De Novo Synthesis and Specific Assembly of Keratin Filaments in Nonepithelial Cells after Microinjection of mRNA for Epidermal Keratin

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Summary

Poly(A)⁺ RNA isolated from bovine muzzle epidermis was microinjected into nonepithelial cells containing only intermediate-sized filaments of the vimentin type. In recipient cells keratin polypeptides are synthesized and assemble into intermediate-sized filaments at multiple dispersed sites. We describe the time course and the pattern of de novo assembly of keratin filaments within living cells. These filaments were indistinguishable, by immunofluorescence and immunoelectron microscopic criteria, from keratin filament arrays present in true epithelial cells. The presence of extended keratin fibrous meshworks in these injected cells is compatible with cell growth and mitosis. Double immunolabeling revealed that newly assembled keratin was not codistributed with microfilament bundles, microtubules or vimentin filaments. We suggest that assembly mechanisms exist which in vivo sort out newly synthesized cytokeratin polypeptides from vimentin.

Introduction

The cytoplasm of most eucaryotic cells contains proteinaceous structures that are characterized by a very low solubility, even when exposed to extreme ionic and pH conditions, to different detergents and to organic solvents. In cells of vertebrates, most prominent among these insoluble structures are the intermediate-sized filaments ("intermediate filaments"); for reviews see Lazarides, 1982; Anderton, 1981; Franke et al., 1981c). It is remarkable that these structural proteins are expressed in patterns related to cell differentiation. For example, different types of intermediate filaments have been found in different cell types: cytokeratins in epithelial cells, vimentin in mesenchymally derived and some other nonepithelial cells, desmin in cells of myogenic differentiation, neurofilaments in neurons, and glial filaments in astrocytes (e.g., Bennett et al., 1978; Franke et al., 1978a, 1978b, 1981c; for reviews see Holtzer et al., 1981; Osborn et al., 1981; Lazarides, 1982). It is known that in vitro all these diverse types of proteins of intermediate filaments spontaneously renature and reassemble into filaments indistinguishable from native intermediate filaments when the denaturing agent is removed (Cooke, 1976; Steinert et al., 1976, 1980, 1981a, 1981b, Small and Sobieszek, 1977; Rueger et al., 1979; Geisler and Weber, 1981a; Liem et al., 1981; Renner et al., 1981). Although molecules renatured in vitro have been described as soluble monomers or oligomers at very low salt buffers and slightly alkaline pH (7–8.5) for some intermediate filament proteins such as desmin (Huitt et al., 1980) and glial filament protein (Rueger et al., 1979), but not for keratin-type proteins, all these proteins tend to form filamentous polymer structures when exposed to salt conditions approximating those present in the living cell. In addition, it has been shown that in vitro diverse intermediate filament proteins can be induced to co-polymerize (Steinert et al., 1981a, 1981b). In certain cells two different types of intermediate filament proteins could be localized in the same filamentous structure. This includes the presence of both desmin and vimentin in certain smooth muscle and BHK cells as well as glial filament protein and vimentin in astrocytes and glioma cell cultures (e.g., Steinert et al., 1981b; Quinlan and Franke, 1982; Sharp et al., 1982).

Microinjection of fluorescently labeled proteins into living cells has become a valuable technique in studying the distribution of a variety of cytoskeletal proteins of the microtubule and microfilament system (Taylor and Wang, 1978; for review see Kreis and Birchmeier, 1982). Such injected proteins can be stably incorporated into existing cytoskeletal structures, hence allowing studies on the dynamics and mechanisms of exchange and polymerization of such proteins (Kreis et al., 1982). Proteins of this category can be applied in physiologically tolerated solutions and can be kept in a soluble state at considerably high concentrations. Because of the practical impossibility of introducing purified intermediate filament proteins that are insoluble in physiological buffers by microinjection we have used a different approach—namely, the microinjection of purified poly(A)⁺ mRNA (Stacey and Allfrey, 1976) highly enriched in mRNA for a specific category of insoluble proteins, in this case epidermal α-keratins. We have studied the appearance and distribution of the newly synthesized proteins by using specific antibodies for detection. For the experiments described here we have chosen cells that express intermediate filaments of the vimentin type only. We show that translation of keratin mRNA in nonepithelial cells of the same or other species results in the production of molecules that assemble into specific keratin filaments and thus establish a novel type of cytoskeletal filament system in these cells.
Results

Characterization of mRNAs by Translation In Vitro
Poly(A)⁺ RNA isolated from stratum spinosum of bovine muzzle epidermis is highly enriched in mRNA coding for all α-keratin polypeptides detected in the tissue. This could be demonstrated by translation in vitro using the reticulocyte lysate system and ³⁵S-methionine as label (Figure 1). The translational products identified are the major basic keratin polypeptides designated la, lb and lc, III and IV, as well as the major acidic α-keratin polypeptides Vla, Vlb and VII and the minor basic components designated 4 and 5. All these polypeptides can be recovered from translational assay mixtures by in vitro filament coassembly with added unlabeled keratins (Experimental Procedures). This production of all known epidermal keratin polypeptides by translation of bovine muzzle poly(A)⁺ RNA is different from the situation in human skin, where Fuchs and Green (1980) have found that two keratin polypeptides present in the tissue are not included among the in vitro translation products. The only other notable component identified in these translation assays is relatively small amounts of actin (A in Figure 1). Quantitation of ³⁵S-methionine-labeled translational products by densitometric scans of autoradiographs (see Experimental Procedures) has shown that in these preparations consistently more than 80% of polypeptide radioactivity is associated with keratin polypeptides.

Injection of Epidermal Poly(A)⁺ RNA into Cultured Lens-Forming Cells
Cultured bovine lens-forming cells have been chosen for the first microinjection experiments because they grow in monolayers with epithelioid morphology but contain only intermediate filaments of the vimentin type and are devoid of keratin and desmosomal components (Barritault et al., 1980; Ramaekers et al., 1980; Franke et al., 1981 b). Actually the relative amount of vimentin present in mammalian eye lens cells is so large that it is frequently used as a rich source for large scale preparations of vimentin (e.g., Geisler and Weber, 1981 b). When stained with various antibodies to epidermal and nonepidermal cyto-keratins these cells are negative (Figure 2a; Ramaekers et al., 1980). However, when individual lens-forming cells are injected with epidermal poly(A)⁺ RNA they acquire positive fibrillar staining with cytokeratin antibodies (Figure 2a), whereas all cells of the culture show strong fibrillar staining with antibodies to vimentin (Figure 2b). Desmosomal plaque proteins (Franke et al., 1981 b, 1983) could not be detected by immunofluorescence labeling of injected cells, in accordance with our failure to detect these components in the in vitro translation products. The newly formed keratin was found exclusively in the cytoplasm and could not be detected in the cell nucleus. Segregation of de novo formed keratin into autophagic vacuoles as described by Stacey and Allfrey (1977) for injected proteins has not been observed.

When cells are simultaneously injected with both fluorescein-labeled bovine serum albumin (BSA) and epidermal poly(A)⁺ RNA and observed at various times after injection, the BSA appears rather diffusely spread in the cytoplasm and is unrelated to the arrays of de novo synthesized keratins or other filaments in the same cells (Figures 3a and 3b). The display of the newly formed keratins in the injected cells is also different from arrays formed by many other fluorescein-labeled injected proteins, cytoskeletal ones included (for review see Kreis and Birchmeier, 1982).
Figure 2. Immunofluorescence Microscopy of Calf Lens-Forming Cells after Staining with Antibodies against Prekeratin and Vimentin

Individual cells within subconfluent monolayers were microinjected with keratin mRNA (see Figure 1), fixed 3 days after microinjection, and stained with antibodies against bovine prekeratin (a) or vimentin (b). Note that only the injected cell shows fibrillar arrays of keratin filaments. Staining of a similar culture with antibodies against vimentin demonstrates the abundance of vimentin filaments in all the cells (b). Bars: 50 μm.
As another type of control we have injected poly(A)+ RNA from rat brain (Figures 3c and 3d), which has not resulted in the appearance of any structure stainable with cytokeratin antibodies.

**Time Course of Appearance of Keratin Filaments Formed De Novo in Injected Cells**

After injection of epidermal keratin mRNA, different structural arrangements of de novo formed keratin filaments and fibrils have been detected (Figure 4). The earliest forms of newly synthesized keratin material detected by immunofluorescence microscopy, as early as 1 hr after injection, consist of small granular or comma-shaped particles randomly distributed throughout the cytoplasm (Figure 4a), similar to the "pieces" of keratin filaments described in PtK cells by Osborn et al. (1980). Occasionally in some individual cells more extended fibrillar formations have also been observed even at these early times.

Typically, upon prolonged incubation of cells injected with keratin mRNA, i.e., for 1–4 hr, considerably longer fibrillar elements (up to about 5 μm long) and tangles of thread-like formations are predominant (Figures 4b and 4c). Such cells often still contain some granular or comma-shaped particles (Figures 4b and 4c). Apparently, these threads are intermediate stages of further fibrillar growth and, after periods of 4 hr to 3 days following injection, often develop into massive, usually extended arrays of fibrils (Figures 4d and 4e). These structures, as revealed by immunofluorescence microscopy, resemble typical arrays of keratin fibrils in true epithelial cells such as rat kangaroo PtK cells, human HeLa cells or keratinocytes (Franke et al., 1978a, 1978b, 1979a, 1979b; Sun and Green, 1978; Sun et al., 1979). In some cells injected with keratin mRNA, excessive amounts of keratin fibrils can accumulate revealing bizarre forms of fibrillar branching and anastomosing (Figure 4f). Frequently,
Keratin Filaments from Microinjected mRNA

Figure 4. Time Course of Keratin Filament Assembly in Calf Lens Cells Microinjected with Prekeratin mRNA

Cells were fixed at different intervals after microinjection and immunofluorescently labeled for prekeratin. (a) One hour after injection; early pattern of newly synthesized keratin in the form of small granules or short comma-shaped particles randomly distributed in the cytoplasm. (b) and (c) Four hours after injection; newly synthesized prekeratin is revealed as larger granules or thread-like structures. (d) Twelve hours and (e) 24 hours after injection newly synthesized keratin is mostly organized in fibrillar meshworks, often showing enrichment in the perinuclear region. (f) Extended fibrillar keratin structures seen in some cells 24 hr after microinjection of prekeratin mRNA. Bars: 20 μm.
we have noted a tendency of keratin fibrils formed in these injected cells to concentrate, in variously shaped bundles and whorls, near the nucleus (Figure 4d and 4e; see also Figures 6a, 6c and 7a-7e), a phenomenon which has also frequently been noted in epithelial tissues or cell cultures (Franke, 1971; for references see Franke et al., 1981c).

The presence of relatively large amounts of keratin fibrils is perfectly compatible with the viability and proliferation of the injected cells. Mitotic cells containing keratin fibrils have frequently been observed (Figure 5), and the changes of distribution of these fibrils during mitosis are similar to those described for endogenous cytokeratin or vimentin filaments in relevant noninjected cells of various kinds (e.g., Blose and Chacko, 1976; Franke et al., 1978b; Aubin et al., 1980; Blose, 1981). Whether or not certain small granular particles still seen in cells after injection are structurally related to the cytokeratin-containing spheroidal aggregates observed in mitoses of certain epithelial cells (Horwitz et al., 1981; Franke et al., 1982) remains to be examined in more detailed electron microscopic analyses.

De Novo Synthesized Keratins Do Not Colocalize with Other Cytoskeletal Meshworks

Using double immunofluorescence microscopy we have shown that the keratin structures produced in cells injected with mRNA coding for epidermal keratins, be they comma-shaped or fibrillar, are distributed differently from arrays of microtubules visualized by tubulin antibodies (Figures 6a and 6b) and vimentin filaments decorated by vimentin antibodies (Figures 6c and 6d). Similarly, we have verified that the display of these newly formed keratin filaments is fundamentally different from the arrays of typical "stress fibers" containing actin and α-actinin typical of these cells (not shown). Furthermore, the keratin filaments in injected cells remain dispersed and are not significantly affected by colchicine treatments that induce aggregation of vimentin filaments.

Electron Microscopic Identification of Keratin Filaments Formed after Microinjection of Keratin mRNA

The cytoplasm of cultured lens cells is densely packed by flocules of vimentin filaments (Ramaekers et al., 1980). We have used two different labeling techniques to characterize the structures containing the newly synthesized keratins in the injected cells at the electron microscope level. The first method is the direct inspection of cell structures after labeling with two antibodies in sequence (e.g., Sharp et al., 1982)—that is, the same procedure as used for indirect immunofluorescence microscopy (Figure 7a)—followed by fixation in glutaraldehyde and processing for electron microscopy of thin sections. The second method involves the immunoperoxidase technique, which allows direct comparison of light microscopy in 1–2 μm thick sections (e.g., Figure 7a, inset) and subsequent electron microscopy using 50–60 nm sections (Figure 7b). With the immunoperoxidase technique we have identified the structures positive for keratin antibodies as distinct, densely stained fibrillar aggregates (Figure 7b) in which, however, individual filaments usually could not be resolved. Direct visualization of the structures decorated by two antibodies in sequence (Figures 7c–7e) has shown bundles of distinct filaments specifically stained with cytokeratin antibodies as distinct, densely stained fibrillar aggregates (Figure 7b) in which, however, individual filaments usually could not be resolved. Direct visualization of the structures decorated by two antibodies in sequence (Figures 7c–7e) has shown bundles of distinct filaments specifically stained with cytokeratin antibodies as distinct, densely stained fibrillar aggregates (Figure 7b) in which, however, individual filaments usually could not be resolved. Direct visualization of the structures decorated by two antibodies in sequence (Figures 7c–7e) has shown bundles of distinct filaments specifically stained with cytokeratin antibodies as distinct, densely stained fibrillar aggregates (Figure 7b) in which, however, individual filaments usually could not be resolved. Direct visualization of the structures decorated by two antibodies in sequence (Figures 7c–7e) has shown bundles of distinct filaments specifically stained with cytokeratin antibodies as distinct, densely stained fibrillar aggregates (Figure 7b) in which, however, individual filaments usually could not be resolved.
nuclear envelope (Figures 7d, 7e). With this technique, higher longitudinal and cross-section magnification allows the identification of the individual intermediate filaments within these antibody-decorated bundles (inset in Figure 7c and Figures 7d and 7e). With this technique the dense decoration of the keratin filaments with antibodies results in an accumulation of immunoglobulin protein between the filaments, sometimes suggestive of dense and regular lateral extensions, similar to those described for vimentin decoration in the experiments of Blose et al. (1981) and Sharp et al. (1982). Control experiments showing decoration of the endogenous vimentin filaments present in these cells has shown similar intense labeling with immunoglobulins (not shown).

**Injection of Keratin mRNA into Other Cultured Cells of Mesenchymal Origin**

We have also used heterologous mesenchymal cells such as rat RMCD fibroblasts for injection with epidermal poly(A)^+ RNA and have obtained essentially similar results, i.e., appearance of keratin-positive fibrils within several hours after injection (not shown) as well as mitotic distribution of keratin filaments in nearly equal proportions to daughter cells. Keratin filaments formed de novo in injected RMCD fibroblasts are also positively stained with the various cytokeratin antibodies (Experimental Procedures) including those that are selective for certain bovine epidermal pre-keratin polypeptides and the monoclonal antibody K8.13, which does not react with most cytokeratin polypeptides of rodents (Gigi et al., 1982).

**Discussion**

Various investigators have shown that foreign mRNA injected into living cultured cells can be translated by the protein-synthesis machinery of the recipient cells (Graessmann and Graessmann, 1976; Stacey and Allfrey, 1976; Liu et al., 1979; Lin et al., 1982).
Figure 7. Immunoelectron Microscopy of Keratin Filament Bundles in Lens-Forming Cells Injected with Keratin mRNA

 Cultured lens cells injected with epidermal keratin mRNA have been processed, at various times after injection, for indirect immunofluorescence microscopy (a) or immunoperoxidase labeling (inset in upper right of a; cf. Experimental Procedures) and then examined by light microscopy (a) or by electron microscopy of thin sections (b–e). In the examples shown (12 hr after injection), cells containing keratin fibrils in the juxtanuclear cytoplasm have been selected. Bars: 20 μm. (b) Immunoelectron microscopy by the peroxidase technique showing specific reaction of prekeratin
Soluble proteins such as globin produced in this way appear to spread diffusely over the cytoplasm and are not associated with specific structures (Stacey and Alfrey, 1976). Other proteins such as SV40 T-antigen accumulate in the nucleus (Graessmann et al., 1978). In this study we present the first example of an insoluble structure, i.e., intermediate filaments of the keratin type, formed de novo upon microinjection of mRNA coding for keratins. Specifically, we show the assembly of an extended cytoskeletal meshwork of keratin filaments in nonepithelial cells injected with this mRNA. These new filament structures are similar, by morphological and immunological criteria, to the keratin filaments present in epithelial cells of various kinds. The only exception is the lack of attachment to typical desmosomes, which are characteristic of most epithelial cells. This proves that not only translation of keratin mRNA but also correct assembly of keratin-type intermediate filaments and fasciation into filament bundles can take place in nonepithelial cells, and that both processes do not require special factors unique to epithelial cells. The apparent absence of cytokeratin polypeptides in nonepithelial cells (Franke et al., 1978a, 1981c; Sun et al., 1979) must therefore be regulated at pretranslational levels.

Our results also show that the newly synthesized keratin molecules are not incorporated into the abundant preexisting vimentin filaments in these cells. Formation of copolymers of vimentin and keratin has been reported to occur in vitro when both kinds of proteins are denatured, mixed and allowed to assemble in the same buffer (Steinert et al., 1981a, 1981b). Therefore, we conclude that in the living cell, mechanisms exist which sort out these two types of intermediate filament proteins and control the formation of distinct filaments of each type. This is in agreement with various observations in cultured epithelial cell lines in which both vimentin and cytokeratin filaments co-exist but are organized in different arrays (Franke et al., 1978a, 1978b, 1979a, 1979b, 1981a; Aubin et al., 1980; Osborn et al., 1980). Limited regions of local alignment or intromingling of both types of intermediate filaments are occasionally noted (e.g., Figure 7c; for related observations in epithelial cells see Henderson and Weber, 1981, and Klymkowsky, 1982), but this, of course, does not indicate the presence of heterogeneous filament formulations and does not prove a direct physical interaction of the two systems. By contrast, formations of intermediate filaments containing both vimentin and desmin or glial filament protein have been described both in vitro and in vivo (Steinert et al., 1981a, 1981b; Quinlan and Franke, 1982; Sharp et al., 1982). The exclusion of keratin molecules from integration into vimentin filaments might be related to the greater differences between the primary structure of keratin polypeptides and the other three types of intermediate filament proteins which apparently display a higher degree of sequence homology (cf. Geisler and Weber, 1981b; Geisler et al., 1982; Hanukoglu and Fuoho, 1982).

Some reports have attracted attention to the close morphological associations of intermediate filaments of the desmin and vimentin type with various other cytoskeletal elements in diverse kinds of cells (e.g., Geiger and Singer, 1980; Singer et al., 1981; Ramakers et al., 1980). Our double-label immunofluorescence microscopic observations have not revealed that the keratin filaments newly formed from injected mRNA are codistributed, to a conspicuous degree, with other cytoskeletal structures. The only association observed in many but not all injected cells is the close apposition of keratin filament bundles to the nuclear envelope, a spatial relationship that has been reported for diverse types of intermediate filaments in other cells (Franke, 1971; Blose and Chaclo, 1976; Starger and Goldman, 1977).

The present study also sheds light on the possible mode of assembly of keratin-type intermediate filaments within the cytoplasm of epithelial cells. The disperse pattern of distribution of short arrays of keratin early after injection (e.g., Figure 4a) further shows not only that the injected mRNA molecules are widely distributed throughout the cytoplasm but also that assembly of keratin molecules into filaments can occur at multiple sites, in an apparently nonoriented fashion, without a predominant "intermediate-filament-organizing center." The existence of such an organizing structure has been suspected by Eckert et al. (1981, 1982) from the finding that keratin filaments reconstituted in vitro tend to be associated with nuclear residues as well as from the collapse of cytokeratin arrays into juxtanuclear aggregates upon microinjection of keratin antibodies.

Our finding of synthesis and assembly of keratins in rat fibroblastoid (RMCD) cells after injection of keratin mRNA also indicates that translation of the bovine mRNA can take place with rat fibroblast polyribosomes. Preliminary biochemical analysis of 35S-methionine labeled cytokeratins isolated from the injected cells by extraction in high salt buffer and Triton X-100 and enriched by filament reconstitution (see Experimental Procedures) as well as two-dimensional gel
electrophoresis of keratin filaments synthesized upon injection of the same epidermal poly(A)+ mRNA into oocytes of Xenopus laevis indicates that the specific bovine epidermal keratin polyproteins are synthesized in such heterologous combinations (U. Jorcano, E. Schmid, M. F. Trendelenburg, H. Soreq and W. W. Franke, personal communication; cf. Stukey and Allfrey, 1976).

Keratin-like polyproteins are a distinct multigene family of proteins, of which the epidermal keratins represent a special subset. Other epithelia contain cytotkeratin filaments formed by various combinations of other polyproteins of this family (for reviews, see Franke et al., 1981c, Mull et al., 1982, Tseng et al., 1982). Microinjection of various combinations of poly(A)+ mRNA coding for other polyproteins of the keratin family into various cells, including those of epithelial origin, may show a way to examine the principles that govern the assembly properties and molecular interrelationships of the diverse cytotkeratin polyproteins within living cells.

Keratin mRNA as well as keratin filaments formed de novo seem to be relatively stable and have a long lifetime in the injected cells, as indicated by the increase of keratin fibrillar masses with time, for at least 12 hr after injection. Experiments to determine directly the half-life times of the injected keratin mRNA and the de novo formed keratin arc under way in our laboratories.

Production of extended meshworks of filaments of the keratin type as a result of microinjection of keratin mRNA does not significantly interfere with cell growth and mitosis. During mitosis the newly developed masses of keratin filaments undergo morphological changes and are distributed to the daughter cells in a similar mode as reported for some dividing epithelial cells (Aubin et al., 1980). Thus accumulation of a large keratin filament pool in the nonepithelial cells does not seem to interfere with functions important for the viability and proliferation of these cells. Whether introduction of new filaments into cells causes differentiation-specific changes in cell physiology such as acquisition of cell polarity or vectorial secretion remains to be seen.

Our demonstration that it is possible, by microinjection of mRNA coding for "insoluble" proteins, to introduce such proteins, and hence such polymer structures, in various combinations and time programs into living cells should encourage experiments attempting to elucidate, in vivo, principles of assembly, disassembly and exchange as well as structural changes and rearrangements of such cytoskeletal elements in living cells.

**Experimental Procedures**

**Cells and Microinjection**

Bovine lens-forming cells and rat fibroblasts (RMCD cells) were grown as described (Ramaekers et al., 1980; Rathke et al., 1975). Microinjection was performed as described (Kreis et al., 1982; Kreis and Birchmeier, 1982) with siliconized glass capillaries filled by negative pressure (cf. Graessmann et al., 1980). Microinjection was controlled manually by the pressure device recently described by Ansgore (1982).

Bovine serum albumin (BSA) was modified with fluorescein isothiocyanate to form fluoresceinated bovine serum albumin (FITC-BSA). The concentration of FITC-BSA in the injection buffer was 10 mg/ml.

**Isolation of Poly(A)+ mRNA**

Total cellular RNA was isolated from bovine muzzles by the guanidinium-HCl technique (Cox, 1968; Deeley et al., 1977; Strohman et al., 1977). Stratum corneum tissues of muzzles was dissected, homogenized and centrifuged to remove nuclei, RNA and proteins. The nucleic acids were then extracted with a 10% (w/v) solution of NaCl and 0.5 M guanidinium-HCl buffer, pH 7.5, and phenol:chloroform (1:1). After precipitation of the DNA with 0.5 vol ethanol, the RNA was dissolved in sterile 0.3 M NaCl, pH 7.5.

**Reconstitution of Keratins from Translation Assays**

Cytokeratins synthesized in cell-free systems have been enriched by one of the two following reconstitution experiments. The first method is a slight variation of the procedure previously used to reconstitute bovine prekeratin filaments (Ronner et al., 1981). Purified muzzles prekeratin was solubilized in 8 M urea, 10 mM Tris-HCl (pH 8.0) and 25 mM 2-mercaptoethanol and dialyzed for 12 hr at room temperature against 1 mM Tris-HCl (pH 7.5). Translation assay mixture (20 μl) was added to 100 μl of the dialyzed solution containing keratin filaments (0.5 mg/ml) and incubated for 30 min at room temperature. After addition of 100 μl of 50 mM Tris-HCl (pH 7.5) and incubation for another 30 min, the mixture was extracted for 30 min at room temperature with 1.5 M KCl, 1% Triton X-100, 0.4 M PMSF and 10 mM EGTA by adding 1 vol 2X of concentrated extraction buffer. Insoluble filaments were recovered by centrifugation, washed with 1 ml of 10 mM Tris (pH 7.5), 10 mM EGTA, centrifuged again and stored at −70°C as a pellet until analyzed. The second method is basically the same used for reconstitution of vimentin synthesized in vitro (cf. Franke et al., 1980b) by addition of partly solubilized vimentin polymeric material. Isolated bovine muzzles tonofibrils were solubilized by homogenization, followed by boiling for 10 min, in 2% SDS,
5 mM Tris–HCl (pH 8.0), 25 mM 2-mercaptoethanol and subsequent dialysis for approximately 12 h at room temperature against 0.1% SDS, 5 mM Tris–HCl (pH 8.0). After dialysis the protein concentration was adjusted to approximately 2 mg/ml. Twenty microfilters of the reticulocyte lysate containing the in vitro synthesized cytokeratin was added to 30 µl of the dialyzed solution containing partly disintegrated tonofilament material and incubated for 15 min at room temperature. After adding 1 vol of 200 mM NaCl, 10 mM sodium phosphate buffer (pH 5.2), and incubating for 30 min at room temperature, the mixture was extracted for 30 min at room temperature in 1.5 M KCl, 10 mM Tris–HCl (pH 7.0), 1% Triton X-100 and 10 mM EDTA and subsequently centrifuged for 10 min at 100,000 x g in a Beckman airfuge. The pellet containing the reconstituted filament material was washed with 0.15 M of 10 mM Tris–HCl buffer (pH 7.2), 1 mM EDTA, centrifuged again and stored at −70°C until use.

Antibodies and Immunofluorescence Microscopy
The following antibodies were used: Guinea pig antibodies raised against total bovine muzzle epidermal prekeratins I–VII obtained after gel electrophoretic purification (Franke et al., 1980a, 1981a). Guinea pig antibodies which react only with bovine epidermal prekeratin polypeptides VI and VII (Franke et al., 1981a). A murine monoclonal antibody (Kc 8.10) with a broad range of cross-reactivity between different cytkeratin polypeptides (Gigl et al., 1982). Guinea pig antibodies against vimentin from cultured cells of mouse (3T3 cells). rat (RWF-SM cells; cf. Franke et al., 1980b) and human (SV40-transformed fibroblasts) origin, which all showed the same specificity for vimentin (for demonstration see Franke et al., 1979b). Rabbit antibodies against vimentin from BHK cells have been described (Ball and Singer, 1981). Rabbit antibodies against tubulin were the same as in the study by Goeller and Singer (1980). Guinea pig antibodies to desmoplakins I and II (Franke et al., 1983) and a minor group of desmosomal plaque-specific proteins (Franke et al., 1981b) have been used. All-antibody structures were visualized with fluorescein-labeled goat antibodies against rabbit IgG were used. Fluorescence micrographs were taken with a Zeiss photomicroscope (Carl Zeiss, Oberkochen, FRG).

Electron Microscopy
Immunoelectron microscopy was performed by the immunoperoxidase procedure (for references see Franke et al., 1982) or according to a protocol modified from that of Sharp et al. (1982). Cover slips bearing cells that had been injected with keratin mRNA were used at various times after microinjection. Cell monolayers were rinsed with phosphate-buffered saline (PBS), immersed for 5 min in methanol of −20°C, followed by dipping three times into acetone of −20°C, and then directly immersed in PBS again. Antibodies (IgG fraction) to cytokeratins or vimentin were then applied (see above), usually at ~200 µg IgG/ml PBS, in PBS for 30 min. After incubation with the first antibody, the coverslips were washed by six sequential immersions in PBS for 2–3 min each. Then the second antibody (in PBS) was added, usually a fluorescein-labeled antibody directed against the species of the first antibody applied, and allowed to react for 30 min. After six washes in PBS as described above, the cells were fixed by immersion in 2.5% glutaraldehyde containing 50 mM KCl, 2.5 mM MgCl2, in 50 mM sodium cacodylate buffer (pH 7.2) for 20 min at 4°C. After three washes in the same buffer (4°C) for 3 min each, cells on coverslips were postfixed in 2% acetic acid, extensively washed in water, stained by soaking in aqueous 0.5% uranyl acetate for 12 h, dehydrated and embedded as described (Franke et al., 1982). Usually 1–2 µm thick sections were made, and suitable groups of injected cells were selected for further processing for electron microscopy of ultrathin sections. Electron micrographs were made with a Siemens EM101.

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