Tissue Type-Specific Expression of Intermediate Filament Proteins in a Cultured Epithelial Cell Line from Bovine Mammary Gland

ERIKA SCHMID, DOROTHEA L. SCHILLER, CHRISTINE GRUND, JOACHIM STADLER, and WERNER W. FRANKE

Division of Membrane Biology and Biochemistry, Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany

ABSTRACT Different clonal cell lines have been isolated from cultures of mammary gland epithelium of lactating cow's udder and have been grown in culture media containing high concentrations of hydrocortisone, insulin, and prolactin. These cells (BMGE+H), which grow in monolayers of typical epithelial appearance, are not tightly packed, but leave intercellular spaces spanned by desmosomal bridges. The cells contain extended arrays of cytokeratin fibrils, arranged in bundles attached to desmosomes. Gel electrophoresis shows that they synthesize cytokeratins similar, if not identical, to those found in bovine epidermis and udder, including two large (mol wt 58,500 and 59,000) and basic (pH range: 7–8) and two small (mol wt 45,500 and 50,000) and acidic (pH 5.32 and 5.36) components that also occur in phosphorylated forms. Two further cytokeratins of mol wts 44,000 (~pH 5.7) and 53,000 (pH 6.3) are detected as minor cytokeratins in some cell clones. BMGE+H cells do not produce vimentin filaments as determined by immunofluorescence microscopy and gel electrophoresis. By contrast, BMGE-H cells, which have emerged from the same original culture but have been grown without hormones added, are not only morphologically different, but also contain vimentin filaments and a different set of cytokeratins, the most striking difference being the absence of the two acidic cytokeratins of mol wt 50,000 and 45,500.

Cells of the BMGE+H line are characterized by an unusual epithelial morphology and represent the first example of a nonmalignant permanent cell line in vitro that produces cytokeratin but not vimentin filaments. The results show that (*a*) tissue-specific patterns of intermediate filament expression can be maintained in permanent epithelial cell lines in culture, at least under certain growth conditions; (*b*) loss of expression of relatively large, basic cytokeratins is not an inevitable consequence of growth of epithelial cells in vitro; (*c*) vimentin filaments are not necessary for permanent growth of epithelial cells in vitro. Our results further show that, during culturing, different cell clones with different cytoskeletal composition can emerge from the same cell population and suggest that the presence of certain hormones may have an influence on the expression of intermediate filament proteins.

The vertebrate cell, whether grown in the body or cultured in vitro, contains an extensive and complex cytoskeleton in which three different major categories of filamentous structures are integrated: microfilaments formed by actin, microtubules containing α- and β-tubulin, and intermediate-sized filaments composed by a class of structurally related proteins collectively described as intermediate filament proteins. These structures can be made in different cells by different proteins of the same class. Especially heterogeneous are the intermediate filaments which display cell-type specific patterns of composition (4, 21, 40, 51, 57; for reviews see references 1 and 39); (*a*) α-keratin proteins in epithelial cells ("cytokeratins"; 25, 55, 56); (*b*) vimentin of mol wt 57,000 in filaments of various non-epithelial cells, in particular those of mesenchymal origin; (*c*) desmin in

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various types of muscle cells; (*d*) glial filaments in certain glia cells, notably astrocytes; and (*e*) neurofilaments in neuronal cells. Of these intermediate filament classes the cytokeratin filaments again show cell type-specific diversity in that different epithelial cells form structurally identical tonofilaments from different combinations of cytokeratin subunit polypeptides (10, 13, 15, 22, 26–29, 41, 43, 52, 63). The various cytokeratin polypeptides differ by molecular weight from 40,000 to 68,000, and by electrical charge, with isoelectric values ranging in denatured molecules from a pH of ~5.0 to 8.5 (15).

When tissue cells are dissociated and grown in vitro they often cease to synthesize certain proteins characteristic of the state of cell differentiation in the tissue ("dedifferentiation"). On the other hand, they may start to produce proteins not found in the corresponding tissues. In relation to the expression of intermediate filament proteins in cultured epithelial cells two remarkable phenomena have been observed: (*a*) Most permanent cell lines derived from epithelial cells still express cytokeratin type filaments but, in addition, also synthesize vimentin filaments not found in the epithelia of the tissue of their origin (14, 16, 20–24, 54, 60). (*b*) Epithelial cells grown in culture often express other cytokeratin patterns than their parental cells in the tissue (10, 14–16, 27–30, 37, 56). In particular it has been noted by Green and co-workers that keratinocytes put into culture discontinue the production of certain relatively large keratins characteristic of epidermal differentiation *in situ* ("keratinization"; 27–29, 37, 55, 56). These observations suggest that epithelial cells change their cytoskeletal composition during culturing, and that the maintenance of synthesis of the cell type-specific cytoskeleton is under environmental control. In the present study we show that culturing in vitro of epithelial cells from the same tissue, lactating udder of cow, can give rise to diverse, clonally stable cell lines that grossly differ in cytoskeletal composition and morphology. In particular, we describe a permanent epithelial cell line (BMGE+H) continuously grown in the presence of high concentrations of hormones (insulin, hydrocortisone, and prolactin) that has maintained the tissue-specific cytokeratin pattern and does not produce vimentin.

MATERIALS AND METHODS

Cells

Mammary gland epithelial cells were obtained from lactating cow udder using the dissociation procedure recommended by Kerkhof and Abraham (35) and modified as previously described (25). Cell cultures of passage 10 were divided and grown under different culture conditions. One portion was propagated in Dulbecco's minimal essential medium (DME) supplemented with 20% fetal calf serum and hormones (insulin, hydrocortisone, and prolactin; 1 μg/ml each) for about four years (passage No. 380 at the time of submission of this paper). Cell clones were isolated with the use of cloning cylinders and, alternatively, clones were established by using microtiter plates: some hours after seeding of highly diluted cell suspensions the micro-wells were checked under the microscope, wells containing only a single cell were labeled and only clones derived from a single cell were selected. 18 clonal lines of these hormone-adapted cells (BMGE+H) were propagated and grown under identical conditions. The cells became confluent after 3–4 d and the subcultivation ratio was 1:4 or 1:5. We also established a permanently growing cell line by separating the other portion of the cell population at passage 10 and by growing it continuously in DME without hormones added. This cell line (BMGE−H) is distinguished from BMGE+H cells by its morphology, by presence of vimentin and by its different cytokeratin polypeptide pattern.

Bovine kidney epithelial cells of the MDBK line (42; ATCC CCL 22) were obtained from Flow Laboratories (Bonn, F. R. G.) and grown in Eagle's MEM supplemented with nonessential amino acids and 10% fetal calf serum.

For radioactive labeling cell cultures grown to half confluency were washed with methionine-free MEM and labeled for 18–24 h with 50 μCi ^{35}S-methionine/ml (spec. radioactivity 1,000 Ci/mmole, New England Nuclear, Boston, MA) added to the culture medium containing 90% MEM and 10% fetal calf serum but only one fifth of the normal methionine concentration.

Tissues

Lactating udder tissue of healthy cows was obtained at a local slaughterhouse. Bladder, tongue, and snout tissues were freshly taken at slaughter from cows or calves. The urothelial cells were scraped off from the inner surface of the bladder; tongue mucosa and epidermis enriched in *stratum spinosum* from muzzle were prepared as described (15, 19).

Antibodies

The following previously characterized antibodies were used: (*a*) various preparations of guinea pig antibodies to prekeratin from desmosome-attached tonofilaments of bovine muzzle or bovine hoof (13, 15, 17, 25). (*b*) Affinity-purified rabbit antibodies against bovine hoof prekeratin (33). (*c*) Guinea pig antibodies against gel electrophoretically purified polypeptide VII of desmosome-associated tonofilaments from bovine muzzle (17). (*d*) Guinea pig antibodies against desmosomal plaque protein isolated from desmosome-rich fractions of bovine muzzle (19). (*e*) Guinea pig antibodies (IgG fractions and affinity-purified IgG) against human and murine vimentin obtained from cytoskeletons of SV40-transformed human skin fibroblasts (SV-80 cells) or mouse 3T3 cells (21, 24). (*f*) Rabbit antibodies against tubulin from chicken embryo brain (31). (*g*) Rabbit antibodies against actin (17). FITC-labeled goat anti-rabbit IgG and rabbit anti-guinea pig IgG were used as second antibodies (Miles-Yeda, Rehovot, Israel). For double-labeling the second antibodies were passed through the heterologous immunoadsorbent to eliminate any cross-reactivity.

Cytoskeletal Preparations

Confluent cell cultures were extracted with Triton-X-100 and 1.5 M KCl buffers as described (14). Pellets enriched in intermediate filaments were directly used for gel electrophoresis or were frozen at −20°C. Cytoskeletal fractions from tissues were prepared from fresh or frozen samples using the procedures as described (15). Intermediate filament-enriched fractions were either directly applied to gel electrophoresis or were first purified by one cycle of solubilization in urea or guanidinium hydrochloride and reconstitution as described (49).

Gel Electrophoresis and Immunological Identification of Polypeptides

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was according to Laemmli (38). For two-dimensional gel electrophoresis according to O'Farrell (45) samples were solubilized directly in lysis buffer containing 9.5 M urea, or they were first dissolved by boiling in 5% SDS sample buffer precipitated by addition of cold acetone and dried as described (14, 26). Alternatively, for better identification of basic proteins, non-equilibrium pH gradient electrophoresis according to O'Farrell et al. (46) was used. The reaction of antibodies with electrophoretically separated polypeptide bands was visualized by the immunoblot technique on nitrocellulose paper (59) using the modifications described (15, 26).

Analysis of Proteolytic Cleavage Products

Aliquots (100–200 μg) of unlabeled bovine muzzle prekeratin polypeptides I or VII (cf. reference 17) prepared on SDS polyacrylamide gels as described above were homogenized in buffer containing 0.5% SDS as recommended by Cleveland et al. (8) and mixed with ^{35}S-methionine-labeled proteins of excised polypeptide bands from cytoskeletons of the specific cell under investigation. After brief boiling (2 min) V8-protease from *Staphylococcus aureus* (Miles Co., Elkhart, IN; frozen aliquots, dissolved in 2 mM Tris-HCl, pH 7.0) was added to a maximum representing one-tenth (wt/wt) of the protein examined. Incubation was carried out at 37°C for 2 h and was stopped by addition of 2-mercaptoethanol (final concentration: 5%) and subsequent boiling for 2 min. The total digest solution was then examined by SDS polyacrylamide (18%) gel electrophoresis using the conditions described by Thomas and Kornberg (58). For tryptic peptide mapping, individual bands excised after one-dimensional gel electrophoresis or spots excised from two-dimensional gel electrophoresis were iodinated with ^{125}-I (0.2 mCi per gel piece), digested with trypsin and analysed by chromatography and electrophoresis as described by Elder et al. (11).

Microscopy

Phase-contrast photographs were performed with a Zeiss photomicroscope III using glutaraldehyde-fixed cells grown on coverslips. For scanning electron

microscopy, cells grown on coverslips were thoroughly washed with PBS, fixed with 2.5% buffered glutaraldehyde for 1 h at 4°C, postfixed for 1 h with aqueous 2% OsO_4, dehydrated in acetone, dried by the critical point method, and processed as described (47). For transmission electron microscopy, cells grown on coverslips were fixed, dehydrated, embedded, and processed for sectioning as described (20).

Immunofluorescence microscopy was performed using cells grown on coverslips as described (13, 24). For double antibody labeling the specific rabbit and guinea pig antibodies were applied simultaneously; after washing, cells were incubated with a mixture of the corresponding FITC (fluorescein isothiocyanate)- and RH (rhodamine-lissamine sulfonylchloride)-labeled goat anti-species IgG antibody preparations.

RESULTS

We have obtained different cell lines from the same culture of mammary gland cells from lactating cow's udder. Three major cell types have been compared in detail all of which are identified as epithelial by the presence of numerous desmosomes and intermediate filaments of the cytokeratin type: (*a*) Cells of the BMGE–H line also contain filaments of the vimentin type, show cuboidal morphology and, at confluency, make close cell-to-cell contact, similar in growth pattern to the kidney epithelial cell lines MDCK and MDBK (42; cf. references 7 and 44); this line has been established from cultures grown without additions of hormones to the culture medium. (*b*) Several clonal cell lines that have been isolated from cultures grown in the continuous presence of hormones (insulin, hydrocortisone, prolactin) added to the culture medium all express a specific set of cytokeratin polypeptides but lack vimentin filaments. These cells (BMGE+H, clones 1–17) differ in their growth pattern from BMGE–H and MDBK cells and form monolayers of cells that do not present the typical architecture characteristic of polar epithelia and, for most of the lateral surface, are separated by an intercellular space of 0.5–1.0 μm traversed by numerous villuslike projections and desmosome-containing bridges (Figs. 1 and 2). None of these cell lines forms "domes" or "blisters" as described for several epithelial cell cultures, mammary gland cells included (e.g., references 7, 44, and 48). Upon subcutaneous injection into nude mice (cf. reference 19) both BMGE+H and BMGE–H cells form nodules or cysts with lobular structures but not malignant tumors.

In the present study we describe the BMGE+H cell line as a line of special epithelial morphology that has maintained the expression of tissue-specific cytokeratins but, unlike most other epithelial cells in culture, does not form vimentin filaments.

Morphology of BMGE + H Cells

From light microscopy of densely grown monolayers of BMGE+H cells (Fig. 1 *a*) as well as from surface scanning electron microscopy (Fig. 1 *b*) it is evident that the cells are separated by intercellular spaces bridged by numerous projections from both adjacent cells. Transmission electron microscopy of nearly vertical sections through such cell layers (Fig. 2) shows that these projections are of two different kinds: (*a*) Mostly slender microvilluslike protrusions of diameter 50–80 nm are frequent and the tips of some of them appear to make contact with the surface of the neighboring cell (Fig. 2 *a* and *b*). Similar microvilluslike projections are also numerous on the apical cell surface (Fig. 1 *b* and 2 *a*). (*b*) Intercellular bridges of larger diameter (from 0.5 to 1.5 μm) containing typical desmosomes and attached bundles of cytokeratin filaments are less frequent than the microvilli projections but are very characteristic of this cell-type (Fig. 2 *a–c*). Such intercellular bridges contain variable numbers (1–4) of desmosomes

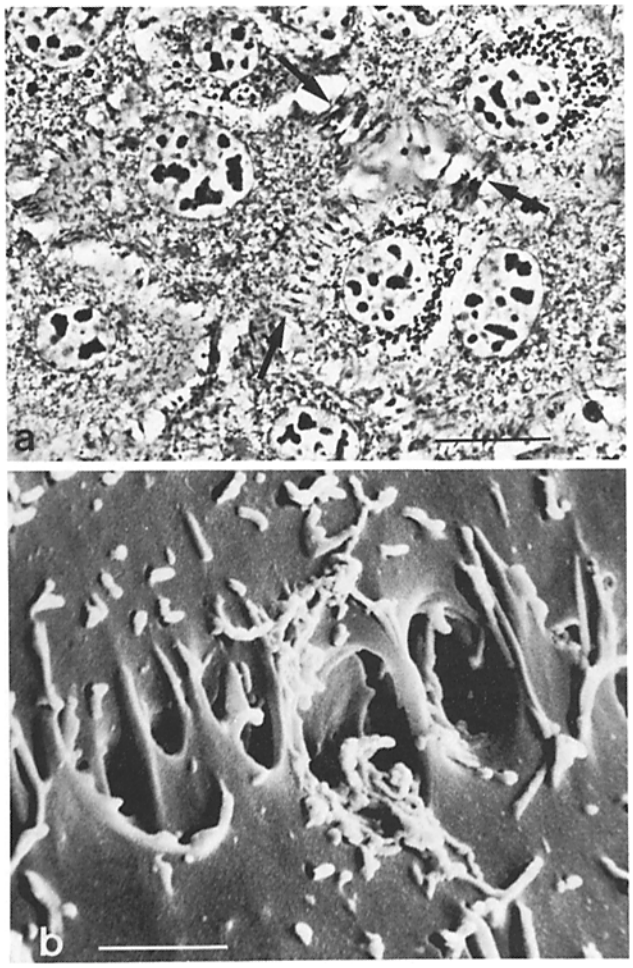

FIGURE 1 Phase-contrast light microscopy (*a*) and surface scanning electron microscopy (*b*) of BMGE+H cells showing the typical epithelial morphology at confluency. The intercellular gaps are bridged by cytoplasmic projections including desmosomal bridges (some are denoted by arrows in *a*). Bars, 50 μm (*a*) and 2 μm (*b*). *a*, × 300; *b*, × 8,500.

that are often closely spaced, sometimes suggestive of fusion of neighboring desmosomes (Fig. 2 *c*). At bottom surfaces typical hemidesmosome attachment sites make contact with the substratum (Fig. 3 *a*), in addition to extensive focal adhesions associated with microfilaments. Symmetrical desmosomelike or asymmetric hemidesmosomelike formations are often also seen deeper in the cell cytoplasm (e.g., Figs. 2 *c* and 3 *b* and *c*), but we have not been able to decide whether a given membrane-bound space represents a deep invagination from the cell surface or an endocytosed desmosome-derived vesicle. Surprisingly, asymmetric attachment plaques of cytokeratin filament bundles are also occasionally seen on apical cell surfaces (Fig. 3 *d*), but in this position do not show the midline-equivalent structures.

Only very rarely have we observed tight and gap junctions between adjacent BMGE+H cells, and extended junctions of the adhaerens type were visualized neither by electron microscopy nor by immunofluorescence microscopy using antibodies to vinculin (not shown).

The cytoplasm of BMGE+H cells is traversed by bundles of microfilaments, most of them located toward the bottom side, by microtubules and intermediate-sized filaments, the latter occurring both in densely fasciated bundles and in individual

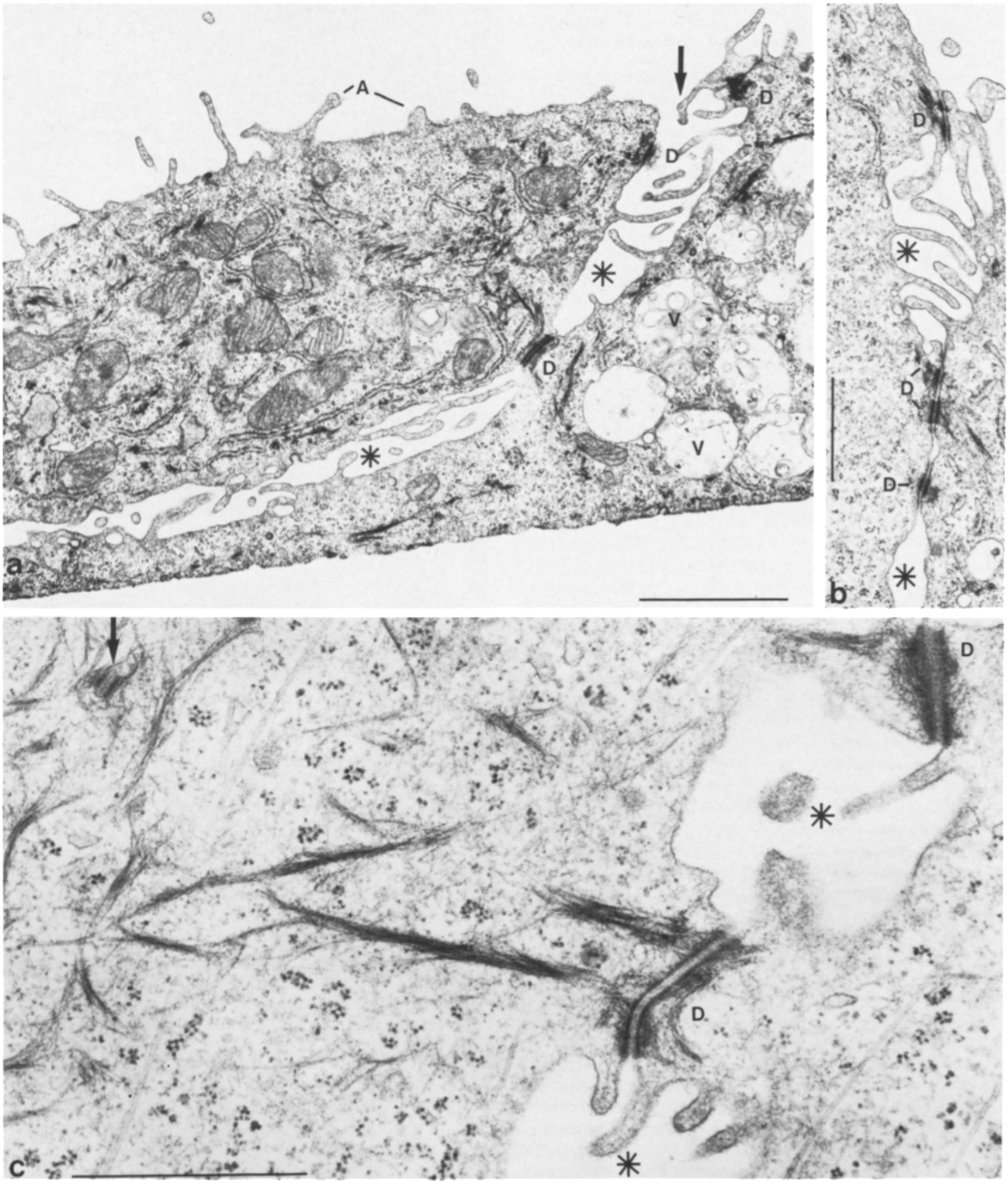

FIGURE 2 Electron micrographs of sections through monolayer cultures of BMGE+H cells fixed on the culture dish. (*a*) Vertical section showing an intercellular space (arrow and asterisks) which is traversed by microvilluslike projections and intercellular bridges containing desmosomes (D). *A*, apical surface; *V*, vacuoles including secondary lysosomes. (*b*) Details of intercellular spaces showing desmosome-containing bridges of various diameters. (*c*) Higher magnification of intercellular space showing desmosomal bridges, the attached tonofilament bundles and the cytoplasmic cytokeratin filament meshwork. Bars, 2 μm (*a*) and 1 μm (*b* and *c*). *a*, × 13,000; *b*, × 18,000; *c*, × 40,000.

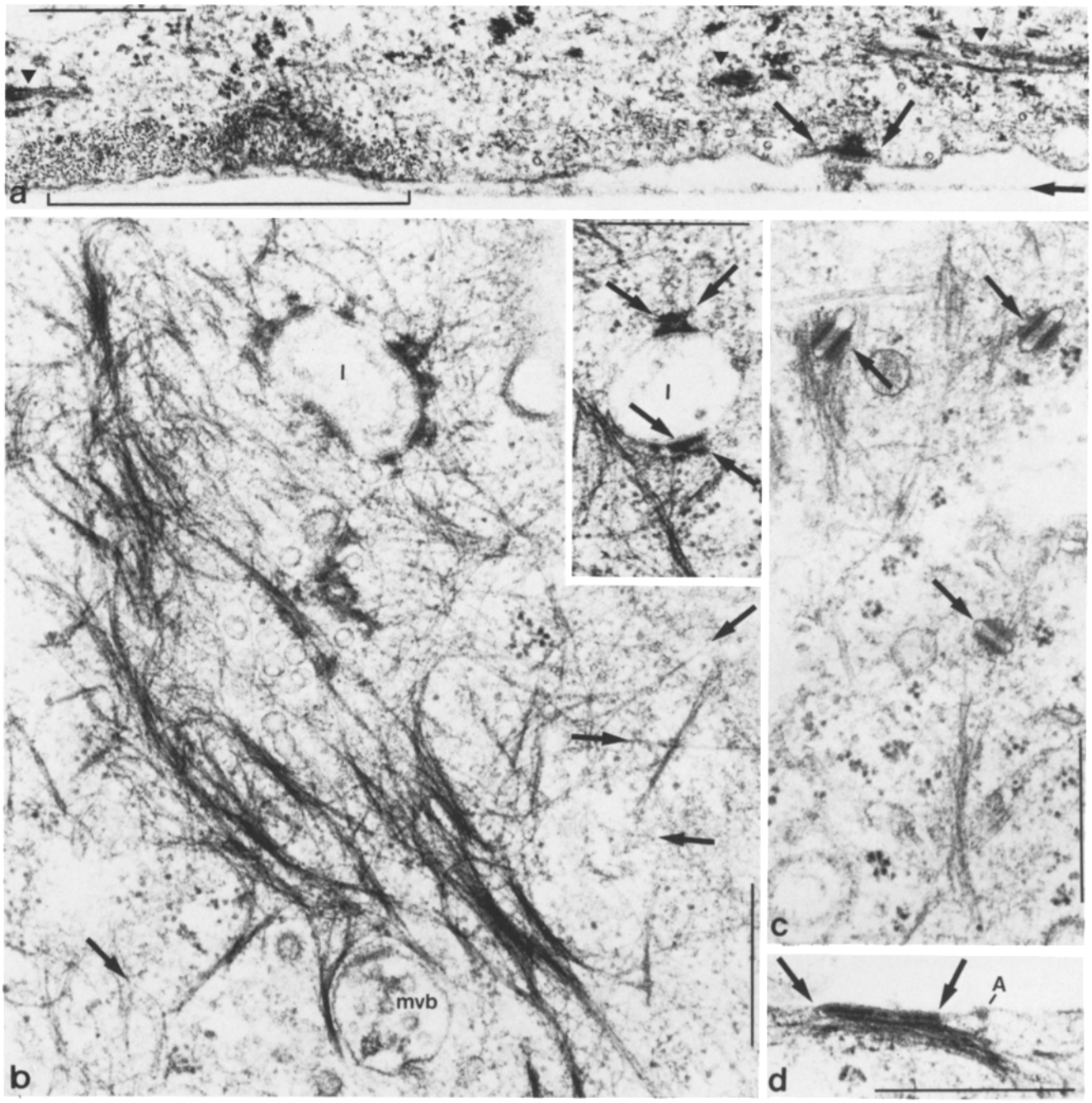

FIGURE 3 Ultrastructural details of BMGE+H cells in sections: (*a*) Contact specializations of the bottom cell surface (substratum denoted by horizontal arrow in the right) such as adhesion plaques (*a*, bracket) associated with bundles of microfilaments and hemidesmosomes associated with cytokeratin-filaments (*a*, pair of arrows). (*b*) Hemidesmosomelike formations at surface invaginations (*I*: arrows in insert of *b*) and cytokeratin filaments either in bundles or as individual filaments (some are denoted by arrows); *mbv*, multivesicular body. (*c*) Intercellular desmosome-equivalent structures (arrows), representing either local invaginations or endocytosed desmosomes, associated with tonofilament bundles. (*d*) Desomosome-equivalent plaque (denoted by pair of arrows) at the apical (*A*) surface and associated bundle of cytokeratin filaments. Bars, 0.5 μm (*a–c*) and 1 μm (*d*). *a–c*, × 50,000; *d*, × 32,000.

filaments (e.g., Figs. 2 *c* and 3 *b*). In addition, numerous coated pits and smooth surfaced cavaeolae are regularly seen, most prominently at bottom surface. BMGE+H cells also form, especially in dense cultures (e.g., 4–5 d after plating) extended cytoplasmic aggregates of glycogen particles (not shown).

Immunofluorescence Microscopy of Cytoskeletal Proteins

The typical display of major cytoskeletal components of BMGE+H cells is illustrated by immunofluorescence microscopy in Figs. 4 and 5. As in other epithelial cell cultures (e.g., reference 9) most of the actin visualized by staining with actin antibodies (Fig. 4 *a*) is associated with typical actin "cables", i.e., microfilament bundles, which are either concentrically arranged, especially in the cell periphery, or form "stress fibers" located toward the bottom part of the cell. Microtubules extend throughout the whole cytoplasm, often showing a higher density in the nuclear vicinity, similarly as described for other cells

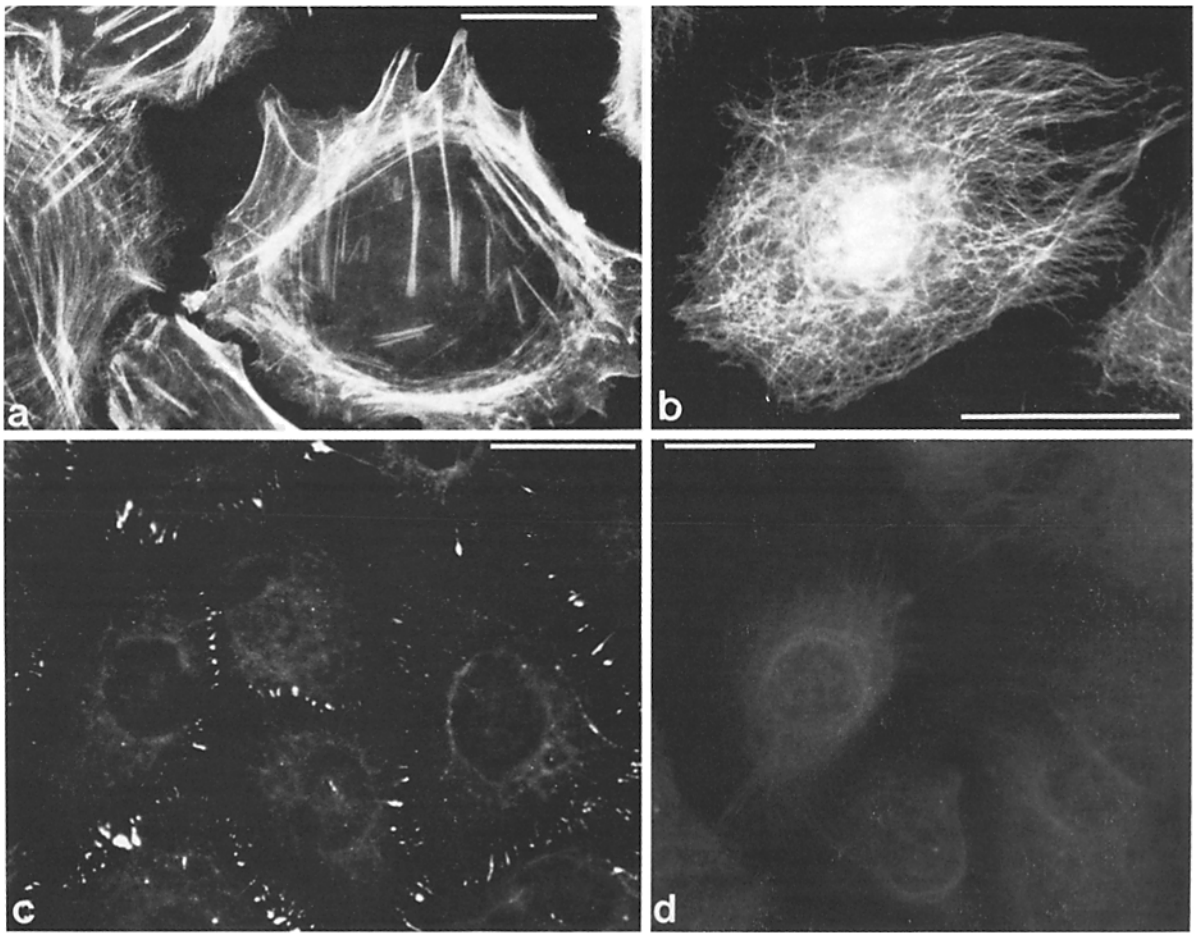

FIGURE 4 Immunofluorescence microscopy of cytoskeletal proteins in BMGE+H cells, showing the display of fibrils stained by antibodies to actin (*a*) and tubulin (*b*) as well as the localization of desmosomes by antibodies to desmosomal plaque protein (*c*). Antibodies to vimentin (*d*) are negative thus demonstrating the absence of vimentin filaments. Bars, 30 μm. *a*, × 600; *b*, × 970; *c*, × 640; *d*, × 670.

grown in culture, epithelia included (Fig. 4 *b*; cf. reference 9). Antibodies to desmosomal plaque protein allow the specific visualization of desmosomal structures (Fig. 4 *c*), irrespective whether they are associated with true desmosomes or hemidesmosomes and other related formations.

Staining of BMGE+H cells with various antibodies against vimentin has not revealed any fibrillar staining in the cytoplasm (Fig. 4 *d*). By contrast, various antibody preparations to epidermal prekeratins have shown intense staining of the cytoplasmic meshwork of cytokeratin fibrils (Fig. 5 *a* and *b*). With these antibodies, the cell colonies usually show correspondence of fibrillar pattern in adjacent cells, obviously reflecting the staining of tonofilament bundles associated with desmosome-containing bridges (Fig. 5 *a* and inset in Fig. 5 *b*). Such associations of adjacent cells through intercellular bridges positively stained for cytokeratin filaments are maintained in mitotic cells (e.g., lower left in Fig. 5 *a*). Single cells, as they are especially frequent in freshly plated cultures, do not exhibit special foci of association of cytokeratin bundles with the cell periphery but rather show a dense uniform cytoplasmic meshwork of cytokeratin fibrils (Fig. 5 *b*). Using double immunofluorescence staining of the same cells with antibodies to cytokeratin and antibodies to desmosomal plaque protein we have traced the spatial relationship of cytokeratin filaments and desmosomes in the intercellular bridges (Fig. 5 *c* and *d*).

We have compared the arrays of cytoskeletal elements of BMGE+H cells with those present in BMGE−H cells, i.e., a cell line derived from the same primary culture, but grown in the absence of hormones. As shown in Fig. 6 BMGE−H cells form epithelial monolayer colonies characterized by dense and direct cell-to-cell contact over most of their lateral cell surfaces (Fig. 6 *a*; electron microscopy not shown). Desmosomal connections are very frequent in these cells as is shown by staining with antibodies to desmosomal plaque protein (Fig. 6 *b*). BMGE−H cells also exhibit a dense meshwork of cytokeratin filaments extending through the whole cytoplasm (Fig. 6 *c*), but in addition contain arrays of filaments stained with vimentin antibodies (Fig. 6 *d*). These vimentin fibrils often appear to be restricted to peripheral portions of the cytoplasm. Not infrequently, situations can be found in which, in adjacent BMGE−H cells, the "bushes" of vimentin filaments appeared to be located to opposite poles, leaving the intercellular boundary practically free of vimentin material (not shown, see Fig. 7 *b* in reference 24). Distributions of actin and tubulin filaments are similar to that described for BMGE+H cells (see above).

The display of intermediate filaments in MDBK cells, which have maintained pronounced polar architecture and function, is very similar to that in the BMGE-H cell line. MDBK cells contain numerous desmosomal structures (22). The meshwork of filaments stained with antibodies to prekeratin consists of somewhat thinner fibrils, mostly concentrated around the nucleus and projecting onto desmosomes (18, 19). Like BMGE-H

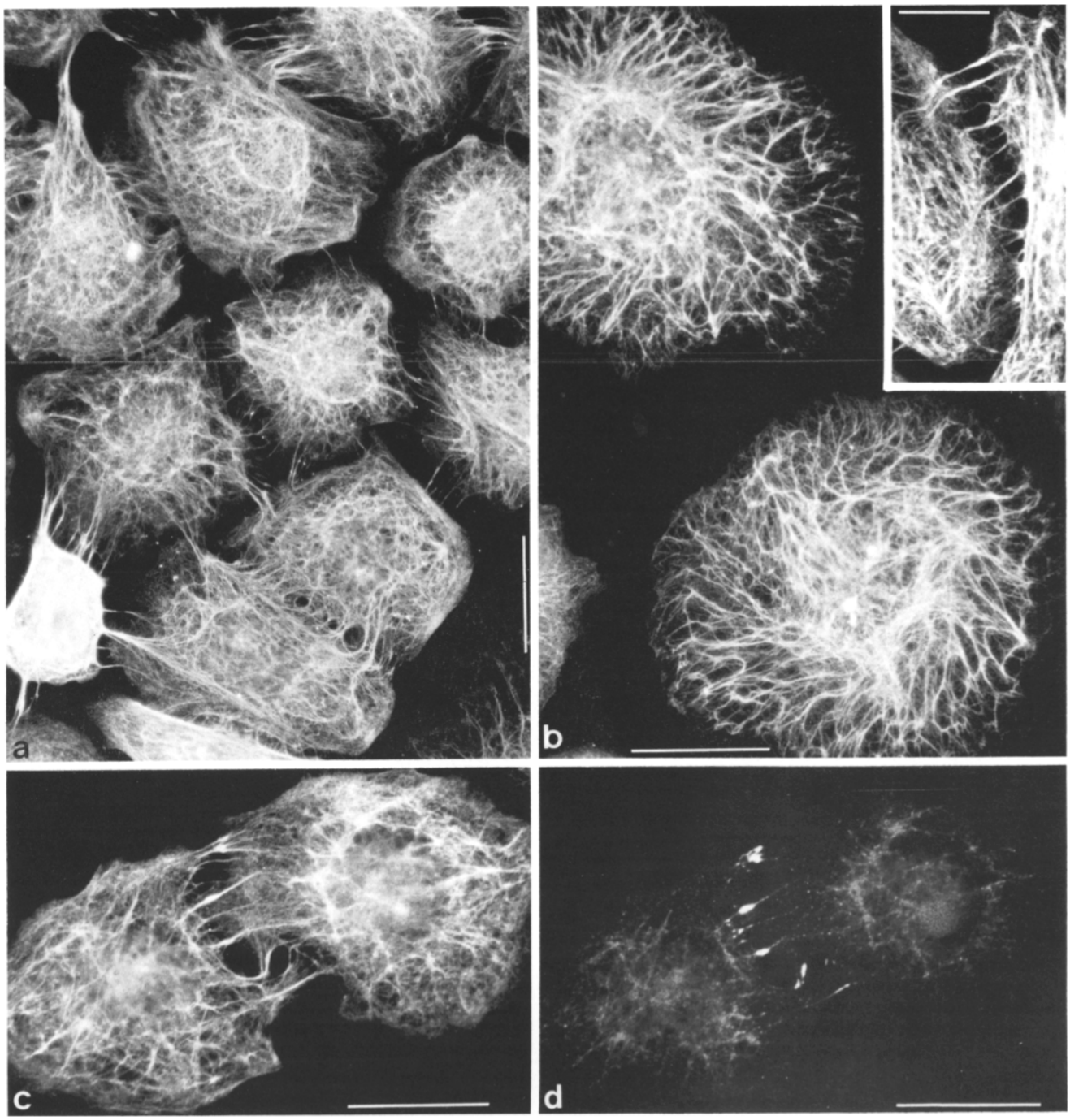

FIGURE 5 Arrangement of cytokeratin fibrils in BMGE+H cells shown by immunofluorescence microscopy using antibodies raised against prekeratin polypeptide VII of desmosome-attached tonofilaments from bovine muzzle (*a* and *b*) and antibodies against total prekeratin from bovine muzzle (inset in *b*) or from bovine hoof (*c*). Note the strong reaction of tonofibrils in the intercellular bridges (*a*, and insert in *b*) which is preserved in some bridges during mitosis (lower left in *a*). The random distribution of cytokeratin fibrils in a cytoplasmic meshwork in single cells of sparse cultures is demonstrated in (*b*). Double-labeling of the same cells with antibodies against cytokeratin (*c*) and antibodies against desmosomal plaque protein (*d*) shows desmosome-specific staining in the central part of the intercellular bridges. Bars, 30 μm (*a–d*) and 20 μm (insert in *b*). *a*, × 600; *b*, × 760; *c* and *d*, × 750.

cells, MDBK cells also produce vimentin filaments, and here also the display of fibrils stained with vimentin antibodies shows accumulation in "bushes" at the cell periphery (22).

Electrophoresis of Cytoskeletal Proteins

SDS PAGE of polypeptides present in cytoskeletal preparations from BMGE+H cells is characterized by three major bands of mol wt 59,000 (K 59), 58,500 (K 58.5) and 50,000 (K 50) and a minor band of mol wt 45,500 (K 45.5; Fig. 7 *b*, slot *3*). By contrast, BMGE-H cytoskeletons contain, in addition to K 58.5 and K 59, which are minor polypeptides in these cells, two major polypeptides of mol wt 53,000 (K 53) and 44,000 (K 44) and one minor band of mol wt 45,000 (K 45), but lack polypeptides K 45.5 and K 50 (Fig. 7 *b*, slot *1*). The polypeptide of apparent mol wt 57,000 co-migrates with vimentin (cf. Fig. 7, slot *4*). Unexpectedly, we have found a striking similarity of

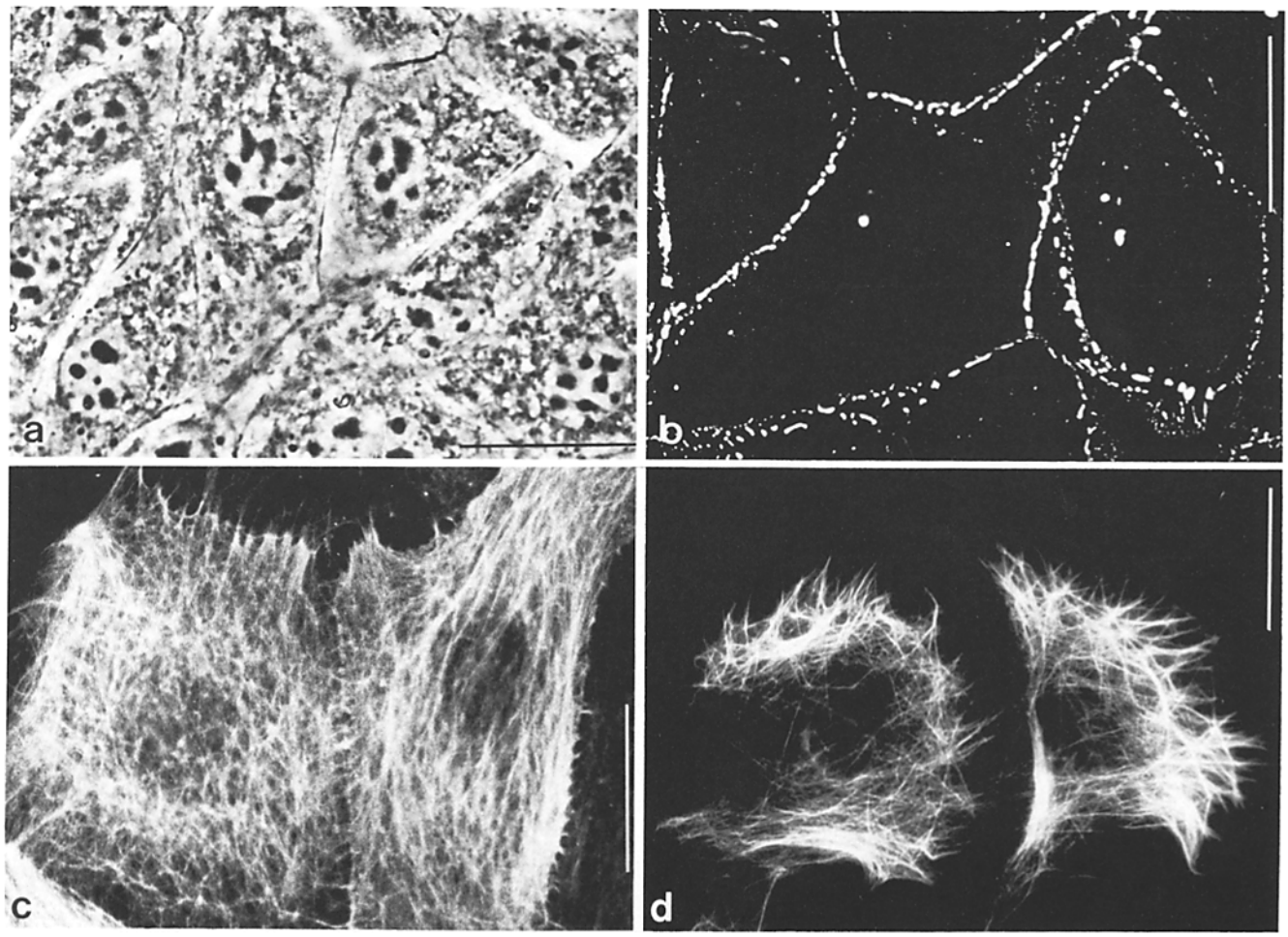

FIGURE 6 Morphology and arrays of cytoskeletal elements of BMGE-H cells, i.e., bovine mammary gland epithelial cells grown without addition of hormones, shown by phase contrast microscopy (*a*) and by immunofluorescence microscopy, using antibodies against desmosomal plaque protein (*b*), bovine hoof prekeratin (*c*), and vimentin (*d*). Note abundance of desmosome-protein containing structures at cell boundaries (*b*). The dense network of cytokeratin fibrils (*c*) extends through the whole cytoplasm, whereas the vimentin filaments (*d*) are enriched in the periphery. Bars, 30 μm. *a* and *b*, × 780; *c*, × 760; *d*, × 640.

cytoskeletal polypeptide patterns between BMGE-H and MDBK cells, i.e., cells derived from different organs (Fig. 7, slots *1* and *4*).

The typical cytoskeletal protein pattern of BMGE+H cells has been found in all 17 cell clones examined, the only difference being that in some clones variable, usually very low amounts of the minor cytokeratin K 53 are also detected (e.g., Fig. 8*b*).

The cytokeratin nature of the cytoskeletal components described above for BMGE+H cells has been identified by immunoblot experiments using cytokeratin antibodies (an example is shown in Fig. 7*b*). The cytokeratin polypeptides K 44, K 45, K 53, and K 59 from BMGE-H cells have also been identified as cytokeratins by immunoblotting (not shown).

We have recently shown that cytokeratin polypeptides from different tissues and cells differ greatly in their isoelectric points over a pH range from approximately 5.0 to 8.5 (15), and that certain cytokeratin bands seen on SDS PAGE can contain several polypeptides of different electrical charges. Therefore, we have analyzed the cytoskeletal proteins of the different cell lines by two-dimensional gel electrophoresis, using both isoelectric focusing and nonequilibrium pH gradient electrophoresis (Figs. 8 and 9). The two large cytokeratins K 58.5 and K 59 are neutral-to-basic (pH 7.0–8.0), whereas cytokeratin components K 50 and K 45.5 are more acidic than actin (Fig. 8*a–c*). A minuscule cytokeratin component K 53, which is almost isoelectric with serum albumin and is observed in some cell clones of the BMGE+H line, appears to be identical to cytokeratin "A" as described in hepatocytes and intestinal cells (13, 15, 26).

All cytokeratin components appear in the form of series of isoelectric variants, which is most prominent for polypeptides K 58.5 and K 59, both allowing the resolution of at least four different variants (Fig. 8*a* and *b*). When cytoskeletal proteins isolated from BMGE+H cells labeled in vivo with ^{32}P-phosphate are examined by gel electrophoresis, it is evident that in each cytokeratin the most basic variant spot represents the nonphosphorylated polypeptide and all the other more acidic isoelectric variants have incorporated phosphate (Fig. 8*c*). Corresponding findings have been made for prekeratins of cultured human and murine keratinocytes (32, 53, 56) and for cytokeratins of murine hepatocytes and human HeLa cells (15).

Vimentin is not detected in BMGE+H cells (Fig. 8*a, b* and *g*), not even in trace amounts in overloaded gels. Comparison with cytoskeletal proteins from lactating udder of cow (Fig. 8*d*) shows that polypeptides similar to cytokeratins K 50, K

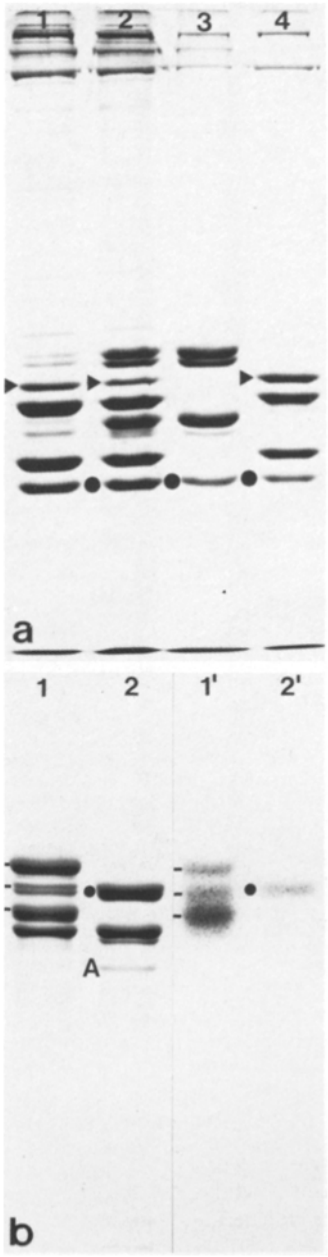

FIGURE 7 (*a*) SDS PAGE of cytoskeletal preparations of three bovine epithelial cell lines: BMGE − H (slot *1*), BMGE + H (slot *3*) and MDBK (slot *4*). Co-electrophoresis (slot *2*) of cytoskeletal proteins from BMGE − H and BMGE + H shows that only polypeptides K 58.5 and K 59 co-migrate. Vimentin (denoted by arrowheads) is found in BMGE − H (slot *1*) and MDBK (slot *4*) cells. (*b*) SDS PAGE of cytoskeletal polypeptides and their reaction with antibodies against bovine muzzle prekeratin using the immunoblot technique. Slot *1*: Coomassie Blue staining of epidermal prekeratin from calf muzzle; slot *2*: Coomassie Blue staining of BMGE + H cytoskeletal proteins; slot *1'* and *2'*: Fluorograph showing reaction of polypeptides shown in slot *1* and *2* with antibodies against epidermal prekeratin. Note that these antibodies react with prekeratins I, III/IV and VI (bars) but not with VII (slot *1'*). They also react with the BMGE + H cytokeratin band containing K 59 and K 58.5 (dot) but not with cytokeratins K 50 and K 45.5. The latter two cytokeratins, however, do react with other cytokeratin antibodies (not shown). *A*, actin.

53, K 58.5, and K 59 are also prominent in this tissue and are located, as judged from immunofluorescence microscopy, in epithelial cells (not shown; see also references 2, 17, 36, 57). Moreover, comparison with epidermal prekeratin from bovine snout (Fig. 8*e*) suggests that the three major polypeptides (K 59, K 58.5, and K 50) are similar in electrophoretic mobility to prekeratin polypeptides III, IV, and VII (13, 25). For direct comparison, cytokeratins of BMGE+H cells labeled in vivo with ^{35}S-methionine are co-electrophoresed with bovine epidermal prekeratin (Fig. 8*f* and *g*). The radioactivity of cytokeratins K 59, K 58.5, and K 50 co-migrates with prekeratins III, IV, and VII, whereas component K 45.5 and K 53 are unique to the BMGE+H cells (Fig. 8*g*). Cytokeratin K 45.5, however, co-electrophoreses with epidermal prekeratin VIII from bovine hoof and hair-follicle root sheath epithelium (24; R. Moll, D. Schiller, and W. W. Franke, in preparation). Comparison of the BMGE+H cytokeratins with those of other bovine tissues has also revealed a high degree of similarity with cytokeratins present in certain stratified squamous epithelia such as mucosa of esophagus and tongue (not shown). The different variants of cytokeratins K 58.5 and K 59 migrate to positions similar to those of cytokeratin components 1 and 2 of tongue, whereas cytokeratins K 45.5 and 50 co-migrate with cytokeratins 3 and 4 of tongue (for designations see reference 15). The minor cytokeratins K 44 and K 53 are also not seen among the major tongue cytokeratins and, vice versa, the small and acidic major cytokeratin 5, which is characteristic for tongue and esophagus, is not found in BMGE+H cells.

Differences of cytoskeletal compositions between BMGE+H cells grown in the presence of hormones and BMGE−H cells established by growth in media without additional hormones are striking (Fig. 9*a–e*). Although cytokeratins K 58.5 and K 59 are also detected in BMGE−H cells, the most striking feature of the BMGE−H cytokeratin pattern is the predominance of cytokeratins K 44 and K 53 and the absence of cytokeratins K 45.5 and K 50 (Fig. 9*a–e*). In addition, BMGE−H cells contain considerable amounts of vimentin (Fig. 9*a, c* and *e*). Direct comparison of ^{35}S-methionine-labeled cytokeratins from BMGE+H cells with unlabeled cytoskeletons of BMGE−H cells shows the relatively high amounts of cytokeratins K 44 and K 53 of BMGE−H cells with special clarity (Fig. 9*c–e*). This cytoskeletal protein pattern of BMGE−H cells is astonishingly similar to that of another bovine cell line, MDBK, derived from an epithelium of a different organ, the kidney (Fig. 9*f* and *g*). In MDBK cells, K 44 and K 53 are also major cytokeratins, and these cells also contain relatively large amounts of vimentin (Fig. 9*g;* see also references 18, 22). In our search for the occurrence of cytokeratins K 44 and K 53 in bovine tissues we have found that both cytokeratins are major cytoskeletal proteins of transitional epithelium of bladder which, however, also contains large amounts of a third major cytokeratin that is smaller (mol wt 40,000) and more acidic than actin (Fig. 9*h*) and is identical, by peptide mapping, to cytokeratin polypeptide K 40 described in intestinal cells of various mammals (15, 26) and certain human squamous carcinoma cell lines (63).

We have also examined the question whether the intermediate filament protein pattern once established in cells grown in the presence of hormones (BMGE+H) is maintained upon further growth in media in which the addition of hormones has been omitted. As is shown in Fig. 9*i*, the typical cytokeratins of BMGE+H cells are still expressed in cultures of BMGE+H cells grown for 40 passages without hormones

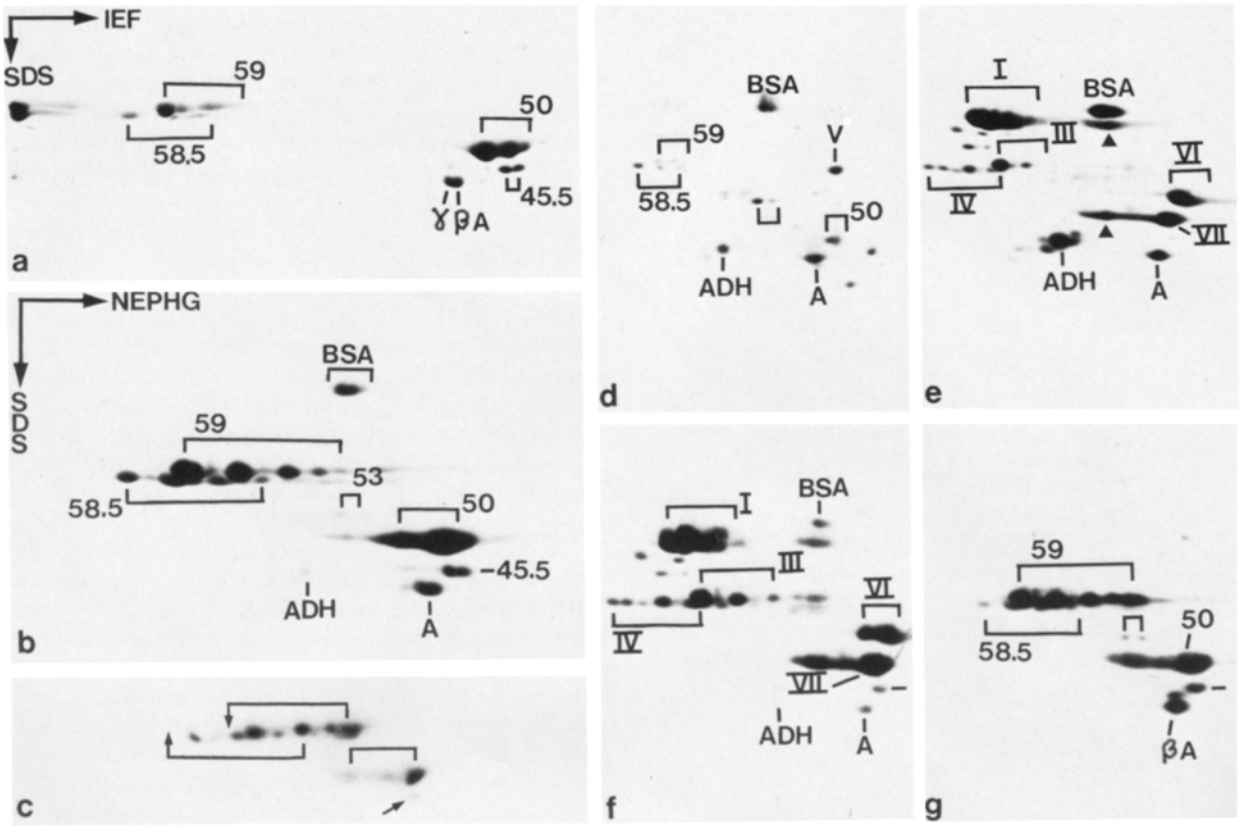

FIGURE 8 Two-dimensional gel electrophoresis (*IEF*, isoelectric focusing in first dimension, *a*; *NEPHG*, direction of nonequilibrium pH gradient electrophoresis: *b–g*; *SDS*, direction of second dimension) of cytoskeletal proteins from BMGE + H cells (*a*, *b*, *c*, and *g*), cow's udder (*d*), and muzzle epidermis (*e* and *f*). Major cytokeratin components of BMGE + H cells are indicated by brackets or horizontal bars and designated by their apparent molecular weights (in units of 10^3), epidermal prekeratin polypeptides are designated by Roman numerals as introduced previously (references 15 and 25). Reference proteins in co-electrophoresis are bovine serum albumin (*BSA*: mol wt 68,000; isoelectric pH of major variant 6.34), α-actin (*A*, mol wt 42,000; isoelectric pH 5.4), and alcohol dehydrogenase (*ADH*; mol wt 43,500; isoelectric pH 7.0). (*a*) Representive BMGE + H cell clone showing major cytokeratins and absence of vimentin (γ- and *βA*, endogenous actins). (*b*) Cytoskeletal proteins of BMGE + H cells showing better separation of the basic cytokeratins. The minor component K 53 is isoelectric with *BSA* and has been detected in some clones but not in others. (*c*) Fluorograph showing cytoskeletal material from BMGE + H cells labeled in vivo with ^{32}P-phosphate. Note that cytokeratins are labeled, including component K 45.5 (arrow), and that radioactivity is associated with the specific more acidic variants but not with the specific most basic variant (arrows at brackets). (*d*) Cytoskeletal preparation from cow's udder. *V*, vimentin, derived from mesenchymal stroma cells (for localization see reference 17). (*e*) Prekeratin polypeptides from bovine muzzle epidermis (arrowheads denote complexes of prekeratins *I* and *VII* not separated under the conditions used). (*f*) Coomassie Blue-stained gel after co-electrophoresis of unlabeled muzzle prekeratins with ^{35}S-methionine-labeled cytoskeletons from BMGE + H cells (*g*; horizontal bar in *f* denotes K 45.5 from BMGE + H cells). (*g*) Fluorograph of gel shown in (*f*), representing comigration of BMGE + H cytokeratins K 50, K 58.5 and K 59 with prekeratins VII, IV, and III. *βA*, endogenous β-actin.

added and these cells also have not begun to synthesize vimentin.

Characterization of Cytokeratin Polypeptides by Peptide Mapping

The relationship of the individual cytokeratin polypeptides expressed in BMGE+H cells to similarly sized cytokeratin polypeptides from other bovine cells and tissues has been examined by peptide mapping analysis on one-dimensional separation according to Cleveland et al. (8) and by two-dimensional separation according to Elder et al. (11). The similarity of the two major BMGE+H cytokeratins, K 50 and K 59, to certain bovine epidermal prekeratins has first been observed by analysis of the cleavage products obtained after limited digestion using protease V8, of ^{35}S-methionine labeled BMGE+H cytokeratins mixed with unlabeled prekeratins from bovine muzzle (Fig. 10*a–c*). More detailed analysis, using tryptic peptides from individual cytokeratin "spots" excised from gels after two-dimensional electrophoresis shows, for example, that BMGE+H cytokeratin K 59 is very similar, albeit not identical, to cytokeratin III from epidermis (Fig. 10*d* and *e*) and bovine udder (not shown). Comparison of the predominant acidic cytokeratin K 50 with epidermal prekeratin VII (Fig. 10*f* and *g*) and cytokeratin K 50 from cow's udder (not shown) illustrates the close similarity of these polypeptides, but also indicates the existence of some minor, yet unknown differences of the same polypeptide in the mammary gland-derived cells and in epidermis.

DISCUSSION

Adaptation of cells to growth in culture, in particular establishment of permanent cell lines, not only results in profound

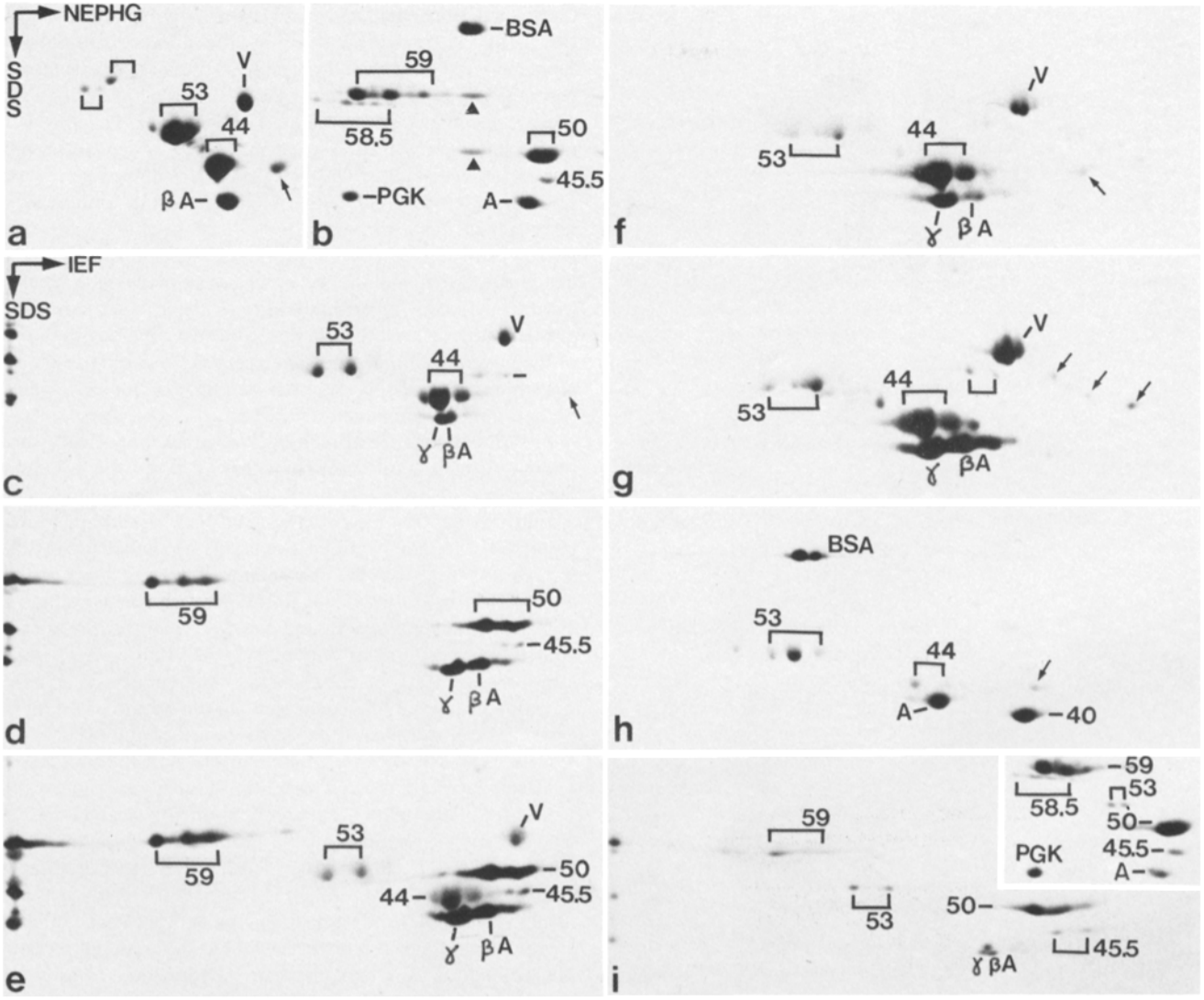

FIGURE 9 Two-dimensional gel electrophoresis (NEPHG in *a*, *b*, and inset in *i*, IEF in *c*–*i*) of cytoskeletal polypeptides from BMGE + H cells, BMGE − H cells, MDBK cells and urothelium from bovine bladder (symbols as in Fig. 10, if not indicated otherwise). (*a*) BMGE − H cytoskeletons showing predominance of polypeptides K 44 and K 53 and vimentin (*V*); the brackets in the upper left denote cytokeratins K 58.5 and K 59 which are only minor components in these cells; the arrow denotes an unusual, very acidic cytoskeletal component of mol wt 45,000 the significance of which is still unclear; *βA*, endogenous *β*-actin. (*b*) BMGE + H cytoskeletons (clone 10) for comparison (PGK, 3-phosphoglyceric kinase; mol wt ~42,000; isoelectric at pH 7.5; *A*, *α*-actin added). (*c*) Co-electrophoresis of relatively large amount of BMGE − H cytoskeletal proteins with relatively small amount of BMGE + H cytoskeletal proteins from cells radioactively labeled with ^{35}S-methionine (Coomassie Blue staining; the horizontal bar denotes a small amount of K 50 from BMGE + H cells visible in this mixture; other symbols as in *a* and *b*). (*d*) Fluorograph of gel shown in (*c*). Note different composition of BMGE + H (*d*) and BMGE − H (*c*) cytoskeletons. (*e*) Photographic superposition of photograph and fluorograph shown in (*c*) and (*d*). (*f*) Co-electrophoresis of cytoskeletal proteins from BMGE − H cells with small amounts of cytoskeletal material from MDBK cells labeled with ^{35}S-methionine (Coomassie Blue staining). (*g*) Fluorograph of gel shown in (*f*), demonstrating identity of cytokeratins K 44 and K 53 and of vimentin in both cells. In addition, a minor MDBK cytokeratin (bracket below vimentin) is seen. Arrows denote proteolytic degradation products from vimentin. (*h*) Coomassie Blue staining of cytoskeletal proteins from urothelium of cow's bladder, showing predominance of cytokeratins K 40, K 44 and K 53 (other symbols as in *a* and *b*; *A*, *α*-actin added). (*i*) Cytoskeletal polypeptides of BMGE + H cells after 40 passages of growth in medium without hormones added (inset shows the same material after nonequilibrium pH gradient electrophoresis in first dimension). Note that no drastic change in polypeptide pattern occurs after withdrawal of hormones.

changes of cell morphology and secretory functions, but often is also accompanied by changes of cytoskeletal composition. In cultures of cells derived from epithelia and epithelial tumors synthesis of relatively large amounts of vimentin filaments, in addition to maintained production of cytokeratin filaments, is a common phenomenon. This is not only observed in long-term cultures (16, 23, 54, 60), but in some cells such as rat hepatocytes begins during the first days of primary culturing, even before mitoses are noted (14, 20). The functional implications of this change in cytoskeletal composition are presently unclear.

In this study we have shown that, under certain conditions, lines of epithelial cells can be derived from bovine mammary gland that are characterized not only by the maintained syn-

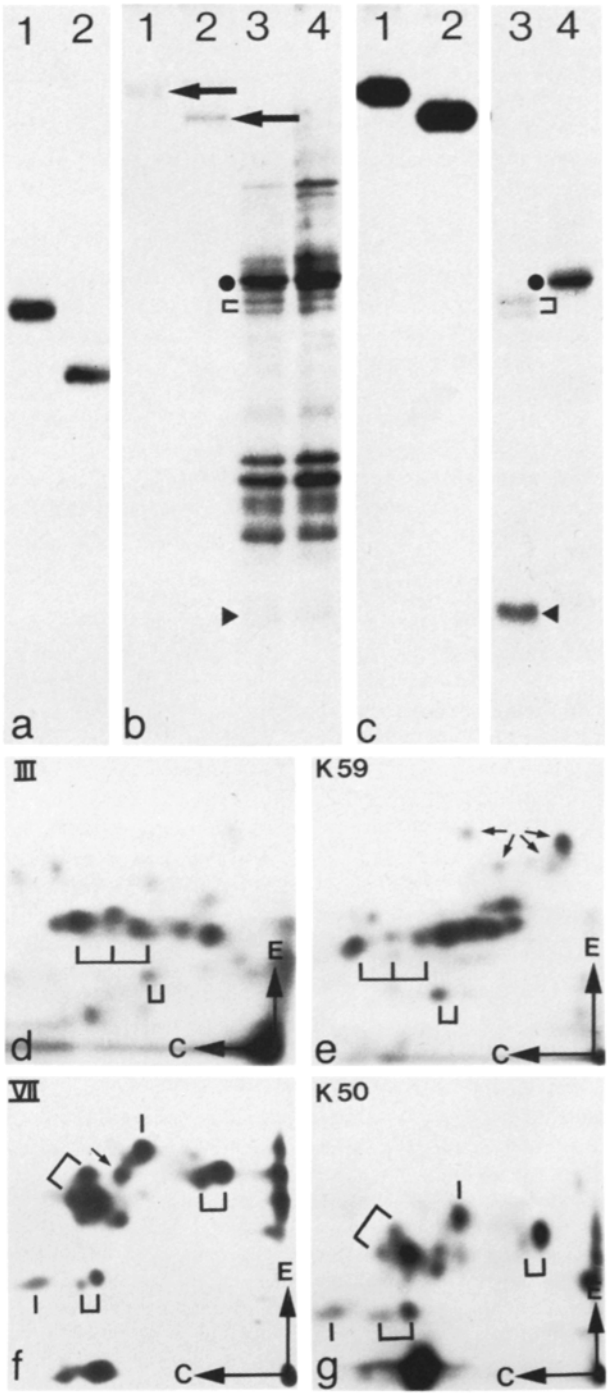

FIGURE 10 (*a–c*) SDS PAGE of ^{35}S-methionine-labeled cytoskeletal proteins from BMGE + H cells (*a*, 8.5% acrylamide gel, fluorograph; slot *1*, re-electrophoresed purified cytokeratin K 59, slot *2*, K 50) and proteolytic cleavage patterns obtained after treatment with protease V8 (*b* and *c*; 18% acrylamide). (*b*) Slots *1–4*, Coomassie Blue-stained, purified cytokeratins K 59 and K 50 from ^{35}S-methionine-labeled BMGE + H cells (arrows in slots *1* and *2*) and proteolytic fragments from bovine muzzle prekeratins (slot *3* and *4*) mixed with the BMGE + H cytokeratins K 59 and K 50 shown in slot *1* and *2* before digestion (see Materials and Methods). (*c*) Slots *1–4* fluorograph of the gel shown in (*b*) demonstrating the radioactivity of BMGE + H cytokeratins K 59 and K 50 (slots *1* and *2*), and of the major proteolytic fragments derived from K 59 (slot *3*) and K 50 (slot *4*). Note some correspondences in proteolytic cleavage patterns between BMGE cytokeratins and epidermal prekeratins (denoted by brackets, dots and arrowheads) as well as differences of major cleavage products from BMGE + H cytokeratins K 59 (*c*, slot *3*) and K 50 (*c*, slot *4*). (*d–g*) Peptide map comparisons (*E*, electrophoresis; *C*, chromatography) of radio-iodinated BMGE + H cytokeratins K 59 (*e*) and K 50 (*g*) excised after two-dimensional gel electrophoresis with prekeratin III (*d*) and prekeratin VII (*f*) from bovine muzzle epidermis. Some corresponding spots are indicated by brackets and bars, some different spots are denoted by arrows. Note also marked difference in peptide maps between relatively basic and large component(s) III/K 59 (*d* and *e*) and the smaller acidic cytokeratin(s) VII/K 50 (*f* and *g*), demonstrating the differences between these classes of cytokeratins.

thesis of desmosomal and cytokeratin polypeptides found in the tissue of origin, but also by the absence of vimentin filaments. BMGE+H cells represent the first example of a permanent line of a nonmalignant cell maintaining the cytoskeletal specificity of the epithelial cells *in situ*. Together with the recent demonstration of the absence of vimentin in a specific rat hepatoma cell line (MH_1C_1; reference 14) our findings show that vimentin filaments are not obligatory or necessary for cell growth in vitro, as they are also not necessary for cell growth in early embryogenesis (34). Our results further demonstrate that maintained synthesis of tissue-specific cytokeratin filaments is compatible with rapid and permanent growth of epithelial cells in vitro. Thus we conclude that none of the general cellular processes essential for proliferation and growth in vitro is associated with—and depending on—vimentin and vimentin filaments.

BMGE+H cells are also characterized by their special morphology, which is different from that of most other epithelial lines, most notably by the formation of intercellular gaps spanned by desmosome-containing bridges, resembling growth patterns of certain stratified squamous epithelial tissues. Although it is clear that the maintenance in vitro of such special epithelial growth forms does not depend on the formation of vimentin filaments, we cannot yet say whether this specific epithelial morphology is only seen in cells that do not synthesize vimentin. On the other hand, maintenance of a highly specific polar epithelial cell architecture in closely coupled epithelial cell sheets as it has been described for MDCK and MDBK cells (19, 44) is well compatible with the presence of relatively large amounts of vimentin filaments (18, 22, 24).

Our study also shows that an unexpectedly large number of cytokeratin polypeptides can be produced in a single cell. In all 17 BMGE+H cell clones we identified at least four different cytokeratins and two additional minor cytokeratin polypeptides (K 44 and K 53) are found in at least some of these clones. We do not know whether these different cytokeratins are located, as heteropolymers of polypeptides, in only one type of filaments or whether these cells contain different cytokeratin filaments formed by specific subsets of these polypeptides.

Both lines of cells, BMGE + H and BMGE − H, are positively identified as epithelial cells by the presence of filaments of the cytokeratin-type as well as by their desmosomes and desmosomal proteins. All cytokeratin polypeptides found in BMGE + H cells can also be detected in cytoskeletal preparations from cow's udder although some of them represent only minor components in the tissue material (not shown). However, as mammary gland tissue contains at least three morphologically and functionally different epithelial cell types (ductal, myoepithelial, and secretory) and immunological studies have indicated differences of cytokeratin determinants exposed in these three epithelial cell types (2, 17, 36), we presently

cannot decide from which cell type the BMGE + H cells have been derived (for problems of identification of mammary gland-derived cells in vitro see references 5, 6, 12, 50, 61, 62).

We have been successful in establishing a total of 17 different clonal cell lines from BMGE + H cell populations, some of which show minor, albeit stable, differences in morphology and expression of cytokeratins K44 and K53 but which all lack vimentin. By contrast, all cell clones grown from BMGE-H cells, i.e., cells from the same early cultures as used for selection of BMGE + H cells but grown in media without hormonal additions, produce vimentin filaments as well as a clearly different set of cytokeratins lacking polypeptides K 45.5 and K 50. This suggests that the maintenance of the cell type-specificity of expression is under hormonal control (for effects of prolactin, hydrocortisone, and insulin on proliferation and differentiation of mammary gland and cell cultures derived therefrom see references 3, 6, 12, 50). Environmental control on expression of intermediate filament proteins in epithelial cells has also been indicated by the experiments of Doran et al. (10) and Summerhayes et al. (54) who studied the behavior of cultured epithelial cells upon injection into nude mice. Fuchs and Green (30) have recently described drastic effects of vitamin A on the expression of cytokeratin patterns in cultured keratinocytes from human foreskin epidermis and conjunctiva. As shown by our experiment of withdrawal of hormone additions from hormone-adapted BMGE + H cells the specific intermediate filament cytoskeleton once established in this line is rather stable. Only minor quantitative changes of the cytokeratin polypeptide pattern have been noted at 40 passages after transfer of BMGE + H cells from the high hormone concentrations to the relatively low level present in fetal calf serum. We are currently examining hormonal effects on expression of cytoskeletal proteins in detail utilizing serum-free media.

Our study also shows that three different bovine epithelial cell lines (BMGE + H, BMGE − H, MDBK) can be distinguished by their different cytokeratin patterns, partly reflecting different degrees of maintenance of tissue-specific cytokeratins. BMGE cells show that relatively large and basic cytokeratin polypeptides, which tend to disappear upon culturing of various cells from squamous stratified epithelia including epidermis (10, 16, 27–29, 37, 55, 56, 63), can be maintained in at least some epithelial cell types permanently growing in vitro. On the other hand, deviations from the tissue-specific patterns during cell culturing, at least in media without additions of differentiation-influencing hormones, are obvious and the emergence of the BMGE − H cells shows how profound such cytoskeletal changes can be. Interestingly, two cell lines from different bovine organs, BMGE − H from mammary gland and MDBK from kidney, show a striking similarity in their cytokeratin patterns. Loss of certain cell type-specific cytokeratins and concomitant appearance of similar newly synthesized cytokeratins may suggest that during culturing in normal growth media "relaxation" from differentiation control exerted in the body may result in cytoskeletal changes in that certain cytokeratins may be induced *de novo* whereas others, differentiation-dependent ones, are no longer synthesized (for hepatocytes see also reference 14). Thus, the identification of the cell of origin of an epithelial cell growing in culture by the specific cytokeratin polypeptide pattern may be possible in certain cells and under certain growth conditions (e.g., reference 14 and this study); however, in other cell cultures the deviation in cytokeratin pattern may be so extensive that a correlation with the cell type of origin cannot be made.

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