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Translational products of mRNAs coding for non-epidermal cytokeratins

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Total RNA and poly(A)$^+$ RNA were isolated from tissues and cultured cells of various mammalian species (bovine muzzle epidermis and bladder urothelium; rat hepatoma cells; human cell lines HeLa, MCF-7 and A-431) and examined by translation *in vitro* using the reticulocyte lysate system. Polypeptides were separated and identified by two-dimensional electrophoresis and cytokeratins were selectively enriched from the translation assays by co-polymerization with added heterologous cytokeratins. In all three species, non-epidermal cytokeratins A, D and mol. wt. 40 000 (corresponding to numbers 8, 18 and 19 of the human cytokeratin catalog of Moll *et al.*, 1982) were identified as translation products capable of co-polymerization with epidermal keratins. Several other basic and other acidic cytokeratins were also identified as translational products. In addition, two unidentified polypeptides (mol. wt. 52 000 and 43 000) which were minor polypeptides in cytoskeletons and translation assays were found to be specifically enriched in co-polymers with bovine epidermal keratins. The results indicate that many, perhaps all, non-epidermal cytokeratins characteristic of simple epithelia are genuine products of translation and that their diversity is not due to post-translational modification or processing. These findings, taken together with observations of *in vitro* translation of epidermal mRNAs, suggest that the diversity of cell type-specific expression of the different members of the cytokeratin polypeptide family is largely due to the cell type-specific synthesis of diverse mRNAs.

Key words: cytokeratins/translation/intermediate filaments

Introduction

The protein constituents of intermediate-sized (7 – 11 nm) filaments are represented by a class of distinct polypeptides which show some homologies and common structural features (Steinert *et al.*, 1980; Geisler and Weber, 1981, 1982; Lazarides, 1982; Osborn *et al.*, 1982) but, on the other hand, can be distinguished by biochemical and immunological criteria (Davison *et al.*, 1977; Bennett *et al.*, 1978; Franke *et al.*, 1978a, 1982). While some types of intermediate-sized filaments contain only one type of polypeptide such as vimentin, desmin, or glial filament protein (GFP), others reveal two (vimentin heteropolymers with either GFP or desmin) or three (neurofilament polypeptides; Liem *et al.*, 1982; Steinert *et al.*, 1982; Quinlan and Franke, 1982, 1983; Sharp *et al.*, 1982). Cytokeratins, however, are a much more complex family of numerous polypeptides (at least 19 in human cells)

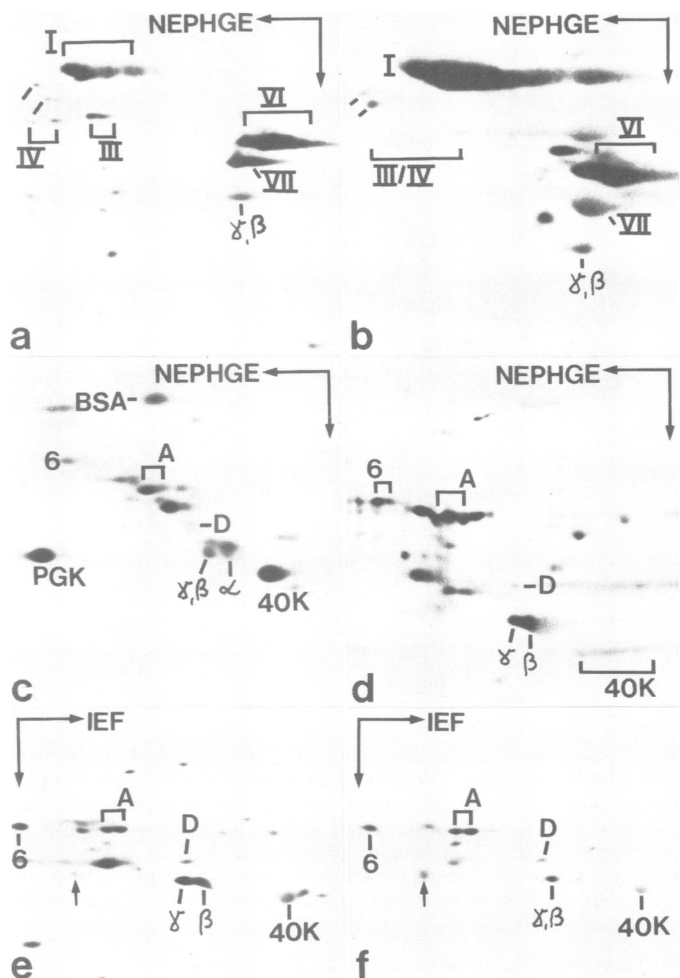

Fig. 1. Two-dimensional gel electrophoresis of cytokeratin polypeptides from bovine muzzle epidermis and bladder urothelium, using isoelectric focusing (IEF; **e,f**) or non-equilibrium electrophoresis (NEPHGE; **a – d**) in the first dimension (SDS, direction of electrophoresis in the second dimension in the presence of SDS). **(a)** Fluorograph showing the *in vitro* translation products of muzzle epidermis mRNA. Roman numerals denote major cytokeratin polypeptides (cf., Schiller *et al.*, 1982). Bars in the upper left denote two minor cytokeratins. **(b)** Fluorograph showing the polypeptides extracted from *X. laevis* oocytes after injection with bovine muzzle poly(A)$^+$ RNA. Designations as in **a**. Components III and IV are relatively under-represented in this analysis but have been more conspicuous in other preparations. **(c)** Coomassie Blue staining of cytokeratins from bladder urothelium. Spots on a diagonal line between cytokeratins A and D represent degradation products of cytokeratin A. **(d,e)** Fluorograph showing the products of *in vitro* translation using mRNA from bladder urothelium (**d**, IEF; **e**, NEPHGE). **(f)** Same experiment as in **d** and **e** but after enrichment of the cytokeratins synthesized *in vitro* by co-polymerization with unlabeled epidermal cytokeratins. No. 6, A, D and 40K are the major bladder urothelium cytokeratins (Schiller *et al.*, 1982). α, β and γ are the respective actin variants. Bovine serum albumin (BSA), phosphoglycerokinase (PGK) and skeletal muscle α-actin have been added as markers. The arrows in **e** and **f** point to a minor polypeptide which is also enriched by co-polymerization. Component no. 6 has migrated unusually fast in the particular gel of **(e)** and **(f)**.

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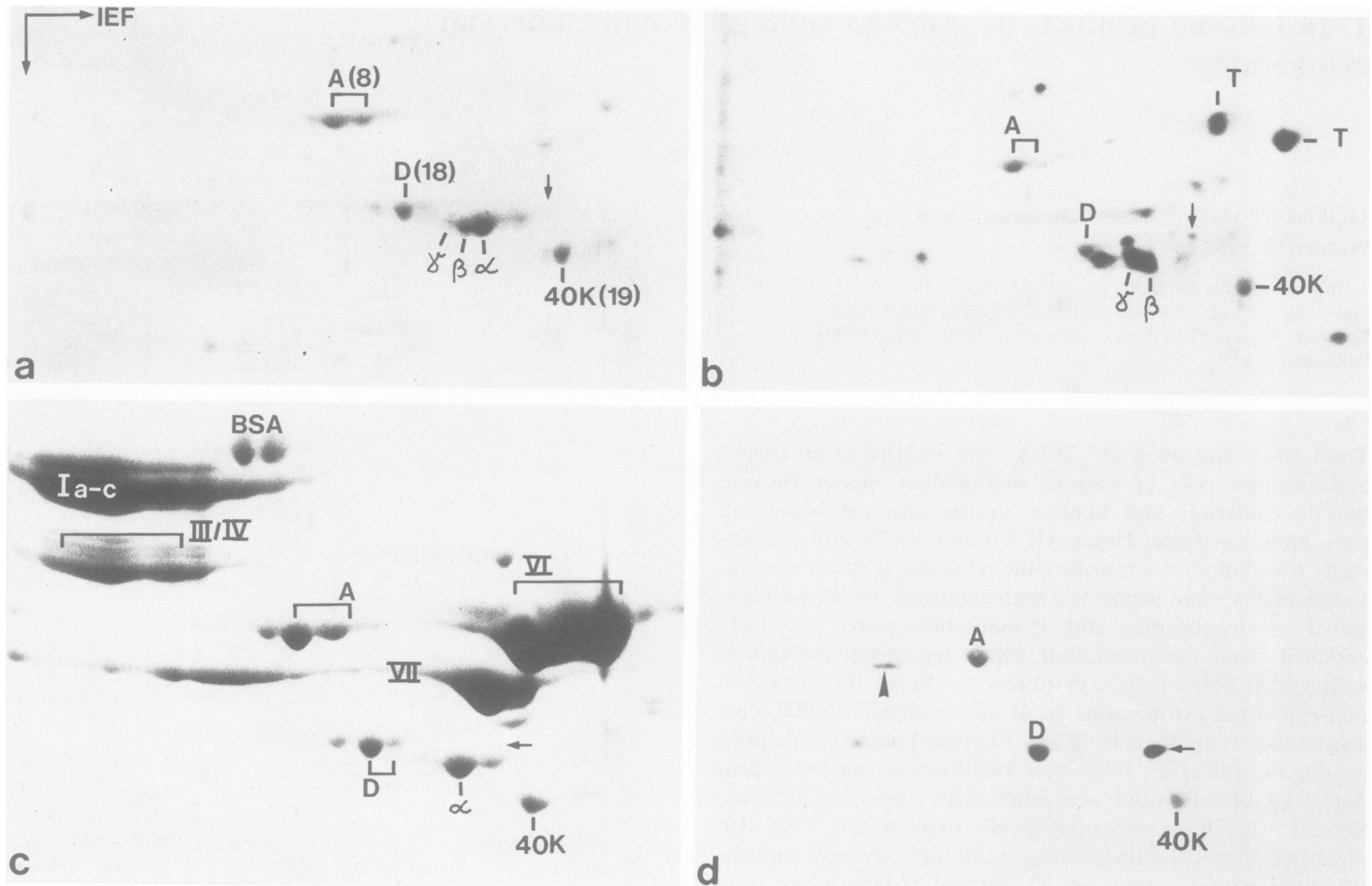

Fig. 2. Two-dimensional gel electrophoresis of cytokeratin polypeptides from human MCF-7 breast carcinoma cells. **(a)** Coomassie Blue staining of MCF-7 cytoskeletal polypeptides. Cytokeratins are denoted A, D and 40K; arabic numerals give numbers in the cytokeratin catalog (Moll *et al.*, 1982). **(b)** Fluorograph of the same gel as in **(a)**, showing total polypeptides translated *in vitro* from mRNA of MCF-7 cells. **(c,d)** Enrichment of MCF-7 cytokeratins translated *in vitro* by co-polymerization with bovine epidermal keratins. **(c)** Coomassie Blue staining showing the unlabeled bovine muzzle cytokeratins and co-electrophoresed MCF-7 cytokeratins. **(d)** Fluorograph of the same gel as in **(c)**, showing the highly enriched MCF-7 cytokeratin polypeptides. Arrows in **a – d** point to a minor cytoskeletal polypeptide which is highly enriched after co-polymerization. The arrowhead on the left margin of **(d)** denotes an as yet unidentified polypeptide (more basic than A) also selectively enriched after co-polymerization.

and a given cell can contain 2 – 10 polypeptides (Franke *et al.*, 1981a, 1981b, 1981c, 1981d, 1983; Moll *et al.*, 1982).

In view of the striking homologies of primary structure as well as common immunologic determinants (e.g., Pruss *et al.*, 1981; Gown and Vogel, 1982) it is important to examine whether the various intermediate filament proteins are products of different mRNAs, i.e., different genes, or whether some of them are derived from common precursors by proteolytic processing. At present it is clear that vimentin and desmin are synthesized by specific mRNAs and that the polypeptides obtained by translation *in vitro* are identical to the vimentin and desmin molecules present in cytoskeletal filaments (Franke *et al.*, 1980b; Schmid *et al.*, 1980; O'Connor *et al.*, 1981; Dodemont *et al.*, 1982; Bladon *et al.*, 1982; McTavish *et al.*, 1983; Zehner and Paterson, 1983). Similarly, it has been shown that all three polypeptides present in neurofilaments can be identified as products of translation *in vitro* using mRNA from rabbit spinal cord (Czosnek *et al.*, 1980). As to cytokeratin polypeptides the situation is more complicated and so far *in vitro* translation has only been examined for mRNAs from epidermal cells. Most of the epidermal keratin polypeptides have also been identified as translational products and appear to be coded by distinct mRNAs (Fuchs and Green, 1979; Schweizer and Goerttler, 1980; Gibbs and Freedberg, 1982; Schiller *et al.*, 1982; Roop *et al.*, 1983). However, two major cytokeratin polypeptides of human epidermis, one of mol. wt. 65 000 and the other of 55 000, have been reported by Fuchs and Green (1980) to be absent from the products of translation of epidermal mRNA, and it has been suggested that they are the result of processing of somewhat larger precursor keratin molecules.

Keratin-like molecules ('cytokeratins') also occur in other, i.e., non-epidermal epithelia where they appear to be expressed in cell type-specific patterns of cytokeratin polypeptides different from epidermal ones (Franke *et al.*, 1978a, 1978b, 1981a, 1981b, 1981c, 1981d, 1982; Sun *et al.*, 1979; Moll *et al.*, 1982). In the present study we describe cytokeratin polypeptides identified after *in vitro* translation of non-epidermal mRNAs from three different species and show that all cytokeratins characteristic of simple epithelia can be identified as translational products, indicating that they are synthesized from different mRNAs. These translational products include the small and acidic cytokeratin polypeptide of mol. wt. 40 000 (Franke *et al.*, 1981d; Wu and Rheinwald, 1981) which, in view of current concepts of intermediate filament organization (Geisler and Weber, 1982), should be near to the minimal size required for a polypeptide constituent of an intermediate filament (Wu and Rheinwald, 1981).

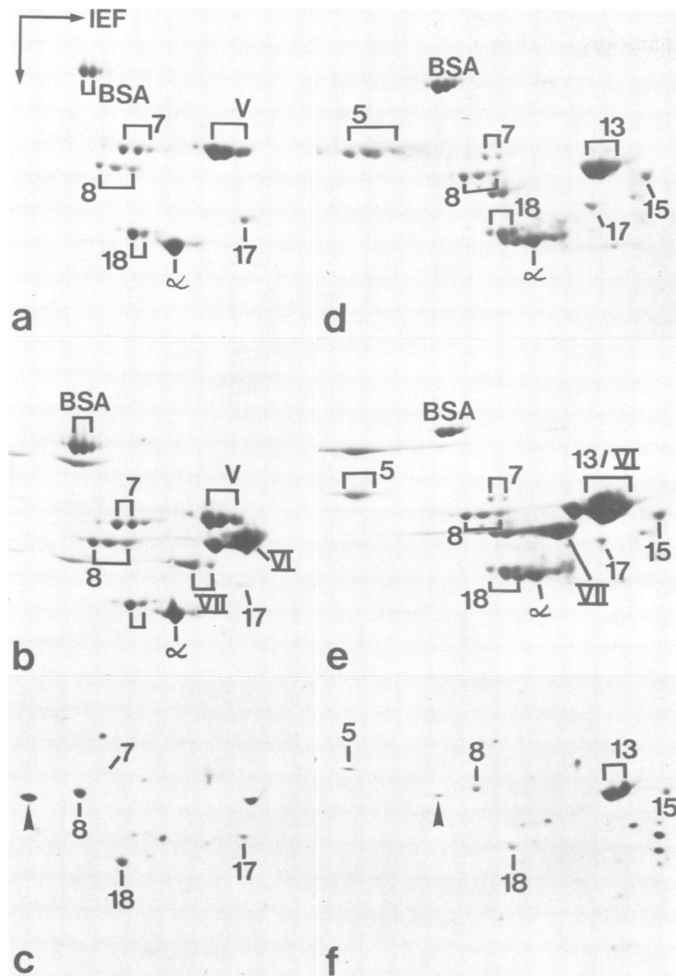

Fig. 3. Two-dimensional gel electrophoresis of cytoskeletal polypeptides from HeLa **(a – c)** and A-431 **(d – f)** cell cultures. **(a,d)** Coomassie Blue staining of cytoskeletal proteins from HeLa **(a)** and A-431 **(d)** cells. **(b)** Coomassie Blue staining of the bovine epidermal cytokeratins used for co-polymerization and of the HeLa cytoskeletal polypeptides added. **(c)** Fluorograph of the same gel as shown in **(b)** presenting the enrichment of HeLa cytokeratins synthesized *in vitro*. **(e)** Coomassie Blue staining of the bovine keratins used for co-polymerization and the added A-431 cytoskeletal polypeptides. **(f)** Fluorograph of the same gel as **(e)**, showing the enrichment of A-431 cytokeratins synthesized *in vitro*. The arrowheads in **(c)** and **(f)** denote a polypeptide present in both HeLa and A-431 cells which co-polymerizes with the bovine muzzle keratins.

Results

When total RNA or poly(A)$^+$ RNA from bovine muzzle epidermis was examined for translation *in vitro*, using the reticulocyte lysate system, and the radioactively labeled products formed were compared with unlabeled cytoskeletal proteins from the same tissue by co-electrophoresis, all keratin components identified in the tissue were also recognized among the translational products (Figure 1a; cf., Schiller *et al.*, 1982; Kreis *et al.*, 1983). The same bovine epidermal cytokeratin polypeptides are recovered in the form of insoluble filaments when stage VI oocytes of *Xenopus laevis* are injected with these RNA preparations (Figure 1b). It is interesting to note in this context that the ^{35}S label in cytokeratins produced from the injected bovine mRNA (40 ng per cell) by far exceeds the incorporation into the endogenous cytokeratins present in these oocytes (Gall *et al.*, 1983; Franz *et al.*, 1983). Apparently both assays, i.e, translation of mRNA *in vitro* and after injection into amphibian oocytes, give identical products. Therefore, we have confined most of our study to translation assays *in vitro* using the reticulocyte lysate system.

When mRNA from bovine bladder urothelium was used for translation *in vitro*, cytokeratins also represented major translational products (Figure 1c, d). Prominent cytokeratins identified are a basic polypeptide (bovine component no. 6 according to Schiller *et al.*, 1982; mol. wt. ~60 000), cytokeratin A (bovine component no. 8; mol. wt. 52 000) and the small cytokeratin of mol. wt. 40 000 which is more acidic than α-actin (Figure 1c, d). Cytokeratin D, a polypeptide (bovine component no. 21; mol. wt. ~43 000) of a slightly higher mol. wt. value and slightly more basic than actin was detected only as a minor cytokeratin component both in cytoskeletons prepared from the tissue and in translational assays (Figure 1c, d). Cytokeratin polypeptides can be specifically enriched from translation assays by two efficient methods, immunoprecipitation and co-polymerization. While immunoprecipitation (for example see Fuchs and Green, 1980) faces the problem of cytokeratin diversity and does not necessarily allow the recovery of all cytokeratin polypeptides from an unknown population of cytokeratin molecules, co-polymerisation of labeled cytokeratins synthesized *in vitro* with an excess of unlabelled cytokeratin material added allows the recovery of all cytokeratins in a filamentous state resistant to extraction in low and high salt buffers and detergents (Schiller *et al.*, 1982). When translational products of total RNA from bovine urothelium (Figure 1e) were allowed to co-polymerize with unlabeled cytokeratins from bovine muzzle epidermis enrichment of bovine cytokeratins nos. 6, A, D and mol. wt. 40 000 was observed (Figure 1f). The relative proportion of cytokeratin D, however, was still very low. In addition, we noticed enrichment of a polypeptide which was slightly larger than actin but much more basic (approximate isoelectric pH 6.5; denoted by arrows in Figure 1e and f). Whether this minor polypeptide is related to cytokeratins remains to be examined.

Cytokeratins also represented major translational products in mRNA isolated from certain cultured epithelial cells. For example, when RNA was isolated from cells of the human breast carcinoma line MCF-7 and examined by translation *in vitro*, the three cytokeratins A (component no. 8 of the human catalog of Moll *et al.*, 1982), D (no. 18) and mol. wt. 40 000 (no. 19) were found in amounts comparable to those of tubulins and actins (Figure 2a and b). Co-polymerization of total translational products with added bovine cytokeratins resulted in the dramatic enrichment of cytokeratins nos. 8, 18 and 19 (Figure 2c, d). In addition, these co-polymers showed enrichment of a polypeptide (arrows in Figure 2a – d) which had an apparent mol. wt. similar to that of cytokeratin D but was much more acidic (approximate isoelectric pH 5.25) and a polypeptide of a mol. wt. value slightly lower than that of cytokeratin A but more basic (approximate isoelectric pH 6.5).

We also found examples in which the relative amounts of the various cytokeratin polypeptides showed differences between total cytoskeleton and cytokeratin polypeptides synthesized *in vitro*. HeLa cells, for example, contain four cytokeratin polypeptides (Figure 3a, Franke *et al.*, 1981c, 1982; Bravo *et al.*, 1982; Fey *et al.*, 1983) which have been identified as cytokeratins nos. 7, 8, 17 and 18 (Moll *et al.*, 1982). In addition, HeLa cells contain considerable amounts of vimentin filaments (Franke *et al.*, 1978a, 1979). When we compared

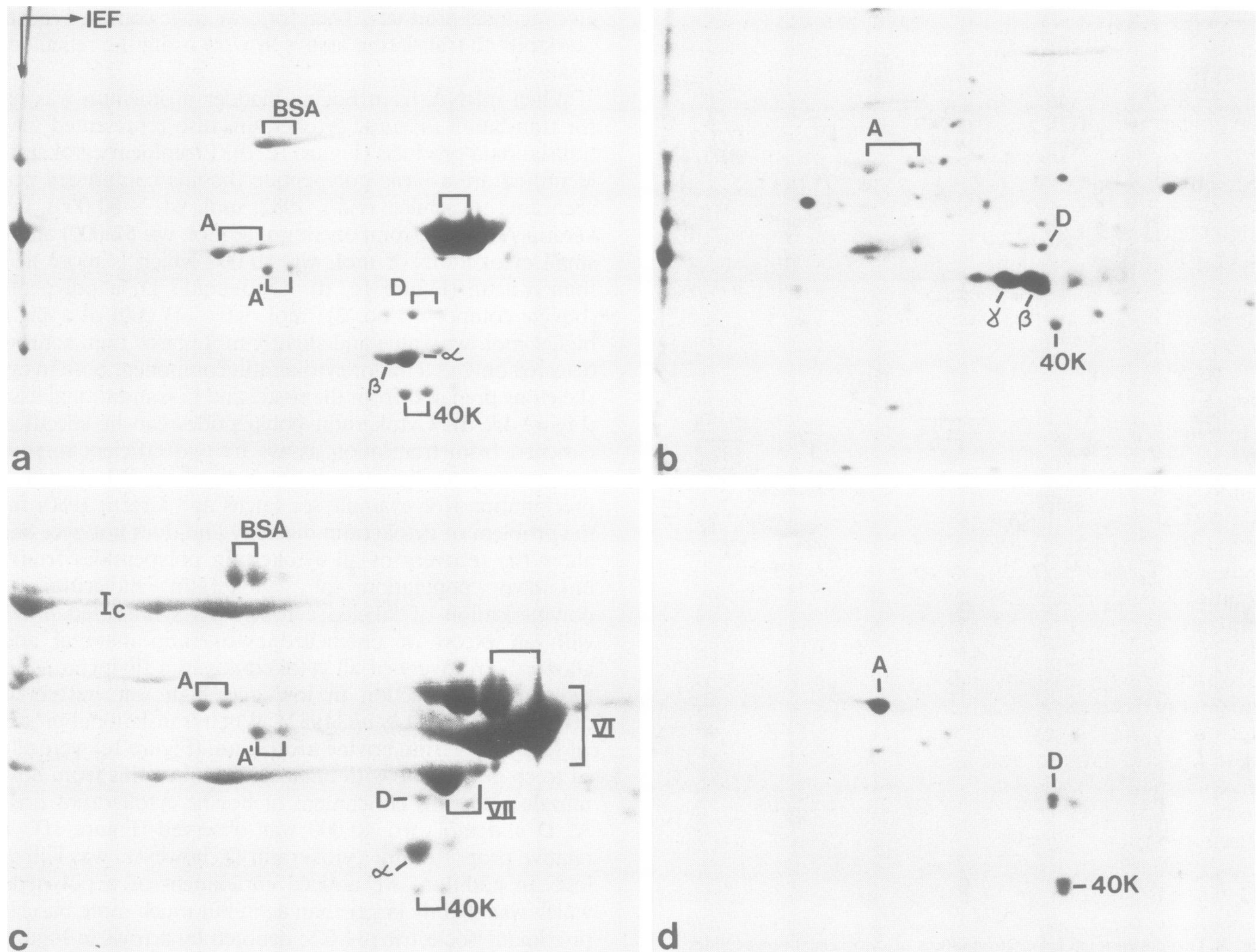

Fig. 4. Two-dimensional gel electrophoresis of cytokeratin polypeptides from rat Novikoff hepatoma cells. **(a)** Coomassie Blue staining of rat Novikoff hepatoma cytoskeletal proteins. **(b)** Fluorograph corresponding to **(a)**, showing the major products of *in vitro* translation using mRNA from the same culture. **(c)** Coomassie Blue staining of protein pelleted after co-polymerization of the [^{35}S]methionine-labelled rat hepatoma proteins translated *in vitro* with unlabelled bovine muzzle cytokeratins added. **(d)** Fluorograph of ^{35}S-labelled *in vitro* translation products of mRNA from rat hepatoma cells (same gel as in **c**). Brackets denotes vimentin. A, D and 40K denote the rat Novikoff cytokeratins (cf., Franke *et al.*, 1981a; Schmidt *et al.*, 1982). A′ is a major degradation product of cytokeratin A (cf., Schiller and Franke, 1983).

the relative intensities of cytokeratin polypeptides present in cytoskeletal filaments (Figure 3a) with those of translational products co-polymerized *in vitro* (Figure 3b, c) sizeable amounts of cytokeratins nos. 8, 17 and 18 were seen whereas only very little radioactivity was associated with the spots of co-electrophoresed unlabeled cytokeratin no. 7 (Figure 3b, c). Again in HeLa cells we observed enrichment of the component slightly smaller and more basic than cytokeratin no. 8 (A) already described for translational products of MCF-7 cells (arrow in Figure 3c).

An even higher degree of disproportionation between cytoskeletal keratin polypeptides and cytokeratins synthesized by translation *in vitro* was noted in human A-431 cells which are characterized by an exceptionally high complexity of 10 different polypeptides (cf., Moll *et al.*, 1982). Here, *in vitro*, one cytokeratin (no. 13) was produced in excessive amounts (Figure 3d, f). Some other cytokeratins such as nos. 5, 8, 15 and 18 were also recovered in appreciable amounts after co-polymerization with bovine muzzle cytokeratins *in vitro* (Figure 3d – f) whereas components nos. 7 and 17 were not recovered in significant amounts. Co-polymerization of the polypeptide slightly smaller and basic than cytokeratin no. 8 was also observed in this cell (arrow in Figure 3f).

Translational products of mRNAs coding for cytokeratins were also identified for cultured rodent cells such as rat hepatoma-derived cell lines MH_1C_1 (not shown) and Novikoff hepatoma cells grown in ascites form. Figure 4 shows the cytoskeletal polypeptides of Novikoff hepatoma cells which, in addition to cytokeratins A and D characteristic of hepatocytes and differentiated hepatoma cells (Franke *et al.*, 1981a, 1981b, 1981c; Schiller *et al.*, 1982; Hubbard and Ma, 1983), contain considerable amounts of the mol. wt. 40 000 cytokeratin (Franke *et al.*, 1981d; Schmidt *et al.*, 1982) and large amounts of vimentin. All three cytokeratins were identified among the major translational products (Figure 4b). Co-polymerization of Novikoff hepatoma cell proteins synthesized *in vitro* with bovine muzzle epidermal keratins showed a specific enrichment for cytokeratins A, D and mol. wt. 40 000 (Figure 4c and d).

In cytoskeletons, cytokeratin polypeptides usually occur as

series of isoelectric variants and major variants have been identified as the specific unphosphorylated (basic) and the mono- and di-phosphorylated forms (Sun and Green, 1978; Gilmartin *et al.*, 1980; Franke *et al.*, 1981c; Steinert *et al.*, 1982). Detailed analysis of the products of translation *in vitro* revealed that generally the degree of phosphorylation was lower in the translational products obtained in the reticulocyte lysate system (Figures 1d – f, 2b and d, 3c and f, 4b and d) although prominent phosphorylated forms were noted in certain cytokeratins (e.g., bovine cytokeratin A in Figure 1d – f). The presence of phosphokinases in reticulocyte lysates and phosphorylation of vimentin newly synthesized *in vitro* has been demonstrated by O'Connor *et al.* (1981).

Discussion

In the three species examined (man, cow and rat) the relatively small non-epidermal cytokeratins characteristic of simple epithelia such as cytokeratins A, D and that of mol. wt. 40 000 (nos. 8, 18 and 19 of the human cytokeratin catalog of Moll *et al.*, 1982) are genuine translational products and not produced by processing or post-translational modification of precursor polypeptides. The cytokeratins positively identified as translational products also include basic non-epidermal cytokeratins such as bovine cytokeratin no. 6 and human cytokeratin no. 5 as well as some very acidic cytokeratins such as human components no. 13, 15 and 17 which occur only in certain types of cells (Moll *et al.*, 1982). These findings suggest that the majority, if not all, of the non-epidermal cytokeratins of mammals are translational products and that the diversity of cell type-specific expression reflects diversity of mRNA synthesis rather than modifications by translational or post-translational processes. These observations, together with the finding that all 10 different cytokeratin polypeptides present in living cells of bovine muzzle epidermis can also be identified as translational products *in vitro*, suggest that post-translational processing of cytokeratins as proposed by Fuchs and Green (1980) to explain their failure to identify two epidermal keratins in their translational assays might be a rather unusual phenomenon possibly specific for the degenerating cells present in the upper strata in the epidermis.

The co-polymerization assay preferentially used in this study allows the detection not only of all cytokeratins but also of specific minor polypeptides (see Results) which are highly enriched in the co-polymer filaments insoluble in low and high salt buffers as well as in Triton X-100. Future experiments will have to show whether these newly detected minor polypeptides are (i) special cytokeratins or (ii) non-keratinous polypeptides that specifically associate with cytokeratin filaments or (iii) degradation products of cytokeratins.

Materials and methods

Isolation and in vitro translation of RNA

Total cellular RNA from various cultured cells (human cell lines: MCF-7, A-431, HeLa), rat Novikoff hepatoma cells grown in ascites form, and tissues (bovine muzzle epidermis and bladder urothelium) was prepared by the guanidinium-HCl-method as described (Kreis *et al.*, 1983), except that cultured cells were directly lysed in 7 M guanidinium-HCl, 0.1 M potassium acetate buffer (pH 5.5) containing 0.2% 2-mercaptoethanol. Generally, a proteinase K digestion step (0.2 mg/ml pre-digested proteinase K in 10 mM NaCl, 10 mM Tris-HCl of pH 7.5, 0.5% SDS, 1 mM $MgCl_2$ for 2 h at 50°C) was included before the extraction in phenol:chloroform. In some experiments poly(A)$^+$ RNA was prepared as described (Franke *et al.*, 1980b; Kreis *et al.*, 1983).

1 – 10 μg of total mRNA and 0.1 – 1 μg poly(A)$^+$ RNA, respectively, were translated *in vitro* using a commercially available reticulocyte system with L-[^{35}S]methionine as radioactive amino acid (1400 Ci/mmol, Amersham-Buchler, Braunschweig, FRG). The products synthesized *in vitro* were analysed by two-dimensional gel electrophoresis as described (Franke *et al.*, 1980 , 1981c; Schiller *et al.*, 1982), using either isoelectric focusing (O'Farrell, 1975) or non-equilibrium pH gradient electrophoresis (O'Farrell *et al.*, 1977).

Oocyte microinjection and high salt extraction

Stage VI oocytes of *X. laevis* were injected with ~40 ng of cow snout poly(A)$^+$ mRNA and incubated for 16 h at 19°C in Barth's modified medium containing 15 μCi [^{35}S]methionine per oocyte as described by Gurdon (1974). In a typical experiment, 15 microinjected oocytes were resuspended in 2 ml of 'high salt-detergent buffer' (Franke *et al.*, 1981a) and incubated for 1 h at 4°C in the same buffer. The pellet recovered after centrifuging for 10 min at 4°C in a laboratory centrifuge (full speed; Eppendorf minifuge) was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and centrifuged as before. This last pellet was dissolved in the corresponding sample buffer and subjected to one- or two-dimensional gel electrophoresis as described (Kreis *et al.*, 1983).

Specific reconstitution of filaments from cytokeratins synthesized in vitro

Cytokeratins synthesized in cell-free systems can be selectively recovered from the translation assay by exploiting its ability to integrate into filments or reconstitute prekeratin filaments in homologous or heterologous combinations of cytokeratins. Purified bovine muzzle prekeratins solubilized in 8 M urea, 10 mM Tris-HCl (pH 8), 25 mM 2-mercaptoethanol were dialyzed against 1 mM Tris-HCl (pH 7.4) and the resulting intermediate filaments have been used as described (Kreis *et al.*, 1983), except that the translation assays were 10-times diluted with 1 mM Tris-HCl (pH 7.4) before adding the solution containing the bovine keratins (20 μl containing 100 – 200 μg protein). For direct identification of the cytokeratins translated *in vitro* the homologous cytoskeletal proteins were added as markers immediately prior to electrophoresis. Alternatively, translational products were immunoprecipitated using guinea pig antisera against bovine epidermal prekeratins previously described (cf., Franke *et al.*, 1980b).

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Note added in proof

After completion of this manuscript a paper by K.H.Kim, J.G.Rheinwald and E.V.Fuchs (*Mol. Cell. Biol.*, **3**, 495-502, 1983) has appeared in which translational products of cytokeratin mRNAs from various cultured human epithelial cells (epidermal, squamous cell carcinoma cells of line SCC-15, conjunctival, mesothelial) are described. Their results, where comparable, are in agreement with those presented here. Translational products of mRNAs from rat spinal cord have been described by P.Strocchi, D.Dahl and J.M.Gilbert (*J. Neurochem.*, **39**, 1132-1411, 1982) who have identified two of the neurofilament polypeptides of mol. wts. 70 000 and 145 000 and glial filament protein.