Our sequence data provide support to Conway and Parry's earlier suggestion [29], which at the time was based on extremely limited data from sheep keratin sequences alone, that within the

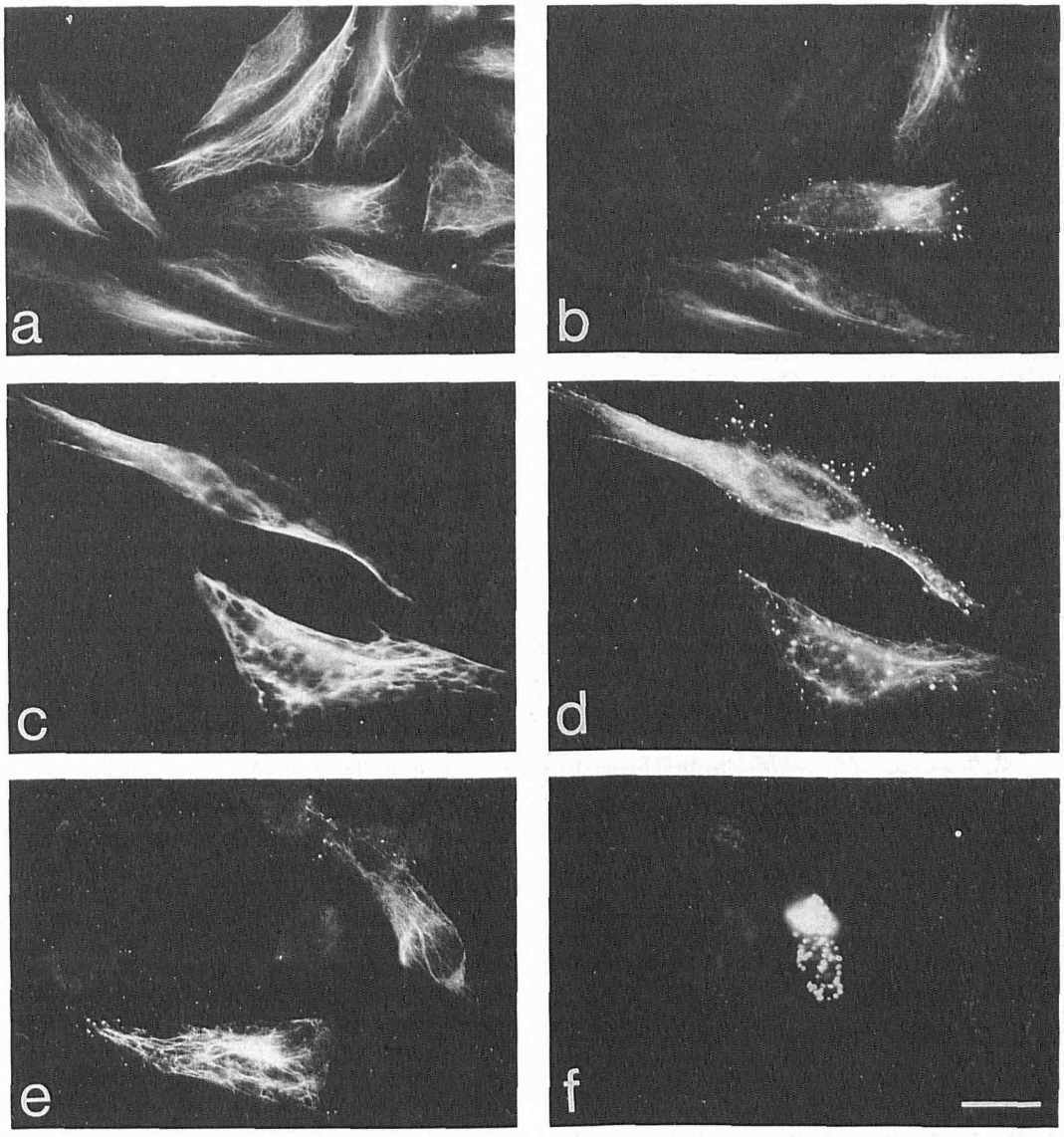

Figure 8. Expression of mouse hair keratin a1 in transfected HeLa cells. HeLa cells were transfected with plasmid pA1, which encodes the acidic hair keratin mHa1, using the calcium phosphate method. Cells were fixed after 48 h. The endogenous keratin network was detected by AK [39], a rabbit polyclonal antibody to total human epidermal (callus) keratins, whereas the alien acidic hair keratin was detected using AE13. (*a*) – (*d*) show double immunofluorescence staining. (*a*) and (*c*) are stained with AK and (*b*) and (*d*) are the corresponding fields stained with AE13. Note the hard keratin filamentous staining in (*b*) and (*d*) indicating that the alien hair keratin can incorporate into the soft keratin network. Some aggregates stained by AE13 are also stained by AK but some are not, indicating that the acidic hair keratin can form aggregates with or without endogenous soft keratin. (*e*) and (*f*) are staining patterns with AE13 showing filamentous networks of the alien hair keratin and aggregates, respectively. All fields are the same magnification; *bar*, 25 μ.

basic type II keratin family the sequences of the hard and soft keratins are divergent enough to warrant their being divided into two subfamilies (IIa for hard and IIb for soft keratins). A similar conclusion can be drawn for the acidic keratins.

(ii) A comparison of the mouse (b4) versus sheep (7c) sequences revealed at least four sites at which a non-conserved amino acid substitution is compensated by a "reversed" substitution in a neighboring amino acid that is only one or two residues away. These include the replacement of arg-thr-gly (bp112-121) in the mouse sequence (MP) by gly-ser-arg in the sheep sequence (SP); thr-arg-ile(MP) by ile-arg-val(SP); glu-leu-glu-gly(MP) by gly-leu-glu-glu(SP); and val-glu-ile-ile(MP) by ile-glu-ile-ala(SP). These "reversed" substitutions raise the possibility that the involved amino acids may play an important role in keratin structure such that their loss must be compensated. Moreover, the fact that a satisfactory substitution can occur in a neighboring amino acid indicates that there exists sufficient flexibility in the involvement of these amino acids in forming a higher-order structure.

(iii) As can be seen in Fig 1, many of the sequence differences between mouse b4 and sheep 7c occur in segments 1B and 2B of the helical rod domain. Most of the resulting amino acid changes can be accounted for by single base substitutions. However, the most significant variation occurs in the C-terminus, where all three currently available type IIa hard keratin sequences (two mouse and one sheep) seem to be unique. Because the C-terminal sequences of soft keratins are also known to be chain-specific but highly conserved across species [9], these results strongly suggest that not only the two mouse keratins but also the sheep keratin represent distinct keratin entities, rather than interspecies counterparts.

Although the C-terminal sequences of the three available type IIa keratins are distinct enough that antibodies to one of these sequences have been shown to be chain-specific, these three available

sequences do share some important features (Fig 2). For example, they all share three amino acids (gly-arg-cys) at the C-termini. In one mouse keratin this consensus segment is broken, however, by a lysine inserted between arginine and cysteine. The 5–12 amino acids adjacent to the GRC consensus sequence and distal to the conserved PCG are rather variable. These results indicate that the chain-specific variation of the C-terminal sequences of keratins, first demonstrated in soft keratins [19], also occurs in hard keratins. It has been suggested that these chain-specific, terminal sequences, which are thought to be exposed on the surface of the assembled filaments, may mediate chain-specific functions including filament-filament and filament-matrix interactions [10]. More data are needed to test this hypothesis and to define these functions.

(iv) An important feature that distinguishes the hard keratins from the soft keratins is that the former contain many more cysteine residues per molecule than the latter. Most of these cysteine residues are known to be involved in the formation of intermolecular disulfide bonds between keratins and either another keratin or a matrix molecule [49]. A comparison of all available hair keratin sequences reveals that these cysteines are extremely conserved in both their number and their positions, consistent with their presumed importance in keratin stabilization.

Co-Assembly of Hard and Soft Keratins Because the primary sequences of hard keratins differ significantly from those of the soft keratins, Conway and Parry [29] suggested that "it would seem likely that only the type Ia/IIa (hard keratins) and type Ib/IIb (soft keratins) chain combinations will be capable of copolymerization in vitro or in vivo." The transfection experiments described here provided a direct test of this hypothesis. Our results clearly showed that the transiently expressed mouse hair keratins, acidic or basic, can incorporate into a filamentous structure indistinguishable by immunofluorescence staining from the pre-existing soft keratin network of HeLa cells (Figs 6 and 8). This result suggests that hard and soft keratins can interact in forming keratin bundles. Because the expression of a hair keratin does not induce a complementary hair keratin, this result implies that the transiently expressed hard keratin must interact with soft keratins in forming these keratin bundles. Although we do not know at what level these hard keratins interact with the soft keratins, recent data suggest that these interactions probably occur at a heterodimer level [10–12]. Nevertheless, this interaction appears to be suboptimal because overproduction of hard keratins can lead to the formation of non-filamentous cytoplasmic aggregates of hair keratin with endogenous soft keratin. This latter observation is consistent with the original Conway and Parry hypothesis [29] and may reflect the predicted incompatibility between the hard and soft keratins. Taken together, our data suggest that small amounts of the newly synthesized hard keratins, possibly in a monomeric state, can incorporate into the "scaffolding" of the preformed filaments possibly through dynamic subunit exchange. However, overproduction of hard keratin can lead to the partial collapse of the soft keratin network. That subunit exchange actually occurs in keratin filaments is supported by some recent data from Miller and Goldman [50] who showed that biotinylated keratins, when microinjected into primary mouse epidermal cells, incorporated rapidly into the existing keratin filaments. Photobleaching experiments have likewise demonstrated subunit exchange in vimentin filaments [51].

Concluding Remarks In this paper, we showed by transient expression studies that hard and soft keratins can interact to form keratin bundles, although overproduction of hair keratins can lead to the partial collapse of an endogenous soft-keratin network. These observations, coupled with our new cDNA and deduced amino acid sequence data, support and extend the concept that hard and soft keratins, although closely related, are divergent enough to justify division into two subgroups. The availability of well-characterized cDNA ([26,30]; present work) and genomic [52] clones will enable us to examine in detail the functional roles and molecular regulation of individual hair-keratin proteins.

We thank Dr. Robert Oshima for useful discussions, Dr. Roland Moll for antibody Ks18.174, and Dr. Kevin Ward for cDNA clone SWK3.

REFERENCES

1. Fuchs E, Coppock SM, Green H, Cleveland DW: Two distinct classes of keratin genes and their evolutionary significance. Cell 27:75–84, 1981
2. Moll R, Franke WW, Schiller D, Geiger B, Krepler R: The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors, and cultured cells. Cell 31:11–24, 1982
3. Schiller DL, Franke WW, Geiger B: A subfamily of relatively large and basic cytokeratin polypeptides as defined by peptide mapping is represented by one of several polypeptides in epithelial cells. EMBO J 6:761–769, 1982
4. Heid HW, Werner E, Franke WW: The complement of native alpha-keratin polypeptides of hair-forming cells: a subset of eight polypeptides that differ from epithelial cytokeratins. Differentiation 32:101–119, 1986
5. Lynch MH, O'Guin WM, Hardy C, Mak L, Sun T-T: Acidic and basic hair/nail ("hard") keratins: their colocalization in upper cortical and cuticle cells of the human hair follicle and their relationship to "soft" keratins. J Cell Biol 103:2593–2606, 1986
6. Sun T-T, Eichner R, Schermer A, Cooper D, Nelson WG, Weiss RA: Classification, expression and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. In: Levine A, Topp W, Vande Woude G, Watson JD (eds.). Cancer Cells, Vol I. The Transformed Phenotype. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984, pp 169–176
7. Steinert PM, Steven AC, Roop DR: Structural features of epidermal keratin filaments reassembled in vitro. J Invest Dermatol 81:86S–90S, 1983
8. Hatzfield M, Franke WW: Pair formation and promiscuity of cytokeratins: formation in vitro of heterotypic complexes and intermediate filaments by homologous and heterologous recombinations of purified polypeptides. J Cell Biol 101:1826–1841, 1985
9. Eichner R, Sun T-T, Aebi U: The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments. J Cell Biol 102:1767–1777, 1986
10. Steinert PM, Roop DR: The molecular and cellular biology of intermediate filaments. Annu Rev Biochem 57:593–625, 1988
11. Steinert PM: The two-chain coiled-coil molecule of native epidermal keratin intermediate filaments is a type I-type II heterodimer. J Biol Chem 265:8766–8774, 1990
12. Hatzfeld M, Weber K: The coiled coil of in vitro assembled keratin filaments is a heterodimer of type I and II keratins: use of site-specific mutagenesis and recombinant protein expression. J Cell Biol 110:1199–1210, 1990
13. Franke WW, Schiller DL, Moll R, Winter S, Schmid E, Engelbrecht I, Denk H, Krepler R, Platzer B: Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. J Mol Biol 153:933–959, 1981
14. Fuchs E, Green H: The expression of keratin genes in epidermis and cultured epidermal cells. Cell 15:887–897, 1978
15. Woodcock-Mitchell J, Eichner R, Nelson WG, Sun T-T: Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies. J Cell Biol 95:580–588, 1982
16. Skerrow D, Skerrow CJ: Tonofilament differentiation in human epidermis: isolation and polypeptide chain composition of keratinocyte subpopulations. Exp Cell Res 143:27–35, 1983
17. Schweizer J, Kinjo M, Furstenberger G, Winter H: Sequential expression of mRNA-encoded keratin sets in neonatal mouse epidermis: basal cells with properties of terminally differentiating cells. Cell 37:159–170, 1984
18. Bowden PE, Quinlan RA, Breikreutz D, Fusenig NE: Proteolytic modification of acidic and basic keratins during terminal differentiation of mouse and human epidermis. Eur J Biochem 142:29–36, 1984
19. Roop DR, Cheng CK, Titterington L, Meyers CA, Stanley JR, Steinert PM, Yuspa SH: Synthetic peptides corresponding to keratin subunits elicit highly specific antibodies. J Biol Chem 259:8037–8040, 1984

20. Rentrop M, Knapp B, Winter H, Schweizer J: Differential localization of distinct keratin mRNA-species in mouse tongue epithelium by in situ hybridization with specific cDNA probes. J Cell Biol 103:2583–2591, 1986
21. Schermer A, Galvin S, Sun T-T: Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103:49–62, 1986
22. Eichner R, Bonitz P, Sun T-T: Classification of epidermal keratins according to their immunoreactivity, isolectric point, and mode of expression. J Cell Biol 98:1388–1396, 1984
23. Galvin S, Loomis C, Manabe M, Dhouailly D, Sun T-T: The major pathway of keratinocyte differentiation as defined by keratin expression: an overview. Adv Dermatol 4:277–300, 1989
24. O'Guin WM, Schermer A, Lynch M, Sun T-T: Differentiation-related expression of keratin pairs. In: Goldman R, Steinert P (eds.). Intermediate Filaments. Plenum, NY, 1990, pp 301–334
25. Tseng SCG, Hatchell D, Tierney N, Huang AJW, Sun T-T: Expression of specific keratin markers by rabbit corneal, conjunctival and esophageal epithelia during vitamin A deficiency-induced keratinization. J Cell Biol 99:2279–2286, 1984
26. Bertolino AP, Checkla DM, Notterman R, Sklaver I, Schiff TA, Freedberg IM, DiDona GJ: Cloning and characterization of a mouse type I hair keratin cDNA. J Invest Dermatol 91:541–546, 1988
27. Heid JW, Moll I, Franke WW: Patterns of expression of trichocytic epithelial cytokeratins in mammalian tissues. II. Concomitant and mutually exclusive sythesis of trichocytic and epithelial cytokeratins in diverse human and bovine tissues (hair follicle, nail bed and matrix, lingual papilla, thymic reticulum). Differentiation 37:215–230, 1988
28. Dhouailly D, Xu C, Manabe M, Schermer A, Sun T-T: Expression of hair-related keratins in a soft epithelium: subpopulations of human and mouse dorsal tongue keratinocytes express keratin markers for hair-, skin- and esophageal-types of differentation. Exp Cell Res 181:141–158, 1989
29. Conway JF, Parry DAD: Intermediate filament structure. 3. Analysis of sequence homologies. Int J Biol Macromol 10:79–98, 1988
30. Bertolino AP, Checkla DM, Heitner S, Freedberg IM, Yu DW: Differential expression of type I hair keratins. J Invest Dermatol 94:297–303, 1990
31. Ward KA, Sleigh MJ, Powell BC, Rogers, GE: The isolation and analysis of the major wool keratin gene families. Proc 2nd World Congr Genet Appl Livestock Prod 6:146–156, 1982
32. Maniatis T, Fritsch EF, Sambrook J: Isolation of bacteriophage and plasmid DNA. In: Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, 1982, pp 88–94
33. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci 74:5463–5467, 1977
34. Bertolino AP, Gibbs PEM, Freedberg IM: In vitro biosynthesis of mouse hair keratins under the direction of follicular RNA. J Invest Dermatol 79:173–177, 1982
35. Previero A, Derancourt J, Coletti-Previero MA: Solid phase sequential analysis: specific linking of acidic peptides by their carboxyl ends to insoluble resins. FEBS Lett 33:135–138, 1973
36. Roop DR, Cheng CK, Titterington L, Meyers CA, Stanley JR, Steinert PM, Yuspa SH: Synthetic peptides corresponding to keratin subunits elicit highly specific antibodies. J Biol Chem 259:8037–8040, 1984
37. Moll R, Achtstatter T, Becht E, Balcarova-Stander J, Ittensohn M, Franke WW. Cytokeratins in normal and malignant transitional epithelium: maintenance of expression of urothelium differentiation features in transitional cell carcinomas and bladder carcinoma cell lines. Am J Pathol 132:123–144, 1988
38. O'Guin WM, Schermer A, Sun T-T: Immunofluorescence staining of keratin filaments in cultured epithelial cells. J Tiss Cult Meth 9:123–128, 1985
39. Sun T-T, Green H: Keratin filaments of cultured human epidermal cells. J Biol Chem 253:2053–2060, 1978
40. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970
41. O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021, 1975
42. Towbin H, Staehelim T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci 76:4350–4354, 1979
43. Sternberger LA: Immunocytochemistry. Prentice-Hall, Inc., Englewood Cliffs, NJ, 1974, pp 104–169
44. Ellis L, Clauser E, Morgan DO, Eder M, Roth RA, Rutter WJ: Replacement of insulin receptor residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 45:721–732, 1986
45. Graham FL, Van Der E: A new technique for the assay of infections of human adenovirus DNA. Virology 52:456–467, 1973
46. Parker BA, Stark GR: Regulation of SENCAR virus 40 transcription sensitive analysis of the RNA species present early in infections by virus or viral DNA. J Virol 31:360–369, 1979
47. Sparrow LG, Robinson CP, MacMahon DTW, Rubira MR: The amino acid sequence of component 7c, a type II intermediate-filament protein from wool. Biochem J 261:1015–1022, 1989
48. Dowling LM, Crewther WG, Inglis AS: The primary structure of component 8c-1, a subunit protein of intermediate filaments in wool keratin. Biochem J 236:695–703, 1986
49. Fraser RDB, MacRae TP, Sparrow LG, Parry DAD: Disulfide bonding in alpha keratin. Int J Biol Macromol 10:106–112, 1988
50. Miller RK, Goldman RD: Biotinylated-keratin is incorporated into the keratin intermediate filament (IF) network of primary mouse epidermal cells. J Cell Biol 109:164a, 1989
51. Vikstrom KL, Lim SS, Borisy GG, Goldman RD: Subunit exchange in the intermediate filaments of living cells as analyzed by FRAP. J Cell Biol 111:260a, 1990
52. Kaytes PS, McNab AR, Rea TJ, Groppi V, Kawabe TT, Buhl AE, Bertolino AP, Hatzenbuhler NT, Vogeli G: Hair-specific keratins: characterization and expression of a mouse type I keratin gene. J Invest Dermatol (in press)