Mouse Differentiation-specific Keratins 1 and 10 Require a Preexisting Keratin Scaffold to Form a Filament Network

Tonja Kartasova,* Dennis R. Roop,‡ Karen A. Holbrook,§ and Stuart H. Yuspa*

*Laboratory of Cellular Carcinogenesis and Tumor Promotion, Division of Cancer Etiology, National Cancer Institute, Bethesda, Maryland 20892; ‡Baylor College of Medicine, Department of Cell Biology, Houston, Texas 77030; and §Department of Biological Structure, University of Washington, Seattle, Washington 98195

Abstract. Keratins 1 (K1) and 10 (K10) are the predominant cytoskeletal intermediate filaments of epidermal cells during transition from the proliferative to the terminal differentiation stage. In situ, formation of the K1/K10 intermediate filament network occurs in the cytoplasm of cells with a preexisting cytoskeleton composed of keratins 5 and 14. To define cytoskeletal interactions permissive for formation of the K1/K10 filamentous network, active copies of mouse K1 and K10 genes were introduced into fibroblasts (NIH 3T3) which do not normally express these proteins. Transient and stable transfectants, as well as heterokaryons produced by fusions with epithelial cells, were evaluated for expression of K1 and K10 proteins and filament formation using specific antibodies. In contrast to keratin pairs K5/K14 and K8/K18, the K1/K10 pair failed to form an extensive keratin filament network on its own, although small isolated dense K1/K10 filament bundles were observed throughout the cytoplasm by EM. K1 and K10 filaments integrated only into the preexisting K5/K14 network upon fusion of the NIH 3T3 (K1/K10) cells with epithelial cells expressing endogenous K5/K14 or with NIH 3T3 cells which were transfected with active copies of the K5 and K14 genes. When combinations of active recombinant gene constructs for keratins 1, 5, 10, and 14 were tested in transient NIH 3T3 transfections, the most intact cytokeratin network observed by immunofluorescence was formed by the K5/K14 pair. The K1/K14 pair was capable of forming a cytoskeletal network, but the network was poorly developed, and usually perinuclear. Transfection of K10 in combination with K5 or K1 resulted in cytoplasmic agglomerates, but not a cytoskeleton. These results suggest that the formation of the suprabasal cytoskeleton in epidermis is dependent on the preexisting basal cell intermediate filament network. Furthermore, restrictions on filament formation appear to be more stringent for K10 than for K1.

KERATINS are a large family of highly conserved epithelial-specific intermediate filaments. Each epithelium is characterized by a unique pattern of keratin expression (46). The intracellular localization of keratin filaments is confined to the cytoplasm, where the filaments copolymerize into an extensive network, encaging the nucleus and spreading towards the plasma membrane (11).

Based on size, charge, and gene structure, keratins have been divided into subfamilies of type I (acidic) and type II (basic) (8, 9, 40, 45). For filament formation in vitro, at least one polypeptide from each subgroup is required to form a heteropolymer (9, 17, 39).

The mechanism(s) that govern filament assembly in vivo are now being unraveled. Microinjection experiments of keratin mRNAs and purified keratin polypeptides demonstrated that a newly synthesized keratin could integrate into the endogenous keratin network of the injected epithelial cells and that the inter-species differences in keratin sequences did not prevent filament formation (12, 24, 30).

The structural requirements for filament assembly and interactions between different keratins have been analyzed using cloned keratin genes expressed in transfected cells and transgenic animals (1–4, 7, 14, 25, 27, 48). When certain keratin genes were introduced into cell lines of nonepithelial origin, an extensive filament network could develop even if the cells lacked an endogenous keratin network (3, 7, 27). Similar studies in epithelial cells, showed that exogenous keratins were able to integrate into a preexisting keratin network (1, 25). Moreover, certain keratin mutants disrupted the endogenous keratin network in vitro (1) and caused remarkable changes in the architecture of the skin in transgenic mice (47).

In interfollicular epidermis, four major keratins (K)[1] are synthesized and form filaments copolymerizing in pairs: K5/K14 and K1/K10. The expression of these two pairs is strictly regulated during normal keratinocyte differentiation

Tonja Kartosova's present address is Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan.

1. *Abbreviations used in this paper*: K, keratin; MT-1, metallothionein-1.

(33, 35, 36, 44). Thus, proliferating basal cells express a K5/K14 pair, and the transition to terminal differentiation and the cessation of cell division coincide with the appearance of a K1/K10 pair (33). Abnormal differentiation of skin in benign and malignant tumors correlates well with an aberrant expression of the differentiation-specific K1/K10 pair (35, 49). A delayed onset of terminal differentiation in benign skin tumors is reflected by a delay in the appearance of the K1/K10 pair. After conversion to malignancy, the K1/K10 pair is either absent or expressed in isolated cell foci (35, 36). This loss of K1/K10 is considered to be an early marker of malignant conversion.

The purpose of this study was to analyze the cooperation between keratins 1 and 10 and other keratins in the formation of a cytoskeletal network in epithelial and nonepithelial cells and in particular to explore the requirements for the formation of the K1/K10 filament network.

Materials and Methods

Cell Lines

NIH 3T3 fibroblasts were cultured in DME supplemented with 10% newborn calf serum. NIH 3T3 (K1/K10) cell lines were isolated after transfection of mouse K1 and K10 constructs along with the plasmid carrying the selectable neomycin-resistance marker into NIH 3T3 fibroblasts in a 10:10:1 ratio, respectively. NIH 3T3 (K5/K14) cell lines were isolated after transfection of human K5 and K14 constructs along with the plasmid carrying the selectable hygromycin-resistance marker into NIH 3T3 fibroblasts in a 10:10:1 ratio, respectively.

The keratinocyte cell line 308 was established from Balb/c mouse skin initiated with 7,12-dimethylbenz[a]anthracene in vivo (50). The SLC-1 cell line was derived from a focus which arose spontaneously in newborn keratinocytes grown in vitro in 0.05 mM calcium for an extended period. The 308 cells form benign papillomas and SLC-1 cells form carcinomas when grafted onto the backs of nude mice. The 308 and SLC-1 cell lines were cultured in EMEM containing 8% chelexed (19) FCS. The concentration of calcium in the medium was 0.05 mM.

Construction of Chimeric Keratin Genes

Full length genomic and cDNA copies of the mouse K1 gene were constructed in several assembly steps using overlapping clones (see Fig. 1) (43), (genomic K1 sequence is unpublished). All but 73 nucleotides upstream from the ATG initiation codon were removed by Bal31 exonuclease digestion and ligated to a HindIII linker. In the 3'-noncoding region a BamHI site was converted into a SstI site. The whole K1 genomic sequence including the polyA signal was ligated to HindIII–SstI digested pGEM7.

To create a cDNA version of the mouse K1 gene, the central part of the genomic K1 sequence between the HincII and BamHI sites was substituted by the sequences of two cDNA clones: p3.2 and p4.2 (43). At the 3'-end 270 nucleotides were left beyond the TAA stop codon, marked by the BglII site. The SV-40 splicing and polyA (4709–4100nt and 2700–2533nt, respectively, on the SV-40 map) signals were cloned 3' to the K1 cDNA sequence.

A complete mouse K10 cDNA copy was reconstructed from the two cDNA clones: p8–37 and p4–35 (42) (see Fig. 1). The sequence of the 5' noncoding region was removed by Bal31 exonuclease digestion, leaving four nucleotides upstream to the ATG initiation codon which were then joined to the pGEM7 polylinker sequence via a HindIII linker. At the 3' noncoding region 85 nucleotides were left downstream from the TAA stop codon and joined to the SV-40 splicing and polyA signals via an SstI linker. The end-sequences of all deletions were confirmed by sequencing, using the dideoxy method (38). The sequence fidelity of the K10 gene was confirmed by sequencing over the restriction sites which were used during the construction of the complete coding region of the K10 gene.

In the polylinker sequence of the K1 and K10 constructs just upstream to the ATG initiation codon, three different promoters were introduced: an SV-40 early promoter (HindIII–PvuII fragment) (31), a human cytomegalovirus IE1 gene (CMV) promoter (Sau3A–Sau3A fragment) (18) and mouse metallothionein-I (MT-I) promoter (EcoRI–HindIII) (6). Plasmids pHCMV and p8MT-CAT, carrying the CMV and MT-I promoters were obtained from Drs. L. G. Hennighausen and D. H. Hamer (National Institutes of Health, Bethesda, MD).

Chimeric constructs of human K5 and K14 genes, pJ2GK5-1 and pJK14, were obtained from Dr. Elaine Fuchs (University of Chicago, Chicago, IL) (14, 26).

Introduction of the K1, K10, K5, and K14 Constructs into Cells by Transfection

Chimeric keratin constructs were transfected into NIH 3T3 fibroblasts via the calcium phosphate precipitation procedure followed by a 25% DMSO shock (15). Typically, 10 μg of plasmid DNA was used to transfect 1×10^6 cells (6-cm dish). K1 and K10 chimeric constructs carrying different promoters, CMV, SV-40, MT-I, were used in transient transfections. For the selection of stable transfectants, a pSV2neo plasmid or a pSV2hygro plasmid was mixed with the chimeric keratin constructs in a 1:10 ratio. Selection was in a standard medium supplemented with G418 (200 μg/ml) or hygromycin (100 μg/ml). Individual clones were isolated by ring cloning and cultured in the continuous presence of the selective medium.

Cell Fusion

Fusions between epithelial cells and fibroblasts were performed on 60-mm culture dishes using polyethylene glycol (37). 1×10^6 SLC-1 or 308 cells were plated 20 h before fusion. 1×10^6 NIH 3T3 (K1/K10) fibroblasts were plated 4 h before fusion over the epithelial cells. The cells were washed with PBS and incubated for 2 min in a serum-free medium containing 50% polyethylene glycol (MW 1000), washed three times with DME and 10% newborn serum and incubated for 16 h before fixation. For fusion between the two different fibroblast lines, NIH 3T3 (K1/K10) and NIH 3T3 (K5/K14), 1×10^6 cells of each type, were plated together 4 h before fusion and further treated as described above. In some experiments a parental cell line was marked by BrdU labeling using 10 μM BrdU in medium for 48 h. Under these conditions, >95% of the nuclei became BrdU positive. In some studies cycloheximide was added to the cells immediately after fusion at 5 μg per ml medium. At this concentration, the protein synthesis was inhibited by 95–98% as judged by [^{35}S]methionine incorporation into the total cellular protein 3 h after the treatment (not shown). The cycloheximide treatment was for 16 h.

Antisera, Immunofluorescence, and EM

Cells were washed twice with PBS, fixed with methanol/acetone (1:1), and processed directly for immunofluorescence or kept dry at −20°C. Removal of proteins not organized into a cytoskeleton was done on living cells by a brief wash with 0.6 mM KCl/1% Triton X-100 and subsequent fixation with methanol/acetone (1:1). Monospecific antisera to mouse K1, K5, K10, and K14 from rabbits (anti-K1, K5, K10) or guinea pigs (anti-K1 and K14) have been described previously (34). Antisera to mouse K5 and K14 cross react with human K5 and K14. Keratin antibodies were used at a 1:500 dilution in PBS containing 12.5% BSA. BrdU incorporated into DNA was detected by a mouse monoclonal antiserum to BrdU (Becton Dickinson, San Jose, CA). Vimentin was detected by a polyclonal goat antiserum (ICN ImmunoBiologicals, Lisle, IL). BrdU- and vimentin-specific antibodies were used at dilutions recommended by the manufacturer.

To visualize the primary antibodies, the following fluorescently labeled secondary antibodies and chemicals were used: FITC-conjugated swine anti-rabbit (Dakopatts, Denmark); biotinylated goat anti-guinea pig (Vector Labs, Inc., Burlingame, CA); Texas Red-conjugated streptavidin (BRL, Gaithersburg, MD); Texas Red-conjugated horse anti-mouse (Vector Labs, Inc., Burlingame, CA); rhodamine-conjugated rabbit anti-goat IgG (H+L) (ICN ImmunoBiologicals, Lisle, IL). The staining with BrdU-specific antibodies was done according to the protocol provided by the manufacturer. Secondary antibodies and streptavidin were used at dilutions specified by manufacturers. Control samples, stained with secondary antibodies only, did not reveal specific immunostaining.

For EM, stable transfected cell lines were grown on 60-mm plastic culture dishes. They were washed several times in PBS, flooded with half-strength Karnovsky fixative (Karnovsky, M. J., 1965. *J. Cell Biol.* 27:137[abstr.]) and sealed for shipping to Seattle. Upon arrival, they were washed again, and fresh Karnovsky fixative was added for 1–2 h followed by postfixation in 1% OsO_4 in distilled water for 2 h at room temperature. The cultures were again washed then carried through a graded series of alcohols into 100% ethanol. Epon 812 was prepared in 25, 50 and 100% concentrations in alcohol and added to the dishes in sequence. Full strength Epon was added to the cells and allowed to polymerize overnight. The disks

of plastic-embedded cells were removed from the dishes and examined under a dissecting microscope to select at least 10 sample regions of the culture that were cut out of the disk and mounted on aluminum stubs for semi-thin and thin sectioning. The semi-thin sections were stained with toluidine blue and examined at the light microscopic level to assure that the sample contained an adequate number of cells for study. Thin sections were cut, stained with saturated uranyl acetate and Reynold's lead citrate (32) and examined in a Phillips 420 S(T)EM operated in the transmission mode at 60 kV. At least 500 cells were scanned in each thin section.

Protein Gel Electrophoresis and Blotting

Cytoskeletal proteins were extracted according to Steinert (41), separated by PAGE, transferred to nitrocellulose paper and assayed for the presence of keratins, using specific antisera, alkaline phosphatase-linked second antibodies and an alkaline phosphatase color reaction (Bio-Rad Labs, Richmond, CA).

Northern Blot Analysis

Total RNA was isolated by ultracentrifugation of guanidine isothiocyanate lysates through a 5.7-M cesium chloride gradient (28). 20 μg of total RNA was loaded per lane and separated by 1% agarose/formaldehyde gel electrophoresis, transferred to nitrocellulose paper and hybridized to specific fragments of keratin genes that were ^{32}P-labeled by nick translation (3–5 × 10^6 cpm/ml) (28). A 600-bp BamHI–SstI fragment of the K1 gene (43) and a 560-bp BglII–SstI fragment of the K10 gene (42) were isolated from the specific keratin constructs. A 430-bp fragment of the K5 gene (starting from amino acid 525 in reference 26) and a 400-bp fragment of the K14 gene (nucleotides 4,304–4,710 in reference 29) were generated by standard polymerase chain reaction (PCR) using specific 30-mers as primers (20). Nick-translated pUC18 vector carrying glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA (10) was used as a control for RNA loading.

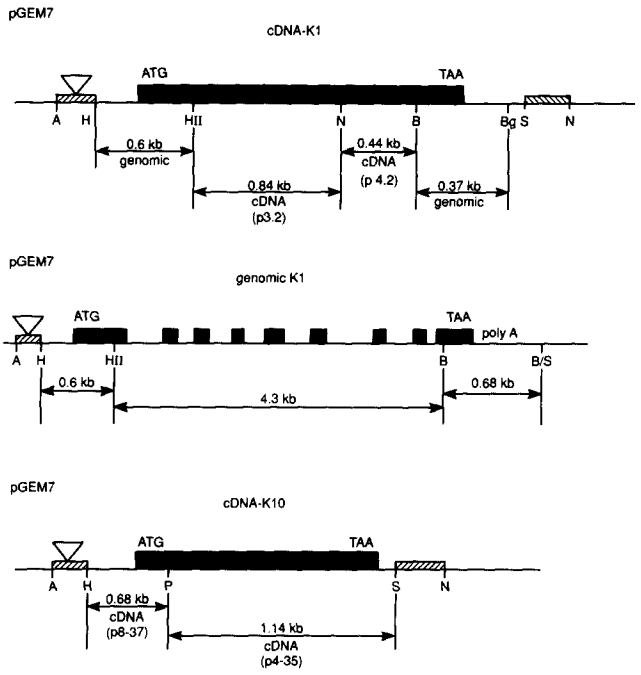

Figure 1. Construction of K1 and K10 chimeric plasmids. (■) coding region; (▨, *left*) pGEM-polylinker; (▨, *right*) SV-40–derived splicing and polyA signals; (▽) substitute for promoters. Only restriction sites used in cloning are indicated by capital letters: *A*, ApaI; *B*, BamHI; *Bg*, BglII; *H*, HindIII; *HII*, HincII; *N*, NsiI; *P*, PstI; and *S*, SstI.

Results

Expression of the Keratin 1/10 Pair in NIH 3T3 Fibroblasts Did Not Result in Formation of a Filament Network

Complete copies of mouse K1 and K10 genes were constructed from overlapping genomic and cDNA sequences and cloned downstream to three heterologous enhancer/promoter elements (Fig. 1). Subsequently, the K1 and K10 constructs were introduced separately or in a 1:1 ratio into NIH 3T3 cells by standard calcium phosphate precipitation. The level of keratin expression and the ability of K1 and K10 to form filaments were analyzed by indirect immunofluorescence in transient (24–48 h after transfection) as well as in stable transfectants isolated after selection of neomycin-resistant clones.

In transient transfectants when introduced separately, K1 and K10 accumulated in agglomerates throughout the cytoplasm and often in the perinuclear region. Surprisingly, in cotransfections no visible filament network was detected by immunocytochemistry, and the keratins agglomerated as in the single transfection experiments. Because of technical limits with regard to transient transfection frequency, the nature of the agglomerates was addressed only in the case of NIH 3T3 cells stably transfected with a K1/K10 pair (see results of electron microscopy below). Rarely, short linear filament fragments were detected in both stable and transient transfectants. Similar results were obtained with all three promoter constructs and several different methods of fixation. To obtain stable transfectants, NIH 3T3 cells were cotransfected with K1/K10 plasmids carrying either MT-I or SV-40 promoters. K1/K10 positive stable clones were detected only with the MT-I constructs.

A cell line, NIH 3T3 (K1/K10), derived from the transfection of the K1 genomic and the K10 cDNA constructs, both under control of the MT-I promoter, was studied in greater detail. In this cell line, K1 and K10 could be detected by indirect immunofluorescence even in the absence of inducing ions (Fig. 2, *A* and *B*). Treatment with $ZnCl_2$ only slightly increased the K1/K10 expression and did not lead to any visible keratin filamentous network. Double immunostaining indicated that K1 and K10 were colocalized to the same cytoplasmic agglomerates (Fig. 2, *C* and *D*) and that all cells positive for K10 were also positive for K1, although some cells were positive only for K1 (the filamentous staining in Fig. 2 *D* is nonspecific background staining with the guinea pig antiserum). The absence of K10 in some K1 positive cells might be due to a masking of the K10 epitope in these agglomerates. The K1 positive staining was not detected in every cell of this line, a finding consistent with previous reports of heterogeneous expression of other keratin pairs in cloned transfectants (3, 25). When NIH 3T3 (K1/K10) were incubated for 24 h with 10 μM BrdU, the K1 and K10 proteins accumulated preferentially in cells with BrdU-negative nuclei (Fig. 2, *E* and *F*). This suggested that the large K1/K10 agglomerates might interfere with a normal cell cycle or that only noncycling cells overproduced K1/K10. Consistent with the second hypothesis, incubation for 2 d in a serum-free medium led to reduction in BrdU labeling and promoted an increase in the number of cells positive for K1 and K10 (not shown).

Costaining studies indicated that the K1/K10 agglomerates did not disturb the cytoskeletal network of actin. Interestingly, a high salt/detergent wash revealed a nesting of the keratin aggregates in the endogenous vimentin network (Fig. 2,

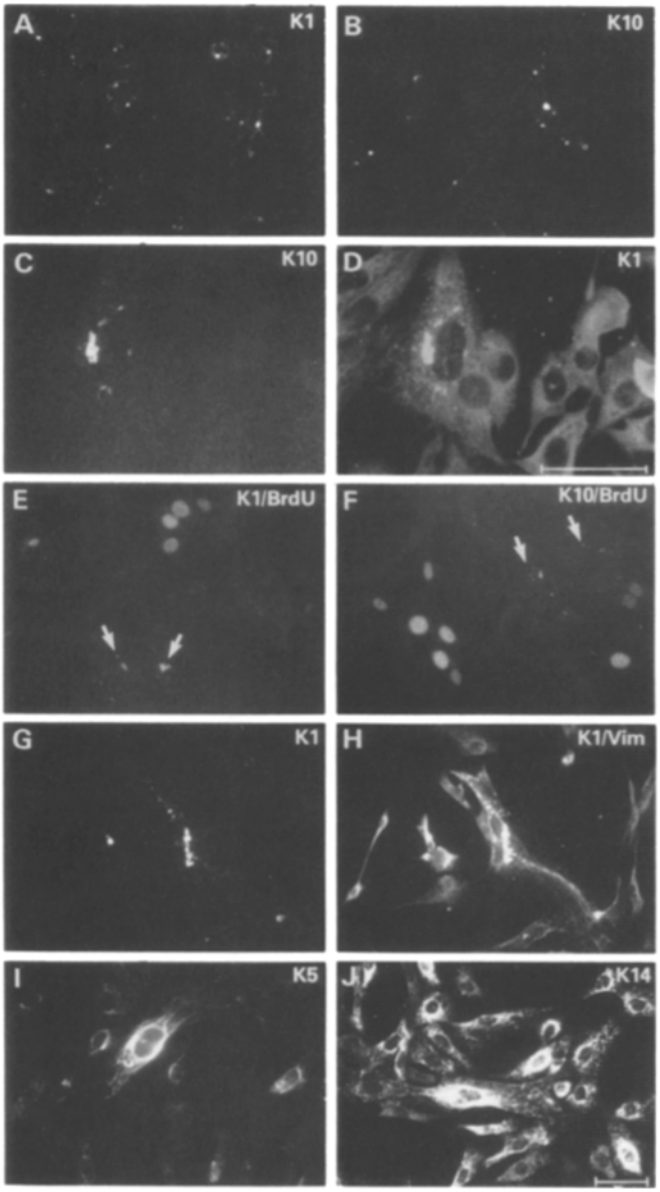

Figure 2. Immunodetection of the K1/K10 and K5/K14 proteins using specific antibodies in NIH 3T3 cells stably transfected with the K1/K10 (*A–H*) and K5/K14 (*I* and *J*) constructs. *C* and *D* are the same field, and *G* and *H* are the same field. Arrows are pointed to K1 (*E*) and K10 (*F*) agglomerates. The antibody specificity is indicated in the upper right corner of the panels. In *D*, the nonspecific staining of the K1-specific guinea pig antibody was high and background fluorescence could not be avoided. The K1 specific staining is clearly distinguished in agglomerates. Bars, 100 μm.

G and *H*). The short linear K1/K10 positive fragments seemed to be closely associated with the vimentin fibers and to resemble their pattern (not shown).

The expression of K1 and K10 in this NIH 3T3 (K1/K10) clone was analyzed further by Northern and Western blots (Fig. 3). The Northern blot showed the presence of full length transcripts (2.4 kb for K1 and 2.8 kb for K10) for the transfected plasmids, as well as shorter K1 and K10 specific transcripts (Fig. 3 *A*), possibly resulting from multiple sites of integration. Reprobing the blots with GAPDH plasmid indicated that the RNA was not degraded. Analysis of the

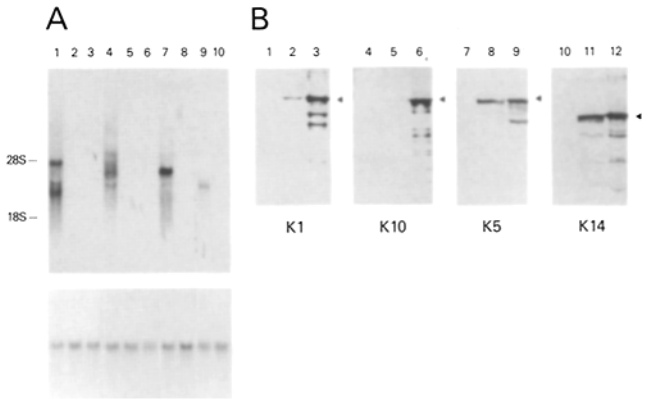

Figure 3. Northern and Western blot analyses of the expression of the K1/K10 and the K5/K14 pairs in NIH 3T3 (*K1/K10*) and NIH 3T3 (*K5/K14*), respectively. (*A*) Northern blot, 20 μg of total RNA was separated on formaldehyde–agarose gel electrophoresis, transferred to nitrocellulose by blotting, and hybridized with radiolabeled probes corresponding to the 600-bp fragment of the K1 genomic clone (lanes *1–3*), the 560-bp fragment of the K10 cDNA (lanes *4–6*), the 430-bp fragment of the K5 genomic clone (lanes *7* and *8*), and the 400-bp fragment of the K14 genomic clone (lanes *9* and *10*). Hybridization to GAPDH probe served as a control (*A*, *lower panel*). (Lanes *1* and *4*) NIH 3T3 (K1/K10); (lanes *2*, *5*, *8*, and *10*) NIH 3T3; (lanes *3* and *6*) NIH 3T3 (neo); (lanes *7* and *9*) NIH 3T3 (K5/K14). (*B*) Western blot, cytoskeletal proteins were resolved by PAGE-SDS, blotted onto nitrocellulose, and the keratins were detected using monospecific antibodies and alkaline phosphatase color reaction: *K1*, 1-3; *K10*, 4-6; *K5*, 7-9; and *K14*, 10-12. (Lanes *1*, *4*, *7*, and *10*) NIH 3T3; (lanes *2* and *5*) NIH 3T3 (K1/K10); (lanes *8* and *11*) NIH 3T3 (K5/K14); (lanes *3* and *6*) newborn mouse skin; and (lanes *9* and *12*) cultured human keratinocytes.

cytoskeletal extracts by Western blot using K1 and K10 specific antibodies revealed protein bands of 67 and 59 kD that comigrated with the control K1 and K10 bands, respectively, of mouse newborn epidermal extract (Fig. 3 *B*).

To determine whether the parental NIH 3T3 cell line was permissive for any keratin network formation, transfections were performed with the human K5/K14 keratin pair. In contrast to the K1/K10 pair, the K5/K14 pair very often formed an extensive filament network in the cytoplasm of both transient and stable transfectants as indicated by indirect immunofluorescence (Fig. 2, *I* and *J*). Short rods were also frequently observed. Whether the K5/K14 network formed independently from the vimentin network was not clear, since there was some cross-reactivity of the rabbit anti-vimentin secondary antibody with swine secondary antibody in double-stained cells. The expression of the K5/K14 pair in one of the clones, NIH 3T3 (K5/K14), was characterized further by Northern and Western blots (Fig. 3). The K5 and K14 RNAs were of predictable sizes: 2.2 and 1.6 kb, (13) respectively (Fig. 3 *A*). The protein bands of 56 and 50 kD detected by specific antibodies comigrated with the K5 and K14 proteins from cultured human keratinocytes (Fig. 3 *B*). The expression of K5 and K14 mRNA was comparable with the level of expression of the K1/K10 pair in the analogous NIH 3T3 (K1/K10) clone (Fig. 3). The amount of the synthesized K5 and K14 proteins in stable cell lines was higher than that of K1 and K10, as judged by Western blot and indirect immunofluorescence (Figs. 2 and 3).

Stable cloned NIH 3T3 cells expressing K1/K10 or K5/K14

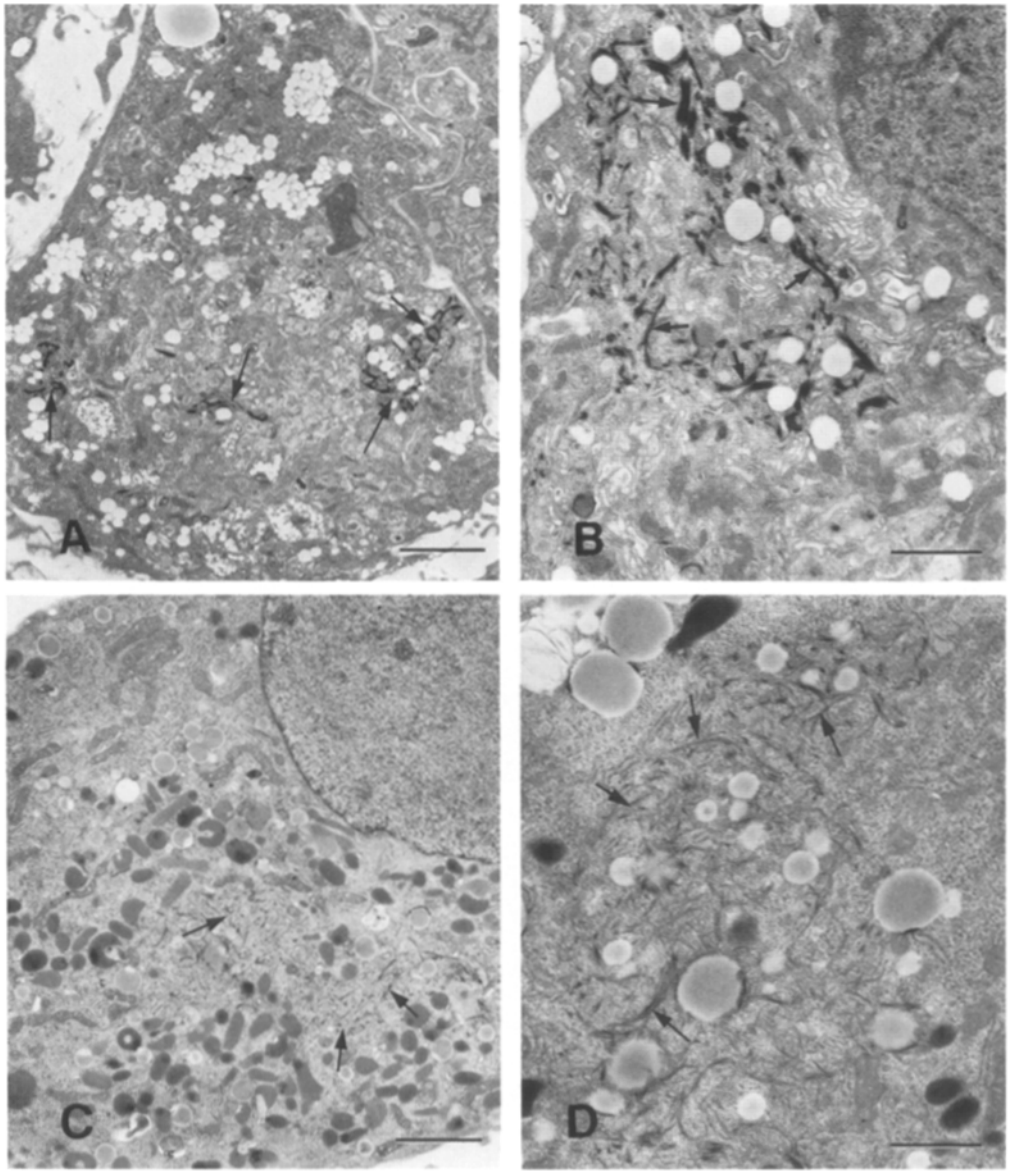

Figure 4. Transmission electron micrographs of segments of NIH 3T3 cells transfected with K1/K10 (*A* and *B*) and K5/K14 (*C* and *D*) recombinant DNA constructs and selected for stable transfected clones which express cytokeratin intermediate filaments. Note the larger and more dense filament bundles (*arrows*) located in Golgi regions in the K1/K10 transfected cells (*A* and *B*) compared with the fine bundles of filaments (*arrows*) in the K5/K14 transfected cells (*C* and *D*). Bars: (*A* and *C*) 2 μm; (*B* and *D*) 1 μm.

were further evaluated by EM. The majority of cells did not appear to express the transfected keratin genes. However, in both the K1/K10 and K5/K14 cultures, numerous cells contained intermediate filaments which were morphologically distinguished from vimentin (Fig. 4). These filaments were assembled in multiple peripheral and perinuclear regions throughout the cytoplasm. The filament bundles in K1/K10 cells appeared to be larger and more densely stained (Fig. 4, *A* and *B*) as compared with those in K5/K14 cells (Fig. 4, *C* and *D*). Thus, the agglomerates detected by indirect immunofluorescence in NIH 3T3 (K1/K10) consisted of short filament bundles. The K5/K14 filament bundles were fine, more reminiscent of filament bundles in basal keratinocytes. If compared with the native keratin network in epithelial cells, the K5/K14 network in NIH 3T3 cells appeared to be more convoluted than extended. This could be due to the lack of a proper anchoring point (through desmosomes) or to an unusual orientation of keratin fibers in fibroblasts.

Fusion between NIH 3T3 (K1/K10) and Two Epithelial Cell Lines, 308 Papilloma and SLC-1 Carcinoma Cells, Leads to Formation of a K1/K10 Filament Network in Heterokaryons

To determine whether the formation of the K1/K10 filament network in NIH 3T3 fibroblasts could be facilitated by addition of components normally absent in these cells, cell–cell hybrids were generated between the NIH 3T3 (K1/K10) line and papilloma (308) and carcinoma (SLC-1) cells. The SLC-1 line does not express K1/K10 in vitro or in vivo (our own unpublished observation), whereas the 308 cells express these keratins in vivo in the benign tumors which form upon grafting but not in the cultured cells (16). The endogenous filament network of both cell lines consists of at least two keratin pairs: K5/K14 and K6/K16. The ability of the K1 and K10 proteins to integrate into the preexisting network contributed by the epithelial donor was analyzed by indirect immuno-

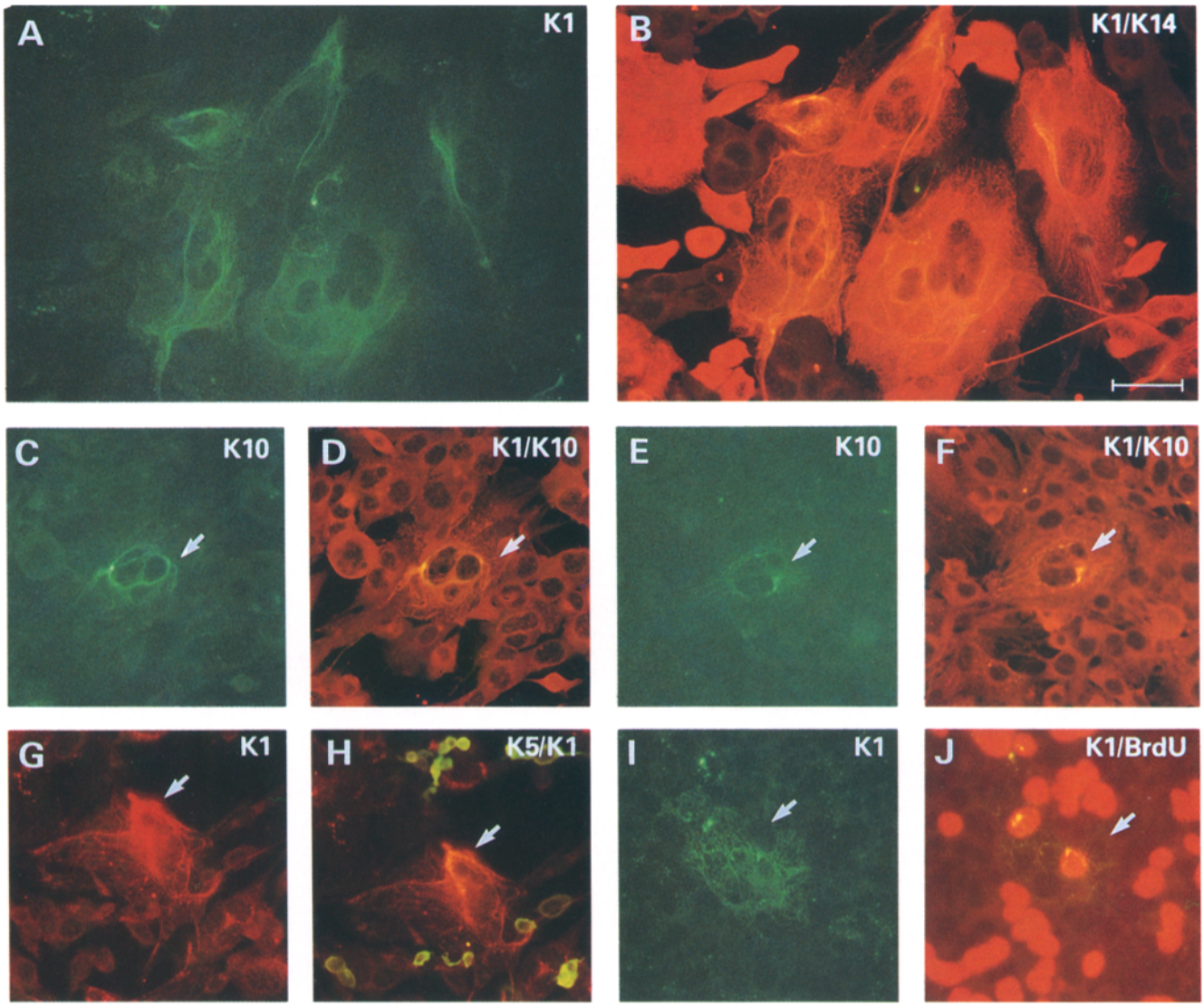

Figure 5. Immunodetection of K1 and K10 filaments in heterokaryons of NIH 3T3 (K1/K10) and epithelial cells: 308 papilloma (*A–D*) and SLC-1 carcinoma (*E–J*) cell lines; *I* and *J*, after cycloheximide. Antibody specificity is indicated in the upper right corner of the panels. (*A*) K1 (green); (*B*) double exposure of K1 (green) and K14 (red); (*C* and *E*) K10 (green); (*D* and *F*) double exposure of K10 (green) and K1 (red); (*G*) K1 (red); (*H*) double exposure of K5 (green) and K1 (red); (*I*) K1 (green); and (*J*) K1 (green) and BrdU (red). Arrows point to heterokaryons. SLC-1 cells in *I* and *J* were prelabeled with BrdU; dark circles represent unlabeled 3T3 nuclei. Dying SLC-1 cells in *H* are due to selection for 2 d after fusion in 0.05 mM calcium medium containing 400 ng/ml G418. Bar, 100 μm.

fluorescence. 6–16 h after fusion, a network containing K1 and K10 filaments could be detected in heterokaryons and colocalized to the endogenous keratin network (Fig. 5, *A, B, G,* and *H*) as well as to each other (Fig. 5, *C–F*). A much larger number of cells contained K1 filaments than K10 filaments. In general, 308 papilloma cells were more permissive for K1 and K10 filament formation than SLC-1 carcinoma cells in which the keratin bundles were also very fine and diffuse. Control fusions of only SLC-1 or 308 cells with themselves or between NIH 3T3 (K1/K10) and primary fibroblasts did not lead to any detectable K1 or K10 filament formation.

To determine if a K1 filament network could be formed from the preexisting agglomerates, cells were treated with cycloheximide to prevent accumulation of newly synthesized keratins directly after fusion and fixed 16 h later (Fig. 5, *I* and *J*). After this treatment, the K1 agglomerates were readily detectable in unfused fibroblasts (noted by BrdU-negative nuclei), whereas in heterokaryons, the K1 protein formed a filament network integrating into the endogenous scaffold derived from either 308 or SLC-1 cells (noted by BrdU-labeled nuclei).

Fusion between NIH 3T3 (K1/K10) and NIH 3T3 (K5/K14) Demonstrates That Preexisting K5/K14 Filaments Are Sufficient to Produce a K1/K10 Network

To determine if the K5/K14 network alone was sufficient to induce the K1/K10 filament network to form in NIH 3T3 fibroblasts, a fusion was performed between two NIH 3T3 cell lines: one expressing the K1/K10 pair as agglomerates

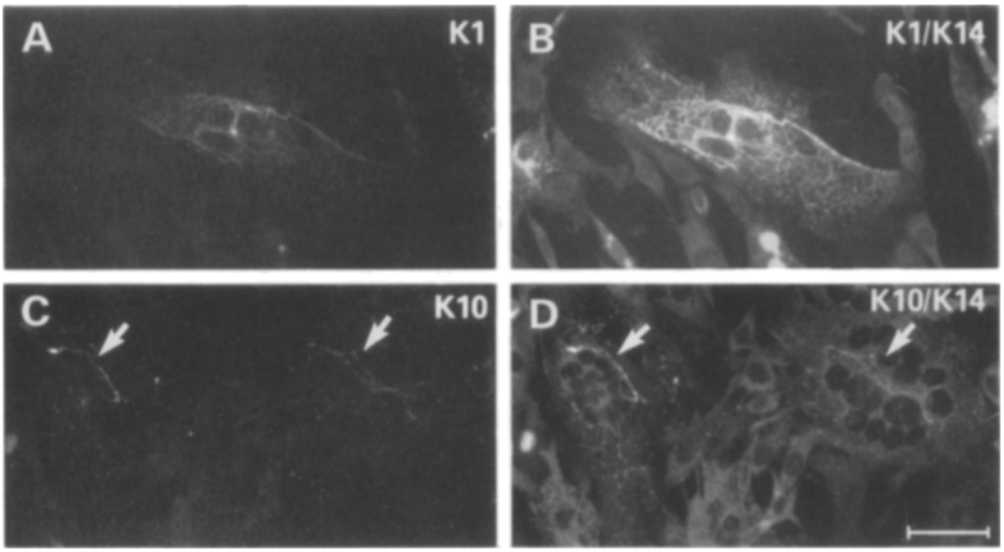

Figure 6. Immunodetection of K1 and K10 filaments in heterokaryons of NIH 3T3 (K1/K10) and NIH 3T3 (K5/K14). (*A*) K1; (*B*) double exposure of K1 and K14; (*C*) K10; and (*D*) double exposure of K10 and K14. Arrows point to heterokaryons. Bar, 100 μm.

and the other expressing the K5/K14 pair. 16 h after fusion, K1 and K10 were integrated into the K5/K14 network in heterokaryons as detected by immunocytochemistry (Fig. 6). Again, as in the case of fusions with epithelial cells, K1 filaments were readily detectable (Fig. 6, *A* and *B*), whereas K10 filaments were rare and not as extended as K1 filaments (Fig. 6, *C* and *D*). K1 agglomerates were not visible in heterokaryons with K1 filament networks.

Transfections of Recombinant Keratin Constructs into NIH 3T3 (K1/K10), NIH 3T3 (K5/K14), or NIH 3T3 Indicate Differences in Filament Compatibility for K1 and K10

To evaluate filament compatibility of keratins from the K5/K14 and K1/K10 pairs, three different sets of transfections were performed. First, NIH 3T3 (K1/K10) cells were used as recipients for transfections of the K5 and K14 constructs. When K14 was transfected alone, a few cells developed cytoskeletal filaments which contained both K1 and K14. However, this network was often fragmented and the K1 staining was very weak (Fig. 7, *O* and *P*). The K1/K14 network was far inferior to that of the K5/K14 network in NIH 3T3 (K5/K14). No K10/K14 positive filaments were detected. In Most K14 positive cells, K14 was detected in agglomerates. The K14 agglomerates were positive for K1 but not for K10. Transfection of K5 alone produced K5 agglomerates but no detectable filament network. The K5 positive agglomerates were negative for K1, but costaining for K10 could not be evaluated due to the lack of antibodies from another species. In K1/K10 cells transfected with the K5/K14 pair, the filament network showed costaining with different sets of antibodies: all cells positive for a K14 network stained positive for K5, some cells positive for a K1 network stained also for K5 (Fig. 7, *C* and *D*) and K14, and none of the cells stained positive for networks with K10 and K14. The K14 agglomerates stained only rarely positive for K1.

Reciprocal transfections of NIH 3T3 (K5/K14) with the K1 and K10 constructs were also performed. K1 transfected alone integrated into a network with K5/K14 (Fig. 7, *A* and *B*). The filaments positive for K1 costained also with K14 and K5 antibodies. However when the K10 construct was transfected alone, the presence of the K10 protein almost always led to the collapse of the endogenous K5/K14 network (Fig. 7, *M* and *N*). K10 and K14 appeared often to collapse independently without intermixing: K14 formed agglomerates in close approximation to the nucleus while K10 was detected throughout the cytoplasm. Only rarely was a fragmented network positive for K10 and K14. Introduction of two keratins, K1 and K10, produced a collapse of the K5/K14 keratin network in NIH 3T3 (K5/K14) cells (Fig. 7, *G* and *H*). Still, in few cells some filaments were observed which costained for K1/K5 (Fig. 7, *E* and *F*) and K1/K14 (not shown). Cells containing K1 filaments were always K10 negative. As a result of the keratin collapse, K14 and K5 were found in perinuclear agglomerates, whereas K1 and K10 were accumulating as agglomerates throughout the cytoplasm.

Finally, transfections of the NIH 3T3 cells with "unnatural" pairs, K1/K14 and K5/K10 were conducted. These two combinations did not lead to formation of an extensive network. In the case of the K1/K14 pair, fragmented filaments or filamentous perinuclear circles were occasionally detected (Fig. 7, *K* and *L*). The K5/K10 pair only formed large cytoplasmic agglomerates (Fig. 7, *I* and *J*).

Discussion

A main goal of this study was to define cytoskeletal interactions permissive for filament assembly of two differentiation-specific keratins, K1 and K10, which are induced during the transition from the proliferative stage to terminal differentiation in epidermis. In vitro assembly experiments have shown that all type I keratins could complex with all type II keratins and form IF filaments (17). This appeared to be valid also for K1 (Type II) and K10 (Type I): in vitro purified K1 and K10 polypeptides were able to form filaments upon mixing in a 1:1 ratio (17, 39). However, as noted by Eichner et al. (9), the 10-nm filaments composed of K5/K14 pair showed little tendency toward filament–filament interactions, whereas filaments enriched in the K1/K10 pair had a marked propensity to form dense filament tangles. In vivo, certain keratin pairs, such as K8/K18, K8/K19, K8/K14, K6/K14, were

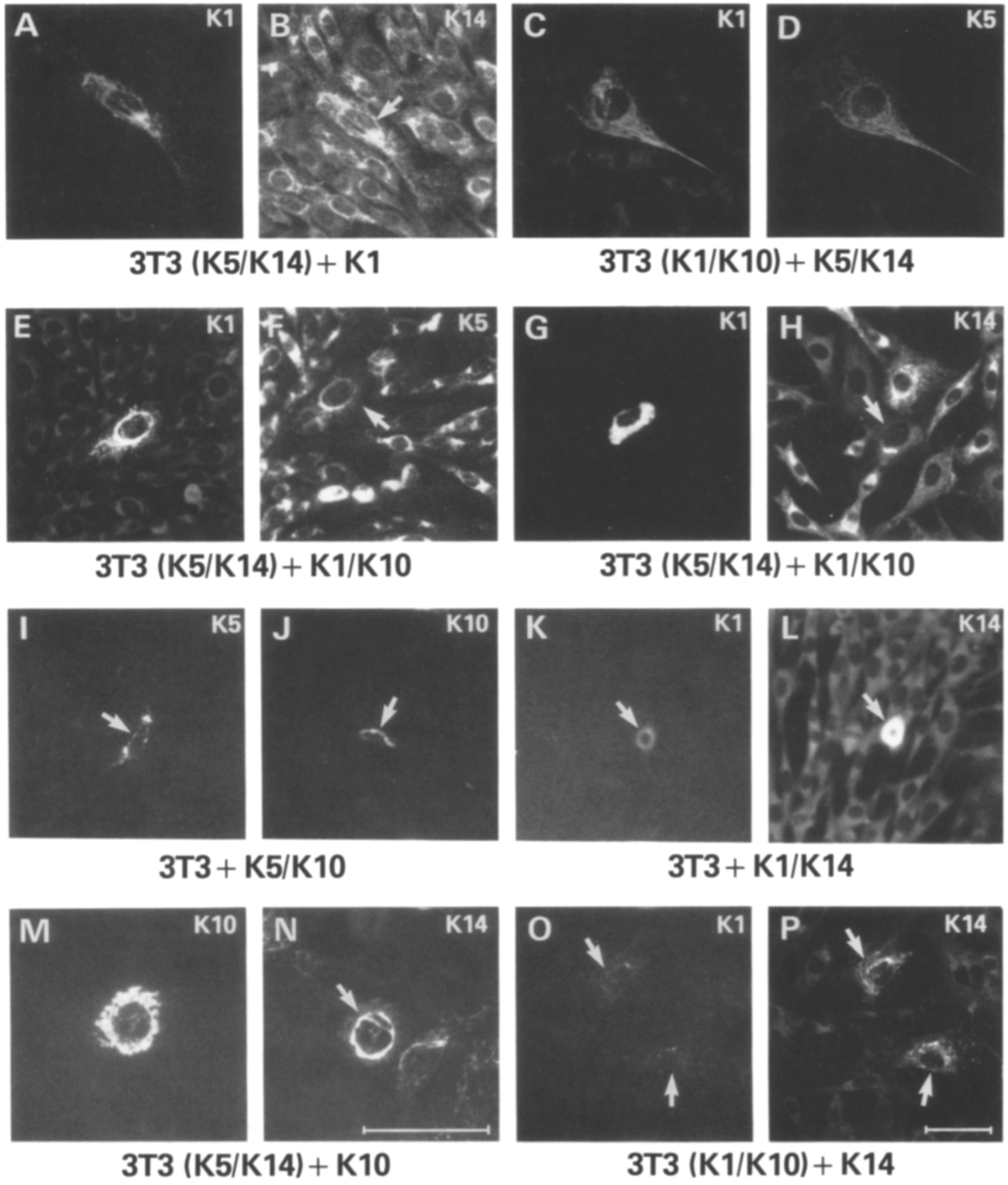

Figure 7. Immunodetection of K1, K5, K10, and K14 24 h after transfection of single or multiple recombinant plasmids into cell lines: NIH 3T3 (*I*, *J*, *K*, and *L*); NIH 3T3 (K5/K14) (*A*, *B*, *E–H*, *M*, and *N*) and NIH 3T3 (K1/K10) (*C*, *D*, *O*, and *P*). The recombinant plasmids encoded: K1 (*A* and *B*); K5/K14 (*C* and *D*); K1/10 (*E* and *F*, and *G* and *H*); K5/K10 (*I* and *J*); K1/K14 (*K* and *L*); K10 (*M* and *N*); and K14 (*O* and *P*). Antibody specificity is indicated in the upper right corner of the panels. *A* and *B*, *C* and *D*, *E* and *F*, *G* and *H*, *K* and *L*, *M* and *N*, and *O* and *P* represent the same fields. *I* and *J* represent two different fields. In *C* the punctate K1 staining originates from an overlying K5 negative cell. Arrows point to transfected cells. Bar, 100 μm.

shown to assemble into an extensive network in NIH 3T3 and other mesenchymal cell lines (3, 4, 7, 14, 25, 27). In NIH 3T3 cells expressing the K8/K18 pair, an organization of the keratin network was indistinguishable from the native epithelial IF cytoskeleton (27).

K1/K10 Requirements for Filament Formation

Our results show that unlike other keratin pairs, the K1/K10 pair is unable to form an independent intracellular network, but requires instead a preexisting keratin scaffold. When introduced into nonepithelial cells, such as NIH 3T3 fibroblasts, which carry an intermediate filament network of a different type (i.e., vimentin), the K1 and K10 proteins were unable to assemble into an extensive network. Instead, agglomerates and, very rarely, short rods accumulated throughout the cytoplasm, often around the nucleus. By double immunofluorescence of cytoskeletal ghosts of NIH 3T3 (K1/K10) cells, these agglomerates appeared to reside on the vimentin scaffold. The short linear fragments seemed to be closely associated with the vimentin fibers and to mimic their pattern. However, our studies could not determine whether vimentin was actually participating in the formation of agglomerates or linear fragments. After transfection, keratins will copolymerize both to the exclusion of vimentin (K8/K18 pair) (3) and to the inclusion of keratins and vimentin in agglomerates (K18 in Cos-1 cells) (4). Association of a vimentin and keratin network has also been observed in cultured simple epithelial cells (23).

Several observations showed that the failure of K1 and K10 to form an extensive filament network on their own was not due to the specific K1 and K10 constructs used or the NIH 3T3 cell line. The size of the K1 and K10 proteins synthesized in NIH 3T3 cells was identical to K1 and K10 from newborn mouse epidermis. However, the presence of truncated K1 and K10 proteins, which potentially could have interfered with the filament extension, as well as modifications (e.g., phosphorylation) of the K1 and K10 proteins which would not change the mobility on the SDS-PAGE, cannot be excluded. When the K1 and K10 constructs were transfected into two different epithelial cell lines, 308 and Cos-1, both K1 and K10 were able to integrate into the preexisting network of cells of epidermal and simple epithelial origins (22 and data not shown). By EM the K1/K10 expressing NIH 3T3 cells were shown to contain dense filament bundles, suggesting that K1 and K10 were able to assemble into filaments in the environment of NIH 3T3 cells although they could not produce a cytoskeletal network. The NIH 3T3 cell line used in the present study was permissive for formation of the cytokeratin network in transient and stable transfectants when the K5/K14 pair was expressed. Finally, fusion and transfection experiments strongly suggested that the K1 and K10 synthesized in NIH 3T3 cells were not "defective" and that they could be recruited into filament network formation under certain conditions. However, it must be emphasized that even under the best conditions of keratin expression, the filament network in NIH 3T3 cells is never as extensive as either the native network in epithelial cells or the network which forms when these same DNA constructs are transfected into epithelial cells (22). Thus, other factors which contribute to filament network formation must be absent from NIH 3T3 cells or the NIH 3T3 cells contain factors detrimental to cytokeratin network development.

Fusions between NIH 3T3 (K1/K10) and two epithelial cell lines led to the appearance of the K1 and K10 filament network which was closely colocalized to the endogenous keratin network of the epithelial donor. Moreover, fusions between two fibroblast cell lines expressing two different keratin pairs, K1/K10 and K5/K14, showed that there was no specific requirement for an epithelial background for the formation of the K1/K10 filament network; the K1/K10 filament network could be "rescued" in NIH 3T3 fibroblasts by the K5/K14 network. The fusion experiments also suggested that the K1/K10 agglomerates could be reused for filament formation: the agglomerates were frequently absent in heterokaryons. However, it is possible that K1 filament network formation developed from newly synthesized K1 in heterokaryons which were negative for K1 agglomerates. This seems unlikely since inhibition of protein synthesis with cycloheximide directly after the fusion did not prevent the appearance of the K1 filaments and was often associated with the absence of the K1 agglomerates. In unfused fibroblasts, the K1 agglomerates were clearly visible 16 h after cycloheximide treatment. This indicated that the K1/K10 agglomerates were stable, at least in NIH 3T3 (K1/K10) cells, and that the absence of the agglomerates in heterokaryons might not be due to the rapid turnover of these structures, but rather to their recruitment into the keratin network.

In Vivo Preference for Filament Formation among Four Different Keratins: K1, K5, K10, and K14

In vitro assembly studies with purified polypeptides of K1, K5, K10, and K14 showed that the combination of K5 and K10 formed a filament complex as stable as that of the native K5/K14 pair (17). The combination of K1 and K14 was less stable than K5 and K14 but similar to K1 and K10. Experiments with NH_2- and COOH-terminal K14 mutants have demonstrated differences between in vitro assembly and in vivo filament formation for some of the mutants. Despite the ability of these mutants to form filaments in vitro, they often perturbed keratin filament formation in vivo (5). Transfection of K7, K8, K19, and K14 into NIH 3T3 cells has indicated that naturally coexpressed keratin pair makes the best-formed higher order filament network (27). Thus, with K7 and K8, K18 is superior to K19, which is better than the keratinocyte K14. From the immunofluorescence pattern it appeared that the network of mismatched keratins is less stable in a living cell (27). Therefore, it was of interest to see whether there was any correlation between the in vitro assembly studies and the in vivo filament formation of K1, K5, K10, and K14.

Transient transfection experiments were performed, using three different fibroblast cell lines: NIH 3T3, NIH 3T3 (K1/K10), and NIH 3T3 (K5/K14), and various combinations of keratins 1, 5, 10, and 14. Coulombe et al. (5) have questioned the validity of expressing keratins in cells that do not have keratin-associated structures, (e.g., desmosomes) since factors which might be important for filament assembly could be missing in nonepithelial cells as well as a proper anchoring of the keratin network might not occur. Nevertheless, in a nonepithelial environment the differences between keratin pairs could be analyzed without the interference of the endogenous keratin network. In combination of two keratins, the only pair of keratins which successfully formed an extensive network by immunofluorescence detection was

K5/K14. The K1/K14 pair formed at most filamentous perinuclear circles. The combinations of the K5/K10 and K1/K10 pairs resulted in cytoplasmic agglomerates without detectable filament network formation by immunocytochemical detection. Our methods cannot exclude the possibility that K5/K14 were expressed to higher concentrations in NIH 3T3 transfectants and this is responsible for the formation of a more extensive network (see Fig. 3). However, in transient transfection experiments, high levels of both K1/K10 and K5/K14 proteins were detected in single cells without altering the relative competence for filament network formation for each pair.

In combination of three keratins, the most successful was the combination of the K5/K14 network and the transiently transfected K1. The K1 filaments were costained for either K5 or K14. Costaining with K1 and K5 indicated that K1 and K5 could coexist in the same network after the integration of K1. This can be relevant to the in vivo situation when during transition of the basal cells to terminal differentiation, the K1 protein is induced first and integrates into the K5/K14 network (36). When K14 cDNA was transfected into the K1/K10 cells, K1 was detected in a filament network which did not contain detectable K10. This suggests a possible exclusion of K10 from filament formation or alternatively a masking of the K10 epitopes. Introduction of K5 into K1/K10 cells did not result in a filament network. From this result as from transfection of the K5/K10 pair, it could be concluded that the K5/K10 pair resembles the K1/K10 pair in its lack of potential to from an extensive network in NIH 3T3. The presence of the transfected K10 in most of the cases interfered with the endogenous network of K5/K14 in NIH 3T3 (K5/K14). Remarkably, K10 and K14 usually collapsed independently without intermixing: K14 in close approximation to the nucleus and K10 throughout the cytoplasm. The fate of K5 was difficult to assess due to the lack of antibodies prepared in a second species.

A combination of 4 keratins produced through the introduction of the K1/K10 pair into NIH 3T3 (K5/K14) or of the K5/K14 pair into NIH 3T3 (K1/K10) gave similar results. The filament network was observed rarely, and when found, it was positive for K1/K5 and K1/K14, but K10 negative. These results are consistent with in vitro assembly studies which indicated that the stability of the K5/K14 pair was higher than the stability of the K1/K10 pair, which was equal to the K1/K14 pair (17). This suggests that the K5/K14 pair could potentially compete K1 from the K1/K10 agglomerates into the filament network. Conclusively, K1 seems to be acceptable as a third partner for the K5/K14 pair, whereas K10 is not. The presence of K10 had a negative effect on the integrity of the K5/K14 network, at least in NIH 3T3 cells and led to its disintegration. It is possible that for a successful K10 integration some sort of "pre-conditioning" of the K5/K14 network by K1 is necessary. This could explain the temporal sequence of keratin expression during epidermal differentiation in vivo, where the appearance of K1 in postmitotic basal cells and its integration into the K5/K14 network precedes the expression of K10.

We are grateful to Drs. Tamar Tennenbaum, Adam Glick, Andrzej Dlugosz, Wendy Weinberg, and Ulrike Lichti for helpful discussions and critical reviews of the manuscript. We are in debt to Dr. Ofelia Olivero for the introduction to the BrdU technique. We would like to thank Dr. Elaine Fuchs for providing us with vectors pJ2GK5-1 and pJK14, Dr. Lothar G. Hennighausen for pHCMV-CAT, and Dr. Dean Hamer for p8MT-CAT. Secretarial and bibliographic assistance of Ms. Margaret Taylor is greatly appreciated. Dr. Ulrike Lichti provided considerable assistance and advice throughout the project and in finalizing the manuscript.

Received for publication 26 December 1991 and in revised form 2 November 1992.

References

1. Albers, K., and E. Fuchs. 1987. The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. *J. Cell Biol.* 105:791–806.
2. Albers, K., and E. Fuchs. 1989. Expression of mutant keratin cDNAs in epithelial cells reveals possible mechanisms for initiation and assembly of intermediate filaments. *J. Cell Biol.* 108:1477–1493.
3. Bader, B. L., T. M. Magin, M. Freudenmann, S. Stumpp, and W. W. Franke. 1991. Intermediate filaments formed de novo from tail-less cytokeratins in the cytoplasm and in the nucleus. *J. Cell Biol.* 115: 1293–1307.
4. Blouin, R., H. Kawahara, S. W. French, and N. Marceau. 1990. Selective accumulation of IF proteins at a focal juxtanuclear site in COS-1 cells transfected with mouse keratin 18 cDNA. *Exp. Cell Res.* 187:234–242.
5. Coulombe, P. A., Y. M. Chan, K. Albers, and E. Fuchs. 1990. Deletions in epidermal keratins leading to alterations in filament organization in vivo and in intermediate filament assembly in vitro. *J. Cell Biol.* 111:3049–3064.
6. Culotta, V. C., and D. H. Hamer. 1989. Fine mapping of a mouse metallothionein gene metal response element. *Mol. Cell Biol.* 9:1376–1380.
7. Domenjoud, L., J. L. Jorcano, B. Breuer, and A. Alonso. 1988. Synthesis and fate of keratins 8 and 18 in nonepithelial cells transfected with cDNA. *Exp. Cell Res.* 179:352–361.
8. Eichner, R., P. Bonitz, and T. T. Sun. 1984. Classification of epidermal keratins according to their immunoreactivity isoelectric point and mode of expression. *J. Cell Biol.* 98:1388–1396.
9. Eichner, R., T. T. Sun, and U. Aebi. 1986. The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments. *J. Cell Biol.* 102:1767–1777.
10. Fort, P., L. Marty, M. Piechaczyk, S. el Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard. 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res.* 13:1431–1442.
11. Franke, W. W., E. Schmid, M. Osborn, and K. Weber. 1978. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. USA.* 75:5034–5038.
12. Franke, W. W., E. Schmid, S. Mittnacht, C. Grund, and J. L. Jorcano. 1984. Integration of different keratins into the same filament system after microinjection of mRNA for epidermal keratins into kidney epithelial cells. *Cell.* 36:813–825.
13. Fuchs, E., and H. Green. 1980. Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell.* 19:1033–1042.
14. Giudice, G. J., and E. Fuchs. 1987. The transfection of epidermal keratin genes into fibroblasts and simple epithelial cells: evidence for inducing a type I keratin by a type II gene. *Cell.* 48:453–463.
15. Graham, F. L., and A. J. Eb. 1973. Transformation of rat cells by DNA of human adenovirus 5. *Virology.* 54:536–539.
16. Greenhalgh, D. A., and S. H. Yuspa. 1988. Malignant conversion of murine squamous papilloma cell lines by transfection with the fos oncogene. *Mol. Carcinog.* 1:134–143.
17. Hatzfeld, M., and W. W. Franke. 1985. Pair formation and promiscuity of cytokeratins: formation in vitro of heterotypic complexes and intermediate-sized filaments by homologous and heterologous recombination of purified polypeptides. *J. Cell Biol.* 101:1826–1841.
18. Hennighausen, L., and B. Fleckenstein. 1986. Nuclear factor 1 interacts with five DNA elements in the promoter region of the human cytomegalovirus major intermediate early gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1367–1371.
19. Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S. H. Yuspa. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell.* 19:245–254.
20. Innis, M. A., D. H. Felfand, J. J. Sninsky, and T. J. White. 1990. PCR Protocols, A Guide to Methods and Applications. Academic Press Inc, San Diego. 1–12.
21. Deleted in proof.
22. Kartasova, T., D. R. Roop, and S. H. Yuspa. Relationship between the expression of differentiation-specific keratins 1 and 10 and cell proliferation in epidermal tumor cells. *Mol. Carcinog.* 6:18–25.
23. Klymkowsky, M. W. 1982. Vimentin and keratin intermediate filament systems in cultured PtK2 epithelial cells are interrelated. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:161–165.
24. Kreis, T. E., B. Geiger, E. Schmid, J. L. Jorcano, and W. W. Franke. 1983. De novo synthesis and specific assembly of keratin filaments in

nonepithelial cells after microinjection of mRNA for epidermal keratin. *Cell.* 32:1125–1137.
25. Kulesh, D. A., G. Cecena, Y. M. Darmon, M. Vasseur, and R. G. Oshima. 1989. Posttranslational regulation of keratins: degradation of mouse and human keratins 18 and 8. *Mol. Cell Biol.* 9:1553–1565.
26. Lersch, R., V. Stellmach, C. Stocks, G. Giudice, and E. Fuchs. 1989. Isolation, sequence, and expression of a human keratin K5 gene: transcriptional regulation of keratins and insights into pairwise control. *Mol. Cell Biol.* 9:3685–3697.
27. Lu, X., and E. B. Lane. 1990. Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: specific domain functions in keratin stabilization and filament formation. *Cell.* 62:681–696.
28. Maniatis, T. E., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. Marchuk, D., S. McCrohon, and E. Fuchs. 1985. Complete sequence of a gene encoding a human type I keratin: sequences homologous to enhancer elements in the regulatory region of the gene. *Proc. Natl. Acad. Sci. USA.* 82:1609–1613.
30. Miller, R. K., K. Vikstrom, and R. D. Goldman. 1991. Keratin incorporation into intermediate filament networks is a rapid process. *J. Cell Biol.* 113:843–855.
31. Mulligan, R. C., B. H. Howard, and P. Berg. 1979. Synthesis of rabbit beta-globin in cultured monkey kidney cells following infection with a SV40 beta-globin recombinant genome. *Nature (Lond.).* 277:108–114.
32. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17:208–213.
33. Roop, D. R., P. Hawley-Nelson, C. K. Cheng, and S. H. Yuspa. 1983. Keratin gene expression in mouse epidermis and cultured epidermal cells. *Proc. Natl. Acad. Sci. USA.* 80:716–720.
34. Roop, D. R., C. K. Cheng, L. Titterington, C. A. Meyers, J. R. Stanley, P. M. Steinert, and S. H. Yuspa. 1984. Synthetic peptides corresponding to keratin subunits elicit highly specific antibodies. *J. Biol. Chem.* 259:8037–8040.
35. Roop, D. R., T. M. Krieg, T. Mehrel, C. K. Cheng, and S. H. Yuspa. 1988. Transcriptional control of high molecular weight keratin gene expression in multistage mouse skin carcinogenesis. *Cancer Res.* 48: 3245–3252.
36. Roop, D. R., H. Nakazawa, T. Mehrel, C. Cheng, J. A. Rothnagel, P. M. Steinert, and S. H. Yuspa. 1989. Sequential changes in gene expression during epidermal differentiation. In The Biology of Wool and Hair. G. E. Rogers, P. J. Reis, K. A. Ward, and R. C. Marshall, editors. Chapman and Hall, London. 311–324.
37. Ruddle, F. H., and R. P. Creagan. 1975. Parasexual approaches to the genetics of man. *Annu. Rev. Genet.* 9:407–486.
38. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.
39. Steinert, P. M. 1990. 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.
40. Steinert, P. M., and D. R. Roop. 1988. Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.* 57:593–625.
41. Steinert, P., R. Zackroff, M. Aynardi-Whitman, and R. D. Goldman. 1982. Isolation and characterization of intermediate filaments. *Methods Cell Biol.* 24:399–419.
42. Steinert, P. M., R. H. Rice, D. R. Roop, B. L. Trus, and A. C. Steven. 1983. Complete amino acid sequence of a mouse epidermal keratin subunit: implications for the structure of intermediate filaments. *Nature (Lond.).* 302:794–800.
43. Steinert, P. M, D. A. D. Parry, W. W. Idler, L. D. Johnson, A. C. Steven, and D. R. Roop. 1985. Amino acid sequences of mouse and human epidermal Type II keratins of Mr 67,000 provide a systematic basis for the structural and functional diversity of the end domains of keratin intermediate filament subunits. *J. Biol. Chem.* 260:7142–7149.
44. Sun, T. T., R. Eichner, W. G. Nelson, A. Vidrich, and J. Woodcock-Mitchell. 1983. Keratin expression during normal epidermal differentiations. *In* Normal and Abnormal Epidermal Differentiation. M. Seiji and I. A. Bernstein, editors. University of Tokyo Press, Tokyo. 277–291.
45. Sun, T. T., R. Eichner, A. Schermer, D. Cooper, W. G. Nelson, and R. A. Weiss. 1984. Classification, expression and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. *In* The Transformed Phenotype. A. J. Levine, editor. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 169–176.
46. Tseng, S. C. G., M. J. Jarvinen, W. G. Nelson, J. W. Huang, J. Woodcock-Mitchell, and T. T. Sun. 1982. Correlation of specific keratins with different types of epithelial differentiation: monoclonal antibody studies. *Cell.* 30:361–372.
47. Vassar, R., P. A. Coulombe, L. Degenstein, K. Albers, and E. Fuchs. 1991. Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. *Cell.* 64:365–380.
48. Yu, D. W., S. Y. Pang, D. M. Checkla, I. M. Freedberg, T. T. Sun, and A. P. Bertolino. 1991. Transient expression of mouse hair keratins in transfected HeLa cells: interactions between "hard" and "soft" keratins. *J. Invest. Dermatol.* 97:354–363.
49. Yuspa, S. H. 1985. Cellular and molecular changes during chemical carcinogenesis in mouse skin cells. *In* Carcinogenesis. E. Huberman and S. H. Barr, editors. Raven Press, New York. 201–209.
50. Yuspa, S. H., and D. L. Morgan. 1981. Mouse skin cells resistant to terminal differentiation associated with initiation of carcinogenesis. *Nature (Lond.).* 293:72–74.