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Type I and type II keratins have evolved from lower eukaryotes to form the epidermal intermediate filaments in mammalian skin

(Southern hybridizations/immunoblot analysis/gene expression)

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ABSTRACT **We have traced the evolutionary origins of keratin-like sequences to the genomes of lower eukaryotes. The proteins encoded by these genes have evolved to form the intermediate filaments that comprise the backbone of vertebrate skin cells. Two related but distinct types of keratins encoded by two separate multigene subfamilies are expressed in the epidermal keratinocytes of vertebrate species from fish to human. Both at the level of protein and at the level of DNA, these two classes of keratins are coordinately conserved throughout vertebrate evolution, indicating the central role that both types of keratins must play in the assembly and structure of the 8-nm filament.**

The epidermal cells of all vertebrates contain a cytoplasmic network of 8-nm intermediate filaments (IFs) that, with microfilaments and microtubules, form the cytoskeletal architecture of these cells (1–6). The 8-nm filaments are especially abundant in mammalian skin, constituting 30% of the total protein of basal epidermal cells and almost 85% of the total protein of fully differentiated cells (stratum corneum). For each mammalian species, the filaments are comprised of a family of about 10–15 closely related fibrous proteins (M_r 40,000–70,000) called keratins (7–14). Not all of these keratins are ever synthesized simultaneously by any one epidermal cell. Basal cells make a subset of smaller keratins, whereas during the course of terminal differentiation, a new subset of larger keratins is synthesized (9, 15, 16). The functional significance of the differential expression of the keratins has not yet been elucidated.

Although the individual keratins are similar, separate mRNAs exist for each of these proteins (17, 18), and mRNA hybridization studies clearly indicate that multiple keratins represent polypeptides of unique, albeit similar, sequence (19, 20). Recently, we have cloned the cDNAs of the human epidermal keratins (19), and hybridization studies with these clones have revealed that the family of keratin mRNAs can be dissected into at least two distinct subfamilies, type I and type II. cDNA sequence analysis has shown that these two subfamilies are distantly related to each other (21–23). The type I keratins are acidic (pK_a 4.5–5.5) and are mostly of size M_r 35,000–55,000, whereas the type II keratins are more basic (pK_a 6.5–7.5) and typically of M_r 56,000–70,000 (24). All human epithelial cells at all stages of differentiation coordinately express mRNA sequences of both major types, suggesting the functional importance of both keratin subfamilies (25).

If these two types of keratins form the foundation for the structure of the epidermal 8-nm filament, then similar proteins should be present in all vertebrate epidermal keratinocytes because all of these cells have been shown to contain 8-nm filaments (2–5). In this paper, we examine the genomes of other eukaryotic species, including yeast, for the presence of sequences similar to the type I and the type II human keratin mRNAs. We also explore the nature of the proteins that are the likely products expressed by these genes.

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MATERIALS AND METHODS

Extraction of IF Proteins. The epidermis was separated from the dermis of fresh vertebrate skins by treatment with 2 M potassium bromide for 2 hr (8). Yeast *Saccharomyces* and *Drosophila* larvae were used directly. Keratins were extracted and purified essentially as described by Sun and Green (13).

Polyacrylamide Gel Electrophoresis and Binding of Anti-Keratin Antibodies to Gel-Fractionated Protein Bands. Samples containing 5 μg of total IF protein were resolved by polyacrylamide gel electrophoresis, and unstained gels were then transferred electrophoretically to nitrocellulose paper. Each blot was first placed in 50 ml of solution containing 50 μl of antiserum specific for human keratin, followed by a solution containing 10^5 cpm of ^{125}I-labeled *Staphylococcus* protein A (Amersham) per ml (26). Three different rabbit antisera were raised against keratins isolated from cultured human epidermal cells: (*i*) total keratins, (*ii*) M_r 50,000, and (*iii*) M_r 56,000.

RESULTS

The DNA from All Vertebrate Species Contain Two Distinct Classes of Keratin Genes. By using positive-hybridization and translation analysis, experiments from this laboratory have shown (25) that two cloned cDNAs for the human M_r 50,000 and M_r 56,000 keratin mRNAs hybridize most if not all type I and type II human keratin mRNAs, respectively. In addition, DNA sequences similar to type I and type II human keratin mRNAs have been found in the genomes of mouse, chicken, and hagfish, suggesting that homologous sequences may be present in all vertebrates (19).

We now have used these two cloned human keratin cDNAs to examine in more detail the genomes of other species for the presence of sequences homologous to the type I and the type II human keratin mRNAs (Fig. 1). DNAs from human, baboon, rat, mouse, rabbit, cow, chicken, turtle, perch, catfish, and yeast (Fig. 1, tracks 1–11, respectively) and frog and *Drosophila* (data not shown) were digested with *Eco*RI, an enzyme that does not cleave within either of the cloned cDNA sequences (19). The DNA was then subjected to agarose gel electrophoresis, transferred to nitrocellulose paper, and hybridized in duplicate with ^{32}P-labeled copies of the two cloned keratin cDNAs (27). The figure shows autoradiograms of the resultant blots. All vertebrate DNAs contained sequences that hybridized with radiolabeled type I (Fig. 1 *Left*) and type II (Fig. 1 *Right*) human keratin cDNA. For a given species, the DNA fragments recognized by the two keratin probes segregated into distinct and

Abbreviations: kb, kilobase(s); IF, intermediate filament.

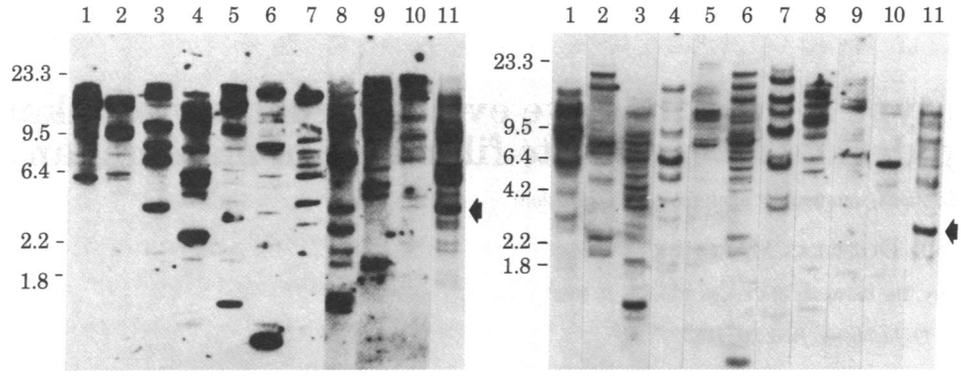

FIG. 1. Presence of sequences homologous to human keratin mRNAs in other eukaryotic genomes. DNAs (2–10 μg) were digested with restriction endonuclease *Eco*RI and resolved by agarose gel electrophoresis. After transferring to nitrocellulose paper (27), the DNA was hybridized (except as noted) under stringent conditions (0.75 M NaCl/50% formamide at 41°C) to a ^{32}P-labeled probe copied from the purified cloned cDNA inserts corresponding to the type I (*Left*) and type II (*Right*) keratin sequences. DNAs in tracks: 1, human; 2, baboon; 3, rat; 4, mouse; 5, rabbit; 6, bovine; 7, chicken; 8, turtle; 9, perch; 10, catfish; 11, yeast. The yeast DNA was hybridized under lowered conditions of stringency (0.9 M NaCl/15% formamide at 50°C). The arrow to the right of track 11 in each diagram indicates a single hybridizing band remaining in yeast DNA when the stringency was raised to 0.9 M NaCl/30% formamide at 50°C. Sizes are shown in kb.

largely nonoverlapping sets of sequences. For most mammalian DNAs, each set contained about 3–7 strongly hybridizing fragments.

Previously, we provided evidence that each of the human genomic fragments that hybridize with either keratin cDNA represent approximately 1–3 copies of intact genes, giving a value of about 4–10 genes for each of the two multigene families of keratins (19). Although it is not possible to accurately determine these numbers when human cDNA probes are hybridized with DNAs of other species, the number of hybridizing fragments suggests that there are multiple genes for both the type I and the type II keratins of mammals, birds, reptiles, amphibians, and possibly even fish.

DNA Sequences Distantly Related to the Human Keratin Genes Are Present in Lower Eukaryotic Organisms. When *Eco*RI-digested *Drosophila* and yeast DNAs were hybridized with ^{32}P-labeled human type I and type II keratin cDNA probes under standard conditions of stringency, no fragments were selected. However, when the conditions of stringency were lowered, 10–12 discrete bands of both genomic DNAs hybridized with both cDNA probes (Fig. 1, tracks 11, for yeast). These conditions of stringency were chosen because they had previously been used to identify the yeast tubulin genomic sequences with chicken α- and β-tubulin cDNA probes (28, 29). Whereas some of the hybridizing fragments seemed to be unique, others appeared to be overlapping. To determine which of these multiple yeast DNA fragments shared the greatest sequence homology with the type I and type II human keratin cDNAs, the blots were rehybridized under varying conditions of stringency. At an intermediate level of stringency, only a single band hybridized at about 3 kilobases (kb) with the type I keratin cDNA probe and 2.5 kb with the type II keratin cDNA probe (marked by the arrows in Fig. 1 *Left* and *Right*). These bands both showed strong hybridization under lowered conditions of stringency, but neither band hybridized at formamide concentrations in excess of 30%. Because of the low stringency conditions, interpretation of these results cannot be made unambiguously. The most likely cause for artifact is possible hybridization of yeast DNA sequences with the 20 G·C base pairs that flank the cloned keratin cDNAs and that were added enzymatically during the construction of the hybrid plasmids (19). This does not appear to be a problem since two different bands were selected by the two keratin cDNAs, even though both cDNAs have this sequence of G·C base pairs.

Recently, a yeast β-tubulin gene was identified by low stringency hybridization with a ^{32}P-labeled chicken β-tubulin cDNA probe (28). The predicted amino acid sequence of the cloned putative yeast tubulin gene showed 70% homology with chicken β-tubulin. Although this finding lends support for the validity of using reduced stringency to identify distantly related sequences, there is still a possibility that nonmeaningful homologies might exist between the human keratin cDNA sequences and the yeast genome.

Finally, although the relationship between the sequences of keratin and those of other IF proteins has not yet been examined for the lower eukaryotes, vimentin, desmin, and neurofilament proteins of mammals share about 30% homology with both types of human epidermal keratins (21, 22). Therefore, we can suggest only that the sequences selected here are likely to belong to the general family of IF proteins observed in the higher eukaryotes. Quite clearly, the cloning and sequencing of these DNA fragments will be necessary before any refined identification can be made.

All Vertebrate Epidermal IFs Are Comprised of a Family of Proteins of M_r 40,000–70,000. As likely candidates for the expressed products of these genes, the presumptive IF proteins of the skin of different vertebrates were investigated. As expected from earlier reports (30–35), the solubility properties of the IF proteins of lower vertebrates paralleled those of the higher vertebrates. IF proteins isolated from different vertebrates were resolved by $NaDodSO_4$ polyacrylamide gel electrophoresis (Fig. 2 *Left*). The patterns indicate that the IFs of all vertebrate epidermis are comprised of a heterogeneous mixture of about 2–10 polypeptides of M_r 40,000–70,000. We have shown (15) that this pattern of keratin polypeptides differs depending on the relative stage of differentiation of the epidermal cell.

For human epidermis, the commitment of basal cells (Fig. 2, track 2) to terminal differentiation and stratum corneum formation (track 1) seems to be correlated with increased synthesis of large keratins of M_r 63,000–67,000 (12, 15, 36). The total epidermal IF proteins of most other mammals (tracks 3–7) include similar, large polypeptides in addition to the smaller ones (M_r 40,000–60,000) typical of basal and spinous cells. It was unusual that the IF-protein profile of the hairless mouse epidermis (track 5) was different from that of normal mouse epidermis (track 4) in that fewer large keratins were produced by these mutant keratinocytes. This finding is consistent with ul-

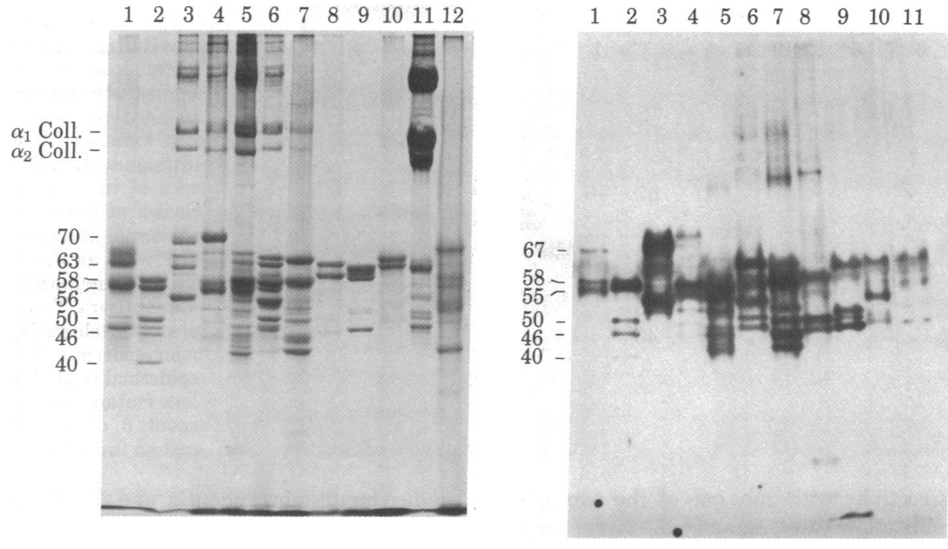

FIG. 2. IF proteins of vertebrate epidermis. Except as noted, IF proteins were extracted from the epidermis of different species. (*Left*) Coomassie blue-stained gel profile of the IF proteins. Tracks: 1, human stratum corneum; 2, cultured human basal epidermal cells; 3, rat; 4, mouse; 5, hairless mutant mouse; 6, rabbit; 7, bovine snout; 8, chicken; 9, turtle; 10, anole molt; 11, frog; 12, catfish fin. Urea/dithiothreitol protein extract from *Drosophila* larvae and yeast (not shown) showed faint bands in the M_r 40,000–70,000 range. M_rs are shown $\times 10^{-3}$; positions of contaminating proteins from the α_1 and α_2 chains of type I collagen are also indicated. The large amount of collagen in frog epidermis was due to incomplete separation of dermis and epidermis. (*Right*) Anti-keratin immunoblot of the gel profile of the IF proteins. A second polyacrylamide gel of the IF proteins was run, and the proteins were transferred electrophoretically from the unstained gel to nitrocellulose paper (26). The blot paper was hybridized first in a solution containing a 1:100 dilution of antiserum specific for human epidermal keratins and then in a solution of 10^5 cpm of ^{125}I-labeled *Staphylococcus aureus* protein A (26). Bands were visualized by autoradiography. IF proteins in tracks: 1, whole human epidermis; 2, cultured human basal epidermal cells; 3, rat; 4, mouse; 5, hairless mutant mouse; 6, rabbit; 7, bovine snout; 8, chicken; 9, turtle; 10, frog; 11, catfish fin. Total protein extracts of *Drosophila* larvae and yeast showed no immunoreactivity with anti-human keratin antisera.

trastructural studies indicating that differentiation and stratum corneum formation in the epidermis of these mutant mice is less pronounced.

The epidermal IF proteins of lower vertebrates (Fig. 2, tracks 8–12) were remarkably similar in their molecular weight and size heterogeneity to those of mammalian epidermis. Preliminary studies on the 8-nm filaments of avian (31, 35), reptilian (32, 34), and amphibian (33) epidermis had indicated more diverse families of IF polypeptides both in size and number. However, our results here show that even fish epidermis (track 12) produced a pattern of IF polypeptides within the size range of M_r 40,000–70,000, similar to that of human skin. This was initially surprising because the superficial cells of fish skin are in contact with an aqueous environment, and their extent of terminal differentiation and keratin filament formation has been thought to be substantially less than it is for reptiles, birds, and mammals (3, 6, 37).

When the procedure used to extract the IF proteins of vertebrates was used for *Drosophila* larvae and for yeast, only a small percentage (<1%) of the total protein of the cell was obtained. Proteins in the M_r 40,000–70,000 range were extracted, but these bands were minor and could not initially be identified (data not shown).

The Epidermal IF Proteins of Other Vertebrates Are Similar to Human Keratins. To determine whether the presumptive IF polypeptides of other vertebrate skin are related to the human keratins, we first examined their immunological crossreactivity with antiserum raised against purified keratin filaments from cultured human keratinocytes. This antiserum has been shown to be specific for all human keratin polypeptides (unpublished data).

Indirect immunoblot analysis (26) of gel-fractionated IF polypeptides allowed identification of proteins that shared immunoreactive determinants that were similar if not identical to those of the human epidermal keratins. All vertebrate IF polypeptides showed crossreactivity with antiserum against human keratin filaments (Fig. 2 *Right*). This effect was specific because no reactivity was observed when serum obtained prior to immunization was used in place of antiserum against keratin. In addition, no crossreactivity was observed between the antikeratin antibody and the marker proteins bovine serum albumin, actin, and collagen.

When the M_r 40,000–70,000 proteins extracted from *Drosophila* and yeast were tested for their immunoreactivity with antiserum against human keratin filaments, they showed no significant crossreactivity. Even when total protein extracts from these organisms were investigated, no polypeptides sharing antigenic determinants closely related to those of the human keratins could be identified. Thus, despite the detection of sequences homologous to the human keratin cDNAs in the genomes of lower eukaryotes, antibodies specific for human keratins did not show immunoreactivity with the M_r 40,000–70,000 IF proteins of these organisms.

The Keratins of All Vertebrates Can Be Dissected into Two Distinct Subfamilies. To determine whether keratins of both sequence types were represented in the IF proteins of other vertebrate epidermal keratinocytes, we raised antibodies against electrophoretically purified M_r 50,000 (type I) and M_r 56,000 (type II) human epidermal keratins. The two resulting anti-keratin antisera were shown to crossreact specifically with most type I and type II human keratins. Neither antiserum showed any crossreactivity with vimentin from human mesothelial cells (unpublished data). Immunoblot analysis using these antisera revealed that all vertebrate epidermis contain keratins of both type I and type II (Fig. 3). Most IF polypeptides of M_r 40,000–55,000 crossreacted with antiserum directed against the type I human keratins (Fig. 3 *Left*), whereas most IF polypeptides of M_r 56,000–70,000 crossreacted with antiserum directed against the type I human keratins (Fig. 3 *Right*). Similar to the human keratins (Fig. 3, tracks 1 and 2), most vertebrate keratins showed

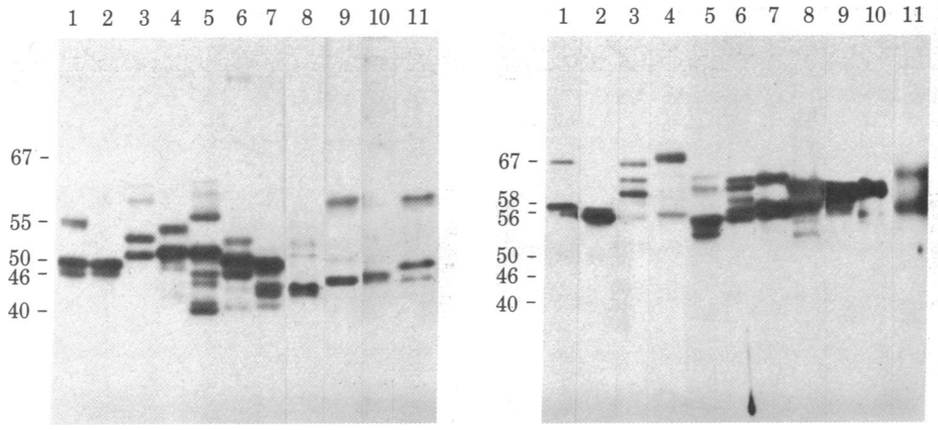

FIG. 3. Keratins of type I (*Left*) and type II (*Right*) are both present in all vertebrate epidermis. Antibodies were raised against electrophoretically purified M_r 50,000 (type I) and M_r 56,000 (type II) human keratins. These antisera were used in immunoblot analysis to detect the presence of immunoreactive forms of both classes of keratins in other vertebrate epidermal keratinocytes. (*Left*) Immunoblot with anti-type I keratin antisera. (*Right*) Immunoblot with anti-type II keratin antisera. Tracks with different samples of vertebrate IF proteins: 1, whole human epidermis; 2, cultured human basal epidermal cells; 3, rat; 4, mouse; 5, hairless mutant mouse; 6, rabbit; 7, bovine snout; 8, chicken; 9, turtle; 10, frog; 11, catfish fin. (M_rs are shown $\times 10^{-3}$.)

immunological crossreactivity with only one of the two antikeratin antisera. In only a few cases, some of the larger (most likely acidic) keratins showed weak immunoreactivity with the type I keratin antisera.

The Keratins of Other Vertebrates Have Similar Peptide Maps. It has been shown (14) that most human keratins give a characteristic and similar fragment pattern when they are subjected to limited proteolysis with *S. aureus* V8 protease followed by electrophoretic separation of the resulting fragments on 15% polyacrylamide gels (38). This pattern consists of 1 or 2 fragments of M_r 23,000–30,000, no fragments of M_r 14,000–23,000, and 3 or 4 fragments of M_r 8,000–12,500 (14). When electrophoretically purified keratins from human, cow, rat, and catfish were subjected to one-dimensional peptide map analysis, a striking similarity in pattern was observed across all species lines (Fig. 4). These results strongly confirm the evolutionary relatedness between the mammalian epidermal IF proteins and those of lower vertebrates.

DISCUSSION

The cytoskeletons of all eukaryotes contain at least two distinct components: 6-nm microfilaments, composed of β- and γ-actin, and 23-nm microtubules, composed of α- and β-tubulin. Throughout the course of vertebrate evolution, the protein subunits of these two structures have been remarkably conserved. The actin amino acid sequence of yeast *Saccharomyces cerevisiae* (40, 41), for instance, shares 85% homology with the sequences of bovine (42) and human (43) cytoplasmic actin. The amino acid sequence of chicken brain α-tubulin (44) differs from human epidermal α-tubulin in only 3 of 451 residues (45). Only in more primitive eukaryotic organisms—e.g., *Dictyostelium* and yeast—are differences in the tubulins substantial (29, 46). For both the actins and the tubulins, this high degree of conservation extends to the level of tissue-specific sequences within a single species. Thus, although the β- and γ-actins and the α- and β-tubulins are all encoded by multigene families whose expression varies with different stages of development and differentiation, the differences in the sequences of the individual members are of a minor nature, resulting only in microheterogeneities rather than major sequence differences (42, 45, 47, 48).

In higher eukaryotes, the cytoskeleton contains a third and more specialized component, the IF, whose diameter is 8–10 nm. In mammals, there are five tissue-specific classes of IF subunits: neurofilament proteins, glial filament proteins, desmins, vimentins, and keratins (for a review, see ref. 49). Although these proteins are clearly related, there are only 60–70% homologies among the partial amino acid sequences determined for desmin, vimentin, and glial filament proteins (50, 51) and even less (30%) homology between these three classes and a type I (21, 23) or a type II (22) human epidermal keratin. Within a class of IF proteins, in particular the keratins, additional and marked heterogeneities exist in the tissue-specific expression of the multiple genes for these subunits, indicating that the IF proteins are significantly more diverse and more specialized than are the actins or tubulins.

Although there is wide variation in the sequences of the different classes of IF proteins, we now have demonstrated that within a particular class (e.g., the keratins) phylogenetic conservation is pronounced. Thus, all keratins from fish to human crossreact immunologically with an antiserum raised against human keratin filaments, and yet this same antiserum does not crossreact with human vimentin. In fact, only a few reports have been made thus far in which an antibody against one IF showed crossreactivity with members of the other classes of IF proteins (52–54). In these cases, it is likely that the general IF antibodies recognize common structural domains rather than specific sequences *per se*.

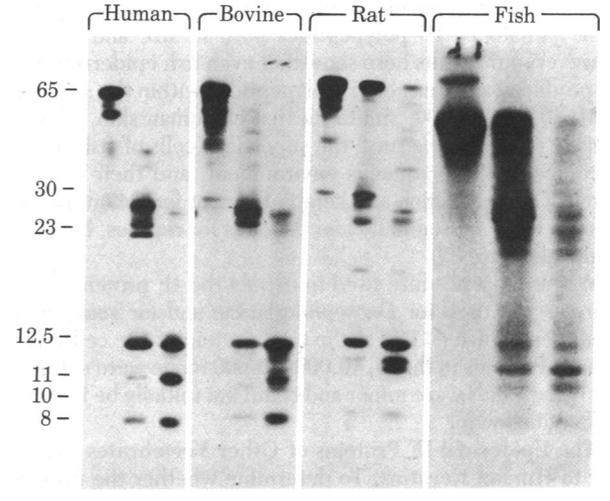

FIG. 4. Keratins of vertebrate epidermis: a comparison of the polypeptides produced from partial proteolytic digestion of the isolated keratins of human, bovine, rat, and fish skin. The lysine residues of extracted keratins were labeled *in vitro* with [^{14}C]formaldehyde (39), and the polypeptides were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis. Individual bands were excised, and each gel slice was placed in triplicate into adjacent sample wells of a 15% gel. *S. aureus* V8 protease was overlaid at amounts of 0, 20, and 300 ng, respectively, and the protease and protein were electrophoresed through the stacking and running gels (38). The M_rs of the proteolytic fragments are shown $\times 10^{-3}$.

In addition to the immunological and protein sequence similarities demonstrated for the keratin polypeptides of different species, genomic and mRNA sequence comparisons also indicate a close relationship for all vertebrate keratins. Moreover, these similarities at the nucleic acid level also appear to be greater across species lines for the keratins than they are for the different classes of IF proteins. Thus, although our specific cDNA probes to human type I and type II keratin mRNAs show strong hybridization with other vertebrate keratin mRNAs and DNAs, they show only weak hybridization with each other or with human vimentin mRNA. Collectively, our results suggest that the keratins are more closely related in different vertebrates than are the different IF proteins from various tissues of the same species.

The primordial origins of the two classes of keratins are still uncertain. At the level of protein and at the level of DNA, sequences similar to both the type I and type II keratins have been found in all vertebrate epidermis. Recently, ultrastructural and immunofluorescence studies have suggested that 7- to 10-nm intermediate filaments might be present in the cytoplasm of lower eukaryotes as well, including *Drosophila* (55, 56), *Tetrahymena* (57), and yeast (58). Our results also indicate that sequences distantly related to the human type I and type II keratin cDNAs may extend to the genomes of *Drosophila* and yeast. Determining whether these sequences actually represent intact type I and type II keratin genes that are expressed in the lower eukaryotes must await the cloning and sequence analysis of these weakly hybridizing DNA fragments.

In summary, type I and type II keratins appear to be similar to α- and β-tubulins in that they form two heterogeneous multigene subfamilies that are coordinately conserved at least throughout vertebrate evolution and possibly even lower. They are coordinately expressed in all vertebrate epidermal keratinocytes, emphasizing the pivotal role that both type I and type II keratins must play in the assembly of the 8-nm epidermal filament.

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