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Biochemical identification and tissue-specific expression patterns of keratins in the zebrafish *Danio rerio*

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Abstract We have identified a number of type I and type II keratins in the zebrafish *Danio rerio* by two-dimensional polyacrylamide gel electrophoresis, complementary keratin blot-binding assay and immunoblotting. These keratins range from 56 kDa to 46 kDa in molecular mass and from pH 6.6 to pH 5.2 in isoelectric point. Type II zebrafish keratins exhibit significantly higher molecular masses (56–52 kDa) compared with the type I keratins (50–48 kDa), but the isoelectric points show no significant difference between the two keratin subclasses (type II: pH 6.0–5.5; type I: pH 6.1–5.2). According to their occurrence in various zebrafish tissues, the identified keratins can be classified into "E" (epidermal) and "S" (simple epithelial) proteins. A panel of monoclonal anti-keratin antibodies has been used for immunoblotting of zebrafish cytoskeletal preparations and immunofluorescence microscopy of frozen tissue sections. These antibodies have revealed differential cytoplasmic expression of keratins; this not only includes epithelia, but also a variety of mesenchymally derived cells and tissues. Thus, previously detected fundamental differences in keratin expression patterns between higher vertebrates and a salmonid, the rainbow trout *Oncorhynchus mykiss*, also apply between vertebrates and the zebrafish, a cyprinid. However, in spite of notable similarities, trout and zebrafish keratins differ from each other in many details. The present data provide a firm basis from which the application of keratins as cell differentiation markers in the well-established genetic model organism, the zebrafish, can be developed.

Key words Fish · Zebrafish · Immunocytochemistry · Keratin · Cytoskeleton · *Danio rerio* (Teleostei)

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Introduction

In vertebrates, cell-specific architecture is maintained by a cytoskeleton consisting predominantly of actin filaments, microtubules, and intermediate filaments (IFs). IF proteins are encoded by a large multigene family and, in the human, include about 50 different proteins, most of which belong to the keratin class. Whereas other IFs are usually homopolymers from a single polypeptide chain, keratins are obligatory heteropolymers and arise by the association of heterodimers composed of two different keratin subclasses, currently referred to as type I and type II keratins (for reviews, see Fuchs and Weber 1994; Klymkowsky 1995). In mammals and birds, keratins are almost exclusively expressed in epithelia, whereas the type III IF protein vimentin is typical for mesenchymally derived cells; a comparable situation is found in the clawed toad *Xenopus laevis*, with the exception that, in adult amphibia, keratins are also expressed in endothelia (Jahn et al. 1987).

In a teleost fish, the rainbow trout *Oncorhynchus mykiss*, we have detected a different IF protein expression pattern, with keratins being present not only in epithelia, but also in many cells and tissues of mesenchymal origin (Markl and Franke 1988; Markl et al. 1989; Markl 1991). The expression of vimentin in trout is restricted to a comparatively small number of cell types (Herrmann et al. 1996). Moreover, data from other fish species, including hagfish and lamprey, indicate differences in IF protein expression programs between fish and terrestrial vertebrates (Alarcon et al. 1993; Glasgow et al. 1994; Arenas et al. 1995; Merrick et al. 1995; Zaccone et al. 1995; Groff et al. 1997). So far, however, many IF protein studies of fish have been limited to a single specialized organ or tissue (e.g., Pankov et al. 1986; Giordano et al. 1989; Frail et al. 1990; Druger et al. 1992; Cohen et al. 1993; Koch et al. 1994; De Guevara et al. 1994; Byrd and Brunjes 1995; Bodega et al. 1995; Cordeiro et al. 1996; Tsai 1996). Comprehensive immunocytochemical keratin surveys are available for several teleosts (e.g., Thompson et al. 1987; Bunton 1993; Ainis et al. 1995; Groff et al.

1997); however, these studies lack biochemical details of the IF proteins. Thus, it remains difficult to present a comprehensive view of the IF protein pattern in fish, although our previous work on the rainbow trout provides a reference model for the biochemistry and immunocytochemistry of fish IF proteins, notably of keratins and vimentin (Markl and Franke 1988; Markl et al. 1989; Herrmann et al. 1996). Our present study of the zebrafish, *Danio rerio*, aims to identify and characterize biochemically individual zebrafish keratins, to analyse their expression patterns, and to link this knowledge to the available trout data.

Materials and methods

Preparation of tissues and cytoskeletal proteins

Mature zebrafish (*Danio rerio*, also called *Brachydanio rerio*) from our laboratory tanks were killed by cutting the neck. For biochemical analysis, whole fish or individual organs were immediately used for the preparation of cytoskeletal proteins according to Achtstätter et al. (1986) and Markl and Franke (1988). For immunofluorescence microscopy, whole fish, isolated eyes, or skin were shock-frozen in isopentane that had been pre-cooled in liquid nitrogen; samples were then stored at –80°C.

Polyacrylamide gel electrophoresis and immunoblotting

Procedures for two-dimensional polyacrylamide gel electrophoresis (PAGE), with sodium dodecylsulfate (SDS) as the second dimension for electrophoresis, were performed as previously described by Achtstätter et al. (1986) and Markl et al. (1989). For the identification of proteins by immunoblotting, polypeptides separated by two-dimensional PAGE were electrotransfered to nitrocellulose membranes, according to the method of Herrmann and Wiche (1987) and exposed to primary antibodies. The alkaline phosphatase system of Promega (Serva, Heidelberg) was used for the detection of bound antibodies.

Complementary keratin blot-binding assay

Cytoskeletal proteins separated by two-dimensional PAGE were blot-transfered to nitrocellulose sheets and exposed for 2 h to 0.01 mg/ml purified human keratins 8 or 18, which bind specifically to the complementary keratin subtype (Hatzfeld et al. 1987). To obtain these probes, pure human keratins 8 or 18 (gifts from H. Herrmann and W.W. Franke) were biotinylated by using chemicals from Boehringer (Mannheim). For the detection of bound biotinylated keratin, the biotin-streptavidin antibody from Promega (Mannheim) was used as directed in the data sheet.

Immunofluorescence microscopy

Indirect immunofluorescence microscopy on 5-μm-thick cryostat sections was performed as described by Markl and Franke (1988). Texas-Red-conjugated goat secondary antibodies were obtained from Dianova (Hamburg).

Antibodies used to detect IFs

From a large antibody panel tested, we finally used six murine monoclonal antibodies raised against human keratins (AE1, C04=K_s18.04, KL1, Pan1–8.136, A45-B/B3, and LE64; for references, see Markl and Franke 1988). AE1, C04, and Pan1–8.136 were purchased from Progen (Heidelberg), and KL1 from Dianova (Hamburg); A45-B/B3 was a gift from P. Stosiek, LE64 was provided by B. Lane, and C10 by J. Bartek. We also used two murine monoclonal antibodies raised against *Xenopus* keratins (79.14 and 68.4: Fouquet 1991; both antibodies were provided by H. Herrmann). In addition, guinea pig antibodies raised in our laboratory against rainbow trout "S" keratins were applied. The following monoclonal antibodies were found to be unreactive to the zebrafish tissues tested here: AE3, K_k8.60, LU-5, 17.2, LE41, VIM 3B4, and DE-B5.

Results

Biochemical analysis of cytoskeletal preparations

Two-dimensional PAGE of cytoskeletal preparations from zebrafish whole body showed a variety of proteins, with molecular masses (M_r) between 55 and 49 kDa and isoelectric points (pI) between pH 6.1 and 5.2, which is well within the range of the values of IF proteins (Fig. 1A). By using biotinylated human keratin 18 in complementary keratin blot-binding (CKBB) assays, a subset of six of these proteins were strongly labeled, thus identifying them as type II keratins (Fig. 1B). *Vice versa*, a subset of four or five different proteins reacted in CKBB assays with biotinylated human keratin 8, which identifies type I keratins (Fig. 1C). Comparable results, but with a smaller number of detected components in the case of the type I keratins, were obtained in CKBB assays with cytoskeletal preparations from zebrafish skin (Fig. 1D–F). Since it can be expected that, in the latter preparations, epidermal keratins clearly predominate, the major skin cytoskeletal proteins were classified as "E" keratins (from "epidermal"; according to Markl et al. 1989). The most prominent epidermal type II keratin (55 kD, pI 5.9) and its two neighbors (pI 6.0, 5.8) were all designated as K1; they probably represent electrophoretic variants of the same keratin (Fig. 1B). The three remaining epidermal type II keratins were termed K2, K3, and K4 (Fig. 1B). The major epidermal type I keratin (50 kDa; pI 5.6) and its two neighbors (at pIs 5.7 and 5.5) were all designated as K10; in this case, we presumed that these were three electrophoretic variants of the same keratin (Fig. 1C).

In cytoskeletal preparations from zebrafish whole body, several components were visible, in addition to those detected in skin, notably a 49-kDa protein of pI 5.2, which was strongly reactive to human keratin 8 in CKBB assays (Fig. 1A, C). It was therefore considered as a type I "S" keratin (from "simple epithelial"; see Markl et al. 1989) and, after further investigations (see below), termed K18'.

A slightly different pattern was obtained from cytoskeletal preparations of zebrafish eye, with various minor and two major components (Fig. 1G). The latter could be identified as K1 (pI 5.9 variant) and K10 (pI 5.6 variant), respectively, by comparing the observed pIs and M_rs, in combination with co-electrophoresis (not shown) and immunoblotting (see below). Indeed, in CKBB assays, both components reacted similarly (Fig. 1H, I). In these exper-

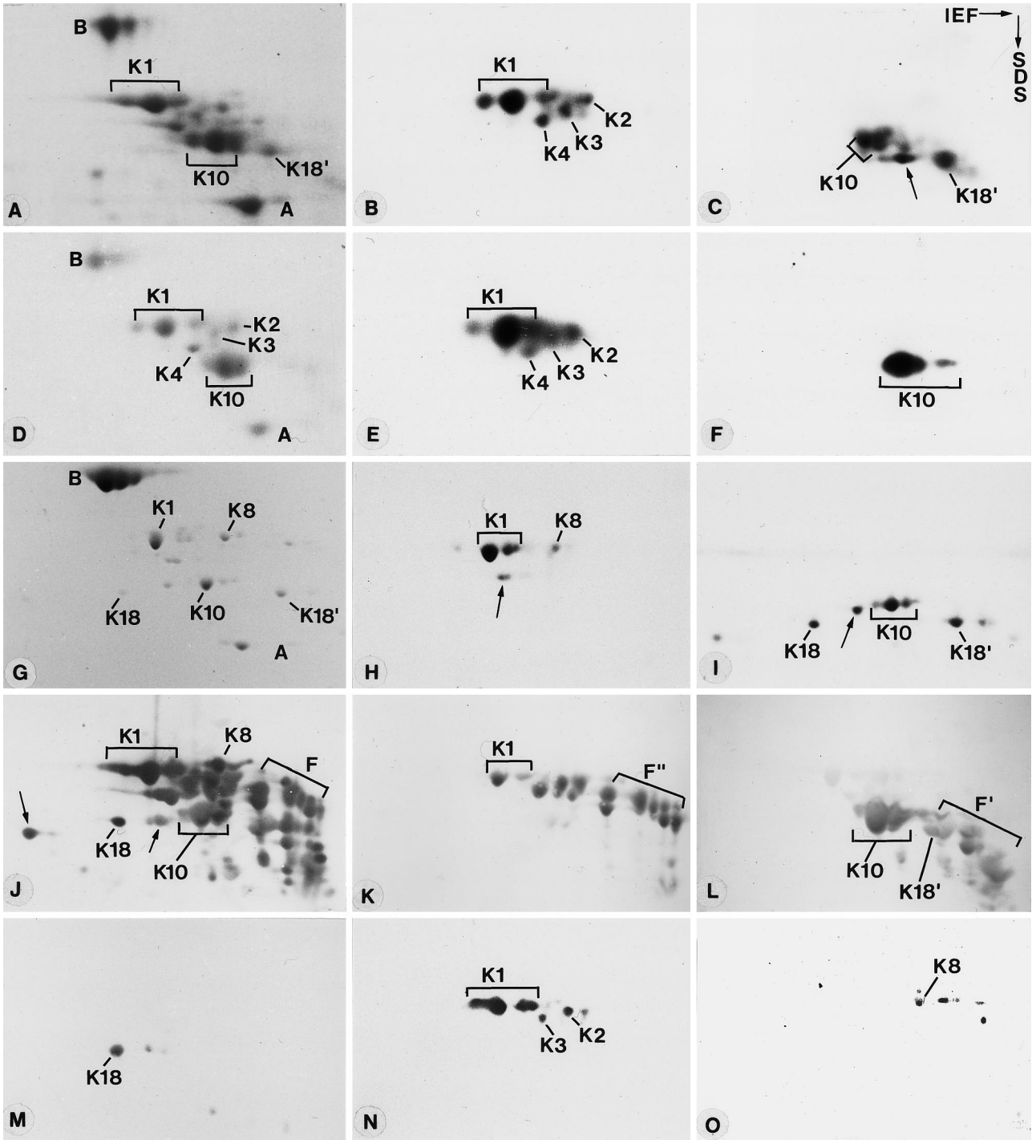


Fig. 1A–O Two-dimensional polyacrylamide gel electrophoreses (PAGE), complementary cytokeratin blot-binding (CKBB) assays, and immunoblots of cytoskeletal preparations from zebrafish. Isoelectric focusing (IEF) was applied in the first dimension, with SDS-PAGE in the second. **A–C, J–L** Whole-body cytoskeleton. **D–F** Skin cytoskeleton. **G–I, M–O** Eye cytoskeleton. **A, D, G** Coomassie-stained gels. **B, E, H** CKBB assays with human keratin 18 to label zebrafish type II keratins. **C, F, I** CKBB assays with human keratin 8 to label zebrafish type I keratins. **J–O** Immunoblots. **J** Anti-keratin guinea pig antibodies raised against trout "S" keratins, staining the entire panel of zebrafish "E" and "S" keratins; note that the large cluster of strongly stained acidic proteins (*F*) is barely visible in the corresponding Coomassie-stained gel shown in **A**; these trace proteins are considered to be keratin fragments. **K** Monoclonal antibody 79.14, here reacting with type II keratins and their putative fragments (*F″*). **L** Monoclonal antibody LE64 reacting with type I keratins and their putative breakdown products (*F′*). **M** Monoclonal antibody C04 specifically reacting with K18; the trace protein, which is also labeled, might be a phosphorylated variant of K18. **N** Monoclonal antibody 79.14 reacting with K1 and several trace proteins, but not with K8. **O** Monoclonal antibody C10 reacting with K8 and several trace proteins, but not with K1

Table 1 Biochemically identified keratins of the zebrafish

Keratin (identified by CKBB assays)	Molecular mass (Dalton)	Isoelectric point(s) (pH)	Sequence type	Expression type	Antibodies[a] (clearly reacting in immunoblots)
K1	55 000	6.0, 5.9, 5.8	II	E	KL1, 79.14
K2	54 000	5.6	II	E	KL1, 79.14
K3	53 000	5.7	II	E	KL1, 79.14
K4	52 000	5.8	II	E	KL1, 79.14
K8	56 000	5.5	II	S	C10
K10	50 000	5.7, 5.6, 5.5	I	E	LE64, 68.4
K18'	49 000	5.2	I	S	LE64, 68.4
K18	48 000	6.1	I	S	C04

[a] The entire panel of keratins was reactive to anti-(trout) keratin guinea pig antibodies (GP_{poly})

iments, in addition to K1, a 56-kDa protein (pI 5.5) was labeled with biotinylated human keratin 18; since it was absent in the skin preparations, it was classified as a type II "S" keratin and, on the basis of results described below, was termed K8 (Fig. 1H). *Vice versa*, in CKBB assays with biotinylated human keratin 8, proteins were also marked and identified as type I keratins; they were not observed in skin and were therefore classified as type I "S" keratins (Fig. 1I). One was identical with K18' (see above); according to the results described below, a second keratin (48 kDa, pI 6.1) was termed K18.

Guinea pig antibodies raised against rainbow trout keratins were used on blotted cytoskeletal preparations of zebrafish whole body and strongly labeled all the components identified as keratins by CKBB assays (Fig. 1J). In addition, numerous acidic proteins were stained that were barely visible in Coomassie-stained gels and that did not react in CKBB assays (see Fig. 1A–C); they most probably represented keratin degradation products. Monoclonal antibody KL1, which decorates a number of epidermal type II keratins in human, labeled the whole series of identified type II "E" keratins in zebrafish; a similar reaction pattern was obtained with monoclonal antibody 79.14 (Fig. 1K). In addition, both antibodies strongly stained a certain fraction of acidic keratin breakdown products, indicating that they stemmed from type II keratins (Fig. 1K). Both antibodies failed, however, to label the 56-kDa type II "S" keratin termed K8 (Fig. 1K). Monoclonal antibody LE64, which decorates type I keratins in human, labeled all K10 variants and the more acidic 49-kDa type I "S" keratin termed K18' in zebrafish (Fig. 1L). Moreover, a certain fraction of the acidic keratin degradation products was strongly decorated by LE64 (Fig. 1L), indicating that they stemmed from type I keratins. A similar reaction to that with LE64 was obtained with monoclonal antibody 68.4, but the immunoblot patterns produced by the latter antibody were much weaker and more diffuse (not shown).

Monoclonal antibody C04, which specifically labels the "S" keratin K18 or its orthologs in man, frog, and trout (see Markl et al. 1989), specifically stained, in immunoblots of cytoskeletal preparations from zebrafish whole body and from zebrafish eye, the 48-kDa type I "S" keratin of pI 6.1, which we consequently also desig-

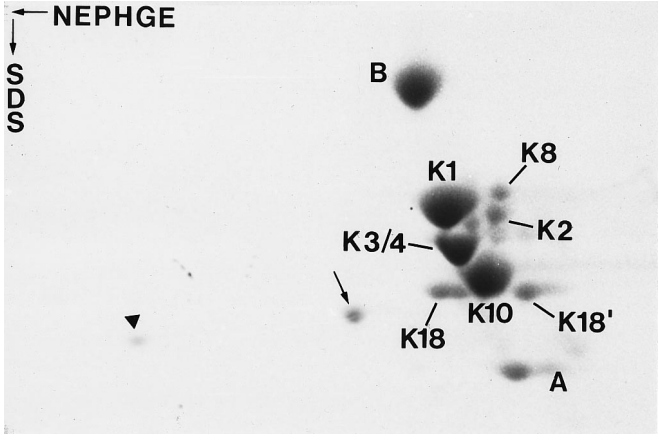
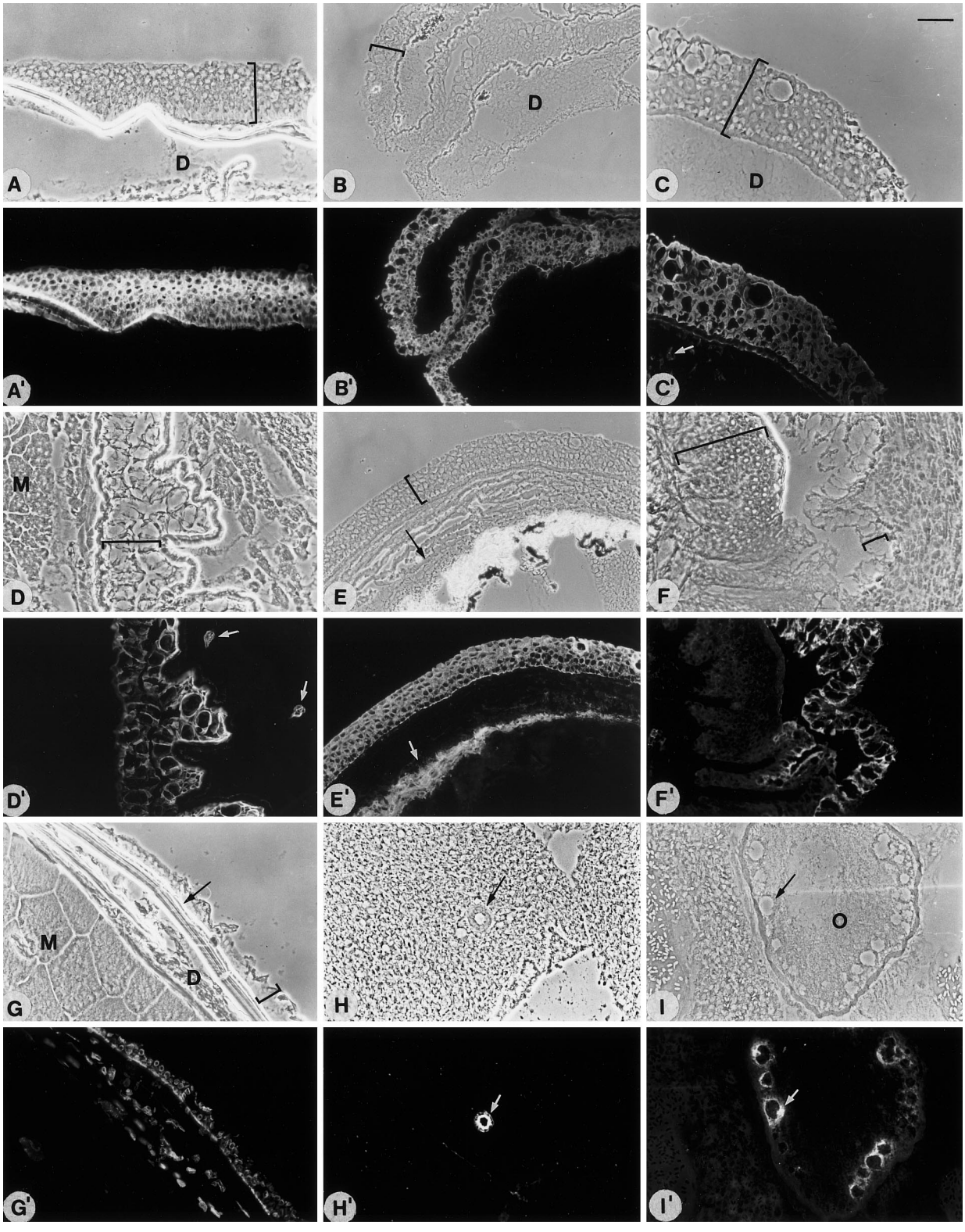


Fig. 2 Coomassie-stained two-dimensional PAGE of zebrafish whole body cytoskeleton. In the first dimension, non-equilibrium pH-gradient electrophoresis (*NEPHGE*) was applied, followed in the second dimension by SDS-PAGE (*SDS*). Only a single protein (*arrowhead*) could be detected, which from its comparatively basic pI value would be lost from gels such as that in Fig. 1A, in which IEF was applied. *B* Bovine serum albumin marker, *A* actin marker, *arrow* unidentified cytoskeletal protein with positive reaction toward guinea pig anti-keratin antibodies (see Fig. 1J)

Fig. 3 Immunofluorescence microscopy of cryostat sections of frozen zebrafish tissues (**A–I** phase-contrast optics, **A'–I'** epifluorescence optics). **A, A'** Antibody 79.14 on skin tissue, exclusively decorating the epidermis (*bracket*). **B, B'** Antibody KL1 on skin tissue, specifically reacting with epidermal cells (*bracket*). **C, C'** Antibody 68.4 on skin tissue, recognizing epidermal (*bracket*) and dermal cells (*arrow*). **D, D'** Antibody 68.4 reacting with epithelial cells of the pharyngeal mucosa (*bracket*); note the positive reaction of single interstitial cells (*arrows*). **E, E'** Antibody LE64 reacting with the corneal epithelium (*bracket*) and endothelium (*arrow*). **F, F'** Antibody AE1 reacting with the simple epithelium (*small bracket*) of the esophageal mucosa; note that the stratified epithelium of esophagus (*large bracket*) is not decorated. **G, G'** Reaction of antibody Pan1–8.136 on skin tissue; note the strong reaction with the epidermis (*bracket*) and dermal interstitial cells; the reaction of scale-associated cells (*arrow*) remains unclear (⌊); *D* dermis, *M* skeletal muscle (⌈). **H, H'** Antibody A45-B/B3 on liver tissue, showing highly selective reaction with the bile duct epithelium (*arrow*). **I, I'** Antibody 79.14 on ovary, with specific staining of the lining of egg yolk granules (*arrow*) (⌈). *O* Oocyte (⌊). *Bar* (in **C**) 10 μm



nated as K18 (Fig. 1M). In the case of zebrafish eye, antibody 79.14 showed a strong reaction with K1 and several other type II "E" keratins (and with several more acidic trace products) but was negative with type II "S" keratin K8 (Fig. 1N). However, monoclonal antibody C10, which is specific for K8 in human (J. Bartek, personal communication) and which specifically stains the putative K8 correlate in trout (Markl et al. 1989), reacted with the 56 kDa protein and several trace components in its vicinity, but not with "E" keratins in zebrafish eye (Fig. 1O).

Table 2 Reactivity patterns of various murine monoclonal anti-keratin antibodies and guinea pig polyclonal anti-keratin antibodies (GP_{poly}) with tissues of the zebrafish as deduced from immunofluorescence microscopy on frozen sections. Reaction patterns of scale-associated cells, spermatids, spermatozoa and lining of the respiratory lamellae remained unclear because of their minute size (# most smooth muscles were negative but a few showed a weak but significant positive reaction, + intense staining, (+) weak but significant staining, – no significant staining)

Tissues	Antibodies									
	68.4	LE64	KL1	79.14	C10	C04	Pan1–8.136	AE1	A45-B/B3	GP poly
Stratified epithelia										
Epidermis	+	+	+	+	–	–	+	+	+	+
Pharyngeal mucosa	+	+	+	+	–	–	+	+	(+)	+
Mucosa of anterior esophagus	+	+	+	+	–	–	+	+	(+)	+
Gill mucosae	+	(+)	+	+	–	–	–	–	–	+
Cornea	+	+	+	+	–	–	–	–	–	+
Simple epithelia										
Intestinal mucosa	+	(+)	–	–	+	+	+	+	+	+
Bile duct cells	+	+	–	–	+	+	+	–	+	+
Hepatocytes	+	–	–	–	+	+	+	–	–	+
Lining of renal tubules	+	–	–	–	+	+	–	–	(+)	+
Endothelia	+	+	–	–	+	+	–	(+)	–	+
Ocular lens epithelium	+	–	–	–	+	+	–	–	–	+
Ocular lens fibers	–	–	–	–	–	–	–	–	–	–
Non-epithelial tissues and cells										
Pillar cells of gills	–	–	–	–	+	+	–	–	–	+
Chondrocytes	+	+	–	–	+	+	–	+	–	+
Interstitial cells / fibroblasts	+	+	–	–	+	+	–	–	–	+
Optic nerve glial cells	+	–	–	–	+	+	–	–	–	+
Smooth muscles	#	#	–	–	#	#	#	–	–	#
Skeleton muscles	–	–	–	–	–	–	–	–	–	–
Heart muscle	–	–	–	–	–	–	–	–	–	–
Blood cells	–	–	–	–	–	–	–	–	–	–
Neurons	–	–	–	–	–	–	–	–	–	–
Ovarial theca cells	+	+	–	–	+	+	+	+	–	+
Lining of egg yolk granules	–	–	–	+	–	–	–	–	–	+

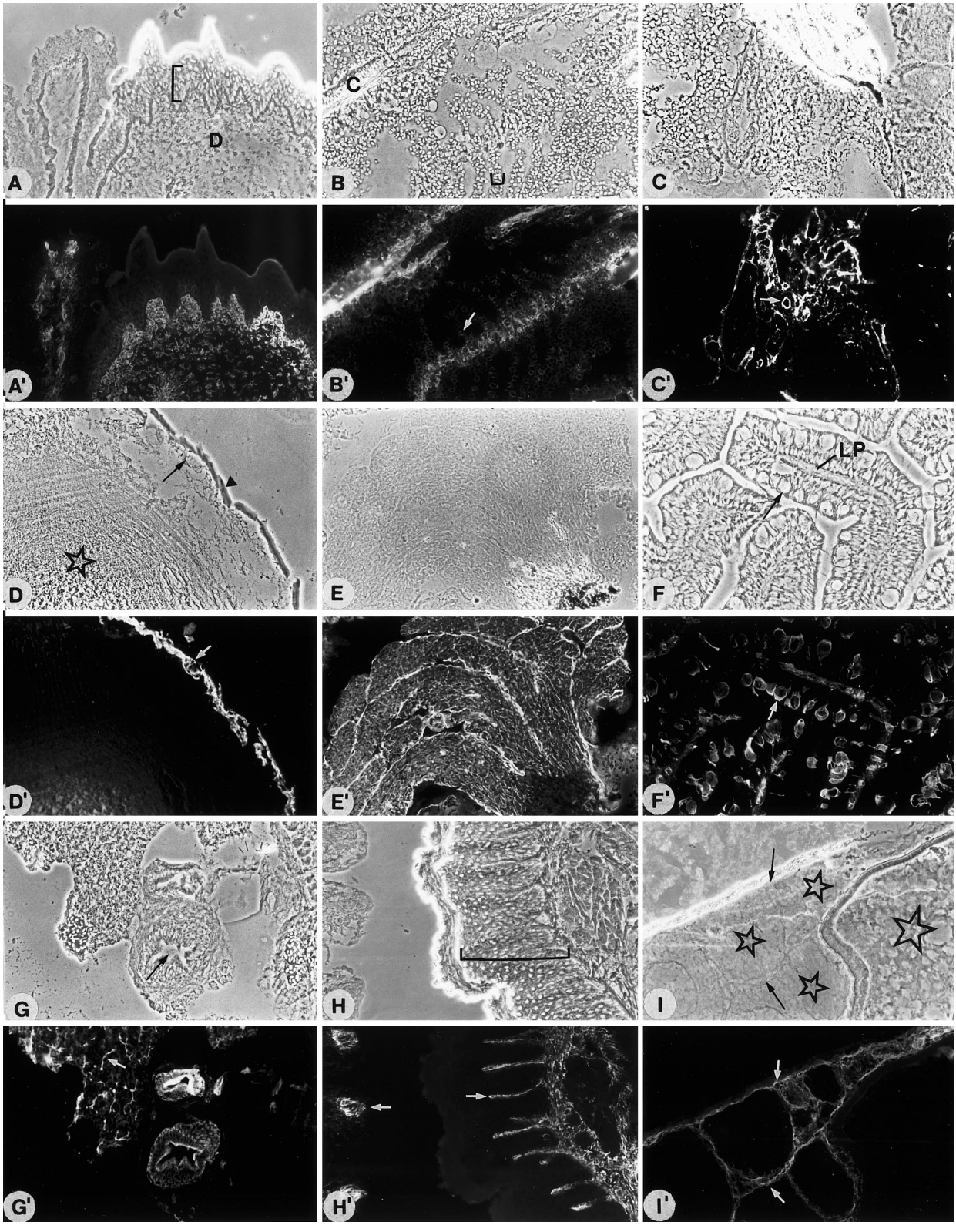
Antibodies Pan1–8.136, AE1, and A45-B/B3, which strongly stained zebrafish tissues in immunofluorescence microscopy (see below), reacted poorly in our immunoblots (data not shown), in spite of many attempts. The biochemical results are summarized in Table 1.

Two-dimensional non-equilibrium pH-gradient electrophoresis of cytoskeletal preparations from zebrafish whole body revealed that most of the proteins were in the range of our molecular markers bovine serum albumin and actin. Only two components were detected with a more basic pI; these may have been additional keratins (Fig. 2).

Immunofluorescence microscopy

Immunofluorescence microscopy of zebrafish frozen tissue sections indicated that antibodies 79.14 and KL1 reacted specifically and exclusively with stratified epithelia, including epidermis (Fig. 3A, A', B, B'), corneal epithelium, gill mucosa, and pharyngeal and stratified esophageal epithelium (Table 2). A similar reaction with stratified epithelia was shown by monoclonal antibody 68.4 (Fig. 3C, C', D, D'), which was originally raised against *Xenopus* type I keratins (Fouquet 1991). However, in contrast to KL1 and 79.14, antibody 68.4 also labeled the simple epithelium of the intestinal mucosa, hepatocytes, and bile duct cells (Table 2). Moreover, it stained a variety of cells and tissues of mesenchymal origin, including

Fig. 4 Immunofluorescence microscopy of cryostat sections of various zebrafish tissues with monoclonal antibody C04 (**A–I** phase-contrast optics, **A'–I'** epifluorescence optics). **A, A'** Skin; note the negative reaction of epidermal cells (*bracket*) and the positive reaction of dermal cells (⌈). *D* Dermis (⌈). **B, B'** Gill tissue, with decoration of pillar cells (*arrow*) in secondary lamellae (*bracket*) and chondrocytes (*C*). **C, C'** Kidney, with the linings of renal tubules having been stained, notably on their luminal side (*arrow*). **D, D'** Ocular lens, with specific decoration of the lens epithelium (*arrow*), and a negative reaction of lens capsule (*arrowhead*) and lens tissue (*star*). **E, E'** Optic nerve, showing labeling of astrocytes and other glial elements. **F, F'** Intestine, with selective decoration of mucous cells (*arrows*) and of cells of the lamina propria (*LP*). **G, G'** Liver, with staining of the luminal portion of bile duct cells (*black arrow*) and the lining of bile canaliculi (*white arrow* in **G'**). **H, H'** Esophagus, with rich decoration of lamina propria cells (*arrow*), and a negative reaction on the stratified epithelium (*bracket*). **I, I'** Ovary, with follicle cells (*arrows*) selectively labeled, whereas egg cells (*star*) are negative. *Bar* (in **C**) 10 μm ▶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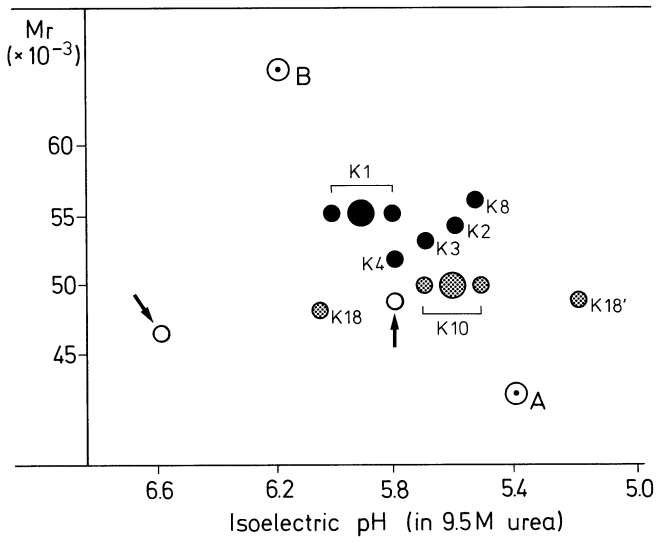

Fig. 5 Schematic catalog of type II (*filled circles*) and type I (*shaded circles*) keratins of the zebrafish, arranged according to their position after separation by two-dimensional PAGE. *K1–K4* type II keratins are exclusively expressed in stratified epithelia ("E" keratins). The three *K10* polypeptides are type I "E" keratins. According to their relative amounts observed in cytoskeletal preparations of zebrafish eye (see Fig. 1G), *K1* and *K10* are probably a heterologous pair in native keratin filaments. *K8* and *K18* are exclusively expressed in simple epithelia and non-epithelial tissues and therefore considered as "S" keratins; they could be heterologous partners in filament formation. An additional positively identified type I "S" keratin is *K18′*. Two further keratin candidates are indicated by *open circles* and *arrows*. *Open circles* with a *central dot* denote reference proteins: actin (*A*) and bovine serum albumin (*B*)

dermal interstitial cells (Fig. 3C, C'), blood vessel endothelia, and chondrocytes, but not pillar cells (Table 2). In a similar manner, monoclonal antibody LE64 gave strong staining with stratified and simple epithelia (Fig. 3E, E'), and also with some mesenchymally derived tissues (Table 2). Differential immunostaining was also obtained with antibodies AE1, Pan1–8.136, and A45-B/B3 (Fig. 3F–H'); the results are summarized in Table 2. Interestingly, antibody 79.14, which otherwise in zebrafish proved to be specific for stratified epithelia, selectively labeled the wall of the yolk granules in egg cells (Fig. 3I, I').

Antibody C04 reaction was found to be negative throughout the stratified epithelia of zebrafish (Fig. 4A, A'). However, it stained a variety of mesenchymal cells, including interstitial cells of the dermis (Fig. 4A, A'), pillar cells and chondrocytes in the gill (Fig. 4B, B'), and endothelial cells (Table 2). C04 was also reactive to renal tubules (Fig. 4C, C'), ocular lens epithelium (Fig. 3D, D'), and glial elements of the optic nerve (Fig. 4E, E'). In the intestinal mucosal epithelium, this antibody specifically reacted with mucous cells (Fig. 4F, F'); it also stained hepatocytes and bile duct cells (Fig. 4G, G'). In addition, it marked interstitial cells of the lamina propria; this was particularly evident in tissue sections of the esophagus (Fig. 4H, H'). In zebrafish ovary, antibody C04 stained theca cells (Fig. 4I, I'); this was also observed with antibodies LE64, 68.4, Pan1–8.136, and AE1 (Table 2). The reactions of antibody C10 corresponded essentially to that of C04 (Table 2).

Discussion

Zebrafish type I and type II keratins

The present study biochemically identifies a number of individual keratin proteins in the zebrafish *Danio rerio*. They were detected by two-dimensional PAGE and positively identified as keratins by their specific reaction in CKBB assays. This test system is particularly useful in that it is based on the ability of keratins to form heterodimers with members of the complementary subclass (Hatzfeld et al. 1987). Therefore, this assay not only unequivocally identifies keratins, but also groups them into sequence type I and type II, based on their specific heterodimer association (Fig. 5, Table 1). As an additional advantage, keratin fragments are not labeled in our CKBB assays; apparently, they fail to dimerize and can therefore be distinguished from intact keratin polypeptides (cf. Fig. 1A–C with Fig. 1J–L). CKBB assays do not allow one to judge structural relationships of the marked keratins, but the latter can be deduced from immunoblotting with specific antibodies. Four of the monoclonal antibodies used in the present study were not found to react mono-specifically but labeled several keratin polypeptides (see Table 1). In cases where such polypeptides show, in addition, a similar M_r, they may represent a genuine keratin and its phosphorylated variants rather than several different translation products. As a consequence, our designations K1 and K10 each include three polypeptides, the structural relationship of which has still to be clarified.

The M_rs and pIs of the zebrafish keratins (Fig. 5, Table 1) are well within the range of keratins found in goldfish optic nerve (e.g., Giordano et al. 1989), loach epidermis (Tsai 1996), mosquito fish testis (Arenas et al. 1995), rainbow trout liver (Pankov et al. 1986), and rainbow trout epidermis (Markl et al. 1989). On average, type II keratins in zebrafish have higher M_rs than type I keratins (Fig. 5, Table 1), corresponding to the situation found in terrestrial vertebrates and in trout. In contrast, charge differences between these two IF protein subclasses are not significant in zebrafish (Fig. 5), and this resembles the situation found in the rainbow trout (Markl et al. 1989) but not that in mammals. In the latter, because of their pI range, type I keratins are generally referred to as "acidic", whereas type II keratins are termed "basic" (Moll et al. 1982), a terminology that is apparently not so useful in fish. For example zebrafish K18, a type I keratin and putative correlate of human keratin 18 (see below), shows a pI of 6.1, which is more basic than virtually all identified type II keratins (Fig. 5). As in trout keratins (documented by Markl et al. 1989), the M_r and pI ranges of zebrafish keratins observed in two-dimensional PAGE are narrow; in contrast, mammalian keratins exhibit much broader

electrophoretic ranges (for a direct comparison of the keratin catalogs of trout and man, see Fig. 1 in Markl 1991).

Keratin expression in mesenchymal tissues

Using various monoclonal anti-keratin antibodies, we have obtained differential immunostaining of zebrafish cell types and tissues. Notably, keratin expression has been detected not only in epithelia, but also in a variety of mesenchymally derived cells and tissues; this demonstrates fundamental differences from mammals in keratin expression, as previously documented in the rainbow trout (see Markl and Franke 1988). The Cypriniformes and the Salmoniformes are distant phylogenetically and became separated within the teleost lineage about 260 million years ago, in the Permian period (see, for example, Gould 1993). However, such variations between the various vertebrate classes do not necessarily become apparent when single organs are interspecifically compared; for example, Byrd and Brunjes (1995) have found that, in zebrafish olfactory bulb, two type III IF proteins, namely glial fibrillary acidic protein and vimentin, have distributions similar to those in the olfactory bulbs of other vertebrate classes. On the other hand, Schechter and his group have demonstrated (e.g., Giordano et al. 1989, 1990; Druger et al. 1992, 1994; Glasgow et al. 1994) that the IF components of the goldfish optic nerve cytoskeleton are different from those found in optic nerves of terrestrial vertebrates (for a review, see Markl and Schechter 1998). Recently, Groff et al. (1997) have published a careful immunocytochemical survey of the keratin expression of another cyprinid, the common carp *Cyprinus carpio*. Although direct comparison with the situation in zebrafish and trout is limited, because of the different tissue fixation procedures and monoclonal antibodies used in the carp, it is clear that, in this teleost as in zebrafish and trout, keratins are expressed in a variety of mesenchymal tissues, including scale-associated cells, fibroblasts, mesothelia, and adipocytes (Groff et al. 1997).

Zebrafish “E” and “S” keratins

In spite of many differences, there are also marked similarities between the keratin systems of fish and terrestrial vertebrates. For example, in all the vertebrates investigated so far, epidermal cells and simple epithelia differ in their immunoreactivity toward anti-keratin antibodies; this is based on two different subsets of keratins being expressed in these tissues (e.g., Moll et al. 1982). Moreover, in the trout, these two subsets have been clearly defined (and termed “E” and “S” keratins, respectively: see Markl et al. 1989). In zebrafish, KL1 and 79.14 exclusively immunostain the epidermis and other stratified epithelia, which indicates that these antibodies specifically react with “E” keratins. Indeed, keratins K1–K4, which have been identified biochemically in CKBB assays as type II polypeptides (Fig. 1E), specifically react with antibodies KL1 and 79.14 in immunoblots (Table 1). In a similar manner, K10 has been identified as type I “E” keratin by CKBB assays (see Fig. 1F) and is labeled by antibody LE 64, which on the other hand reacts strongly with zebrafish stratified epithelia (Table 2). From their relative proportions in cytoskeletal preparations from the eye, K1 and K10 may represent a heterologous keratin pair in IF formation (see Fig. 1G).

Since in addition to labeling stratified epithelia, antibody LE64 marks simple epithelia and various mesenchymal tissues (see Table 2), a reaction with at least one “S” keratin is also suggested. This is apparently K18' (see Fig. 1L). Another zebrafish type I “S” keratin is certainly K18 (48 kDa, pI 6.1), as deduced from its absence in zebrafish epidermis (see Fig. 1D–F) and its selective reaction with antibody C04 (the latter is negative on stratified epithelia; see Table 2). Moreover, C04 is specific for the K18 correlate in mammals, frog, and trout (see Markl et al. 1989). The heterologous partner of K18 and K18' is most probably K8 (56 kDa, pI 5.5), because this type II keratin is unreactive to antibodies KL1 and 79.14 in immunoblots (see Fig. 1K); type II keratins labeled by KL1 or 79.14 can be excluded as candidates, because these antibodies are negative on tissues stained by C04 (see Table 2). Finally, K8 is stained by antibody C10 (Fig. 1O), which is a marker for human K8 and its putative correlate in trout (see Markl et al. 1989). Indeed, by immunofluorescence microscopy, C10 shows a staining pattern comparable to that of C04 (see Table 2). Furthermore, from their relative proportions in cytoskeletal preparations of zebrafish eye, K8 could be the partner of both K18 and K18' for filament formation (see Fig. 1G).

Correspondence between zebrafish K8/K18 and goldfish optic nerve keratins

Two proteins of 58 kDa and 48 kDa (termed ON_3 and GK48) have been detected in optic nerve cytoskeletal preparations from the goldfish (*Carassius auratus*), which like the zebrafish is a cyprinid; these proteins react with antibodies against human keratins 8 and 18, respectively (Giordano et al. 1990). Their sequence as predicted from cDNA clones has indeed identified them as structural correlates of human K8 and K18, respectively (Giordano et al. 1989; Druger et al. 1994). Their electrophoretic position in two-dimensional PAGE, their selective reactions with antibodies C10 and C04 in immunoblots, and the reaction patterns of these antibodies on zebrafish tissues (Table 2) strongly suggest that zebrafish K8 and K18 correspond to goldfish ON_3 and GK48, respectively. Indeed, Asch et al. (1998) describe, in zebrafish optic nerve, two keratins that electrophoretically resemble K8 and K18, as identified in the present paper, and goldfish ON_3 and GK48 (for a comprehensive review of fish keratins and other fish IF proteins, with a discussion of IF protein evolution, see Markl and Schechter 1998).

Anti-keratin antibodies as cell type markers in zebrafish

With respect to the use of keratins as cell differentiation markers in zebrafish, tissues expressing either "E" or "S" keratins can be selectively stained by using the commercially available monoclonal antibodies KL1 and C04; broad-range staining of all cell types expressing keratins is also possible by simply applying a cocktail of both antibodies. However, prior to such experiments, it should be verified that results in controls correspond to those in Table 2, because there are several possible restrictions if different analytical procedures or antibodies other than those introduced in the present paper are applied: when using Bouin's fixative or formalin fixation of tissue sections of medaka (*Oryzias latipes*) and striped bass (*Morone saxatilis*), Bunton (1993) found keratin expression exclusively in epithelia when she applied monoclonal antibodies AE1/AE3, whereas in trout frozen tissue sections, AE3 was also reactive to a variety of mesenchymal tissues (Markl and Franke 1988). In carp, an AE1/AE3 mixture has been found to stain mesenchymal tissues fixed with ethanol and methacarn; however, in this case, skeletal muscle tissue also reacts (Groff et al. 1997). Are such differences species-specific, or do they arise from the different tissue fixation procedures or from varying antibody concentrations? (1) We have found, in preliminary experiments, that immunocytochemistry with zebrafish tissues fixed in Bouin's solution does not produce the same results as presented in Table 2, indicating that the fixation method is a crucial point. (2) We have also observed that the concentration of the primary antibodies is critical, and from commercial sources, they are usually diluted to suit application to mammalian tissues; in some cases, they might be too dilute for fish tissues. (3) Comparing the results obtained from trout and zebrafish on cryostat tissue sections, antibodies AE3 and LU-5, which show strong staining patterns in trout, have been found to be completely negative in zebrafish. A more puzzling result is the specific reaction of antibody 79.14 on zebrafish stratified epithelia (Table 2), because this antibody exclusively stains simple epithelia and certain non-epithelial tissues in trout (our unpublished data). Moreover, in *Xenopus*, antibody 79.14 labels a structural equivalent of human keratin 8 (Fouquet 1991), as is the case in trout (our unpublished data), whereas in zebrafish, it marks the whole panel of type II "E" keratins but not K8 (Fig. 1K). This indicates fundamental species-specific differences between the keratin systems of different teleosts and underlines the advantages of a comparative compilation. Moreover, it stresses the importance of a combined biochemical and immunocytochemical approach, as presented here.

After submitting the present paper, we learnt that Imboden et al. (1997) have recently sequenced a zebrafish cDNA coding for a protein related to human K8, but which, however, is expressed in oocytes and in the epidermis. From this, it appears that the sequenced protein is one of the type II "E" keratins recognized by antibody 79.14 rather then zebrafish "S" keratin K8 recognized by antibody C10, because only the former but not the latter antibody stains oocytes and epidermal cells (see Table 2). If Imboden et al. (1997) have indeed sequenced one of the type II "E" keratins identified in the present study, its structural similarity to K8 orthologs would shed new light on the evolution of the keratin system in fish.

In conclusion, the broad application of the IF protein system in zebrafish requires screening of larger monoclonal antibody panels with different tissue fixation procedures, for which the present study provides a firm basis. Ultimately, striking similarities but also marked differences can be expected from future surveys of fish species, particularly between members from phylogenetically distant families of this enormously diverse class of aquatic animals.

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