Expression of Simple Epithelial Type Cytokeratins In Stratified Epithelia as Detected by Immunolocalization and Hybridization In Situ

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Abstract. Multi-layered ("stratified") epithelia differ from one-layered ("simple") polar epithelia by various architectural and functional properties as well as by their cytoskeletal complements, notably a set of cytokeratins characteristic of stratified tissue. The simple epithelial cytokeratins 8 and 18 have so far not been detected in any stratified epithelium. Using specific monoclonal antibodies we have noted, in several but not all samples of stratified epithelia, including esophagus, tongue, exocervix, and vagina, positive immunocytochemical reactions for cytokeratins 8, 18, and 19 which in some regions were selective for the basal cell layer(s) but extended into suprabasal layers in others. In situ hybridization with different probes (riboprobes, synthetic oligonucleotides) for mRNAs of cytokeratin 8 on esophageal epithelium has shown, in extended regions, relatively strong reactivity for cytokeratin 8 mRNA in the basal cell layer. In contrast, probes to cytokeratin 18 have shown much weaker hybridization which, however, was rather evenly spread over basal and suprabasal strata. These results, which emphasize the importance of in situ hybridization in studies of gene expression in complex tissues, show that the genes encoding simple epithelial cytokeratins can be expressed in stratified epithelia. This suggests that continual expression of genes coding for simple epithelial cytokeratins is compatible with the formation of squamous stratified tissues and can occur, at least in basal cell layers, simultaneously with the synthesis of certain stratification-related cytokeratins. We also emphasize differences of expression and immunoreactivity of these cytokeratins between different samples and in different regions of the same stratified epithelium and discuss the results in relation to changes of cytokeratin expression during fetal development of stratified epithelia, in response to environmental factors and during the formation of squamous cell carcinomas.

EPITHELIA and carcinomas are characterized by the presence of intermediate-sized filaments (IFs)[1] of the cytokeratin type (17, 21, 22, 24, 58, 81, 82). In contrast to the other cytoplasmic IF proteins (vimentin, desmin, glial filaments, and neurofilaments), the cytokeratins are obligatory heteropolymers (16, 37, 49, 79). They form a complex multigene family, comprising in human epithelia at least 19 different polypeptides (58) and at least another eight polypeptides characteristic of hair-forming cells (39, 52). Two subfamilies of cytokeratins can be distinguished, i.e., the acidic (type I) and the basic (type II) cytokeratins, and two polypeptide chains of members of each subfamily are necessary to form the heterotypic tetramer subunit and then the IFs (3, 30, 36, 37, 67, 68, 77, 80, 86, 96). The various cytokeratin polypeptides of both subfamilies are synthesized in patterns that are different in the diverse epithelial cell types, and a certain cytokeratin pattern is characteristic of a given epithelial cell or tissue (10, 20, 58, 68, 83).

Epithelia represent a group of tissues of various histological appearances and are involved in various functions. Grossly, they can be grouped into the one-layered and the multilayered (stratified) epithelia. One-layered epithelia are either simple in cell type complexity, consisting of rather uniform populations of cells, which often display architectural and functional polarity (hepatocytes, pancreatic exocrine cells, tubular epithelia of kidney), or are comprised of different cell types, as in the complex epithelia of, e.g., trachea, lung, bladder, mammary gland, and bladder urothelium. The cytokeratin patterns of "simple epithelia" are also relatively simple, as they form IFs from either only two (8 and 18) or three (8, 18, and 19) or four (7, 8, 18, and 19) polypeptides (46, 58, 68, 83).

Stratified epithelia differ from one-layered epithelia not only by their different cell and tissue architecture but also by

1. *Abbreviation used in this paper*: IFs, intermediate-sized filaments.

their patterns of cytokeratins (9, 10, 58, 68, 83, 86). A number of cytokeratins have been found primarily in stratified tissues, or in tissues with a potential to stratification, including cytokeratins 1–6 and 9–17 (5, 58, 68, 71, 88, 94), suggesting that their production may be somehow related to the stratification process. On the other hand, it is clear that during embryogenesis stratified epithelia are derived from simple epithelia, and these changes of morphology are accompanied by changes of cytokeratin expression (e.g., 4, 46, 62, 71, 84). Similarly, certain forms of localized stratification in a normally one-layered epithelium, be they pathological ("squamous metaplasia") or normal (as in amnion epithelium; 71), are characterized by the advent of certain cytokeratins in these regions.

In agreement with the concept of changes of cytokeratin expression during stratification are reports that the "simple epithelial" cytokeratins 8 and 18 have not been found in most biochemical analyses of cytoskeletal proteins from stratified epithelia (2, 5, 9, 35, 58, 64, 65, 68, 83, 84). Correspondingly, immunocytochemical studies using several monoclonal antibodies specific for the simple epithelial cytokeratins 8 and/or 18 have not shown reactivity in several kinds of stratified epithelia (12, 13, 34, 45, 54, 69, 70), with the exception of the sparse neuroendocrine cells that can occur in some stratified epithelia (e.g., 46, 61, 76). In contrast, cytokeratin 19 has been identified in cytoskeletal extracts and has been localized immunocytochemically in basal cell layers of some stratified epithelia but not of others (8, 19). Remarkably, squamous cell carcinomas derived from stratified epithelia sometimes reveal, over extended regions or only in certain cell clusters, the presence of simple epithelial cytokeratins, including components 8 and 18 (19, 54, 58–60; see, however, reference 12), which may be taken as an indication of changes of cytokeratin expression during malignant transformation. Likewise, simple epithelial cytokeratins have been repeatedly found in cultures of cells derived from stratified epithelia and squamous cell carcinomas (7, 31, 73, 98, 99; for cell lines apparently lacking these proteins see 35, 75) which might reflect changes of expression during culturing in vitro.

In the course of immunocytochemical studies with certain monoclonal antibodies to simple epithelial cytokeratins we have sometimes observed positive reactions in certain cell layers or cell clusters of some stratified epithelia. Because these observations were seemingly at variance with the negative biochemical and immunocytochemical reports mentioned above, we have studied the expression of such cytokeratins in stratified epithelia in greater detail, using various cytokeratin polypeptide-specific antibodies and the cDNA probes to cytokeratins 8 and 18 recently developed in our laboratory (50, 74). We show that simple epithelial cytokeratins can be expressed in stratified epithelia and that the synthesis of cytokeratin 8 is often more prominent in basal cell layer(s). We also emphasize that the pattern of expression of the simple epithelial cytokeratins 8, 18 and 19 can exhibit drastic regional differences in the same epithelium.

Materials and Methods

Tissue Samples

Small pieces of apparently normal ("uninvolved") tissues were obtained from patients during surgical excision of benign or malignant tumors (cf. 58–60) or, in the case of gingiva and oral mucosa, during periodontal surgery (cf. 65). A total of five different samples of esophageal tissue and four vaginal samples was included in the present study. Epidermal samples were taken during surgical removal of tattooed skin (generously provided by Dr. I. Moll, Department of Dermatology, Mannheim Medical School, University of Heidelberg, Mannheim, FRG). In some cases, autopsy specimens were used for controls. For RNA extraction, tissue samples were directly frozen in liquid nitrogen. For immunolocalization and in situ hybridization experiments, tissue samples were preferably frozen in isopentane precooled in liquid nitrogen to ~130°C.

Preparation of RNA

Small pieces of tissue corresponding to 0.5–1 g of wet weight were ground to a fine powder at ~70°C in a Teflon capsule containing a steel bullet inserted into a dysmembrator (Braun, Melsungen, FRG). The powder was then homogenized in 4 M guanidinium isothiocyanate (in 0.1 M Tris-HCl, pH 7.5; 10 mM dithiothreitol (DTT); 5 mM EDTA). Half the volume of cold ethanol was added and RNA was precipitated for more than 6 h at −20°C. The pellet (10,000 *g*; 10 min) was resuspended in 7 M guanidinium-hydrochloride buffer (in 0.1 M sodium acetate, pH 5.5, 10 mM DTT), extensively homogenized twice more, and nucleic acids were precipitated with ethanol. The final pellet was washed with 70% ethanol, dried, and then dissolved in 10 mM Tris-HCl (pH 7.0) containing 0.5% SDS, 10 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, and up to 1 mg/ml proteinase K (Boehringer, Mannheim, FRG) and was incubated for 60 min at 60°C. Residual proteins and proteinase K were removed by three cycles of phenol/chloroform extraction.

Preparation of Radioactively Labeled cRNA Probes and Northern Blot Analysis

These procedures were performed essentially as described (50). As cytokeratin 8 mRNA probes we used either the transcript of the Bgl-linearized clone pKH8[1] as previously described (50) or a shorter Sac I–Eco RI fragment of clone pKH8[1] subcloned into Bluescribe (Stratagene, San Diego, CA) encompassing part of the tail region (for terminology see 84, 93) and the 3′-noncoding sequence, thereby minimizing possible cross-hybridization with other cytokeratin mRNAs. Clones in Bluescribe (pKH8[2]) were linearized with one of several restriction enzymes producing a protruding 5′-end, before transcription in vitro with T7 polymerase. The Bam HI–Eco RI fragment of clone pKH18[1] (50) was cloned in pTZ18 R (clone pKH18[3]), which was linearized. Clones pKH4[1] and pKH15[1] (51) were used for comparison. For "dot blot" hybridization tests fivefold serial dilutions of total RNA were spotted on filters (Gene Screen Plus; New England Nuclear, Boston, MA), using a Minifold II apparatus (Schleicher and Schuell, Dassel, FRG). Hybridization conditions with cRNA probes were as in Northern blot analysis.

Synthetic Oligonucleotides

Our cytokeratin 18-specific cRNA probe (cf. 50) still contains a stretch of ~150 nucleotides, corresponding to the end of the α-helical rod portion. Since theoretically this sequence could cross-hybridize with other type I cytokeratins, albeit only at reduced stringency, we have synthesized a 30 nucleotides–long oligonucleotide 5′ d(GCT CCC CAA AGG GTA CCC TGC TTC TGC TGG) 3′, complementary to the mRNA strand in the noncoding 3′ region, encompassing positions 1278 to 1307 (50). We have also synthesized an oligonucleotide of $n = 30$ complementary to part of the 3′-non-coding region of the mRNA of cytokeratin 14 (55), the sequence being 5′ d(GTG AAG CAG GGT CCA GCT GTG AAG TGC TTG) 3′. The oligonucleotides were labeled at their 5′-ends with γ-^{32}PdATP using T4-polynucleotide kinase and were purified from unincorporated nucleotides by denaturing PAGE, elution through glasswool and lyophilization. Alternatively, they were passed through a SepPak C_{18} Cartridge (Waters Associates, Milford, MA) and lyophilized. The specific activity of freshly labeled oligonucleotides was ~2×10^9 cpm/μg oligonucleotide. For filter hybridizations, using the same hybridization solution as for cRNA probes, the hybridization was at 37°C, and filters were washed first at room temperature in 4× SSC and 0.1% SDS, and finally in 0.1× SSC and 0.1% SDS at 37°C.

In Situ Hybridization

The procedure using cRNA probes was as described (50), except that the hybridizations were at 50°C. When oligonucleotides were used as probes, the hybridization solution was the same but the temperature of hybridization was 37°C, and the temperature during post-hybridization washing did not

exceed 37°C. RNase A treatment had to be omitted, a general draw back in the use of oligonucleotides. An advantage of using oligonucleotides instead of cRNA probes for in situ hybridization protocols seems to be that proteinase K treatment of the sections, including postfixation, is not required, provided that sufficient detergent (0.1–0.5% SDS) is contained in the hybridization solution.

Gel Electrophoresis

Frozen sections of esophageal tissue were used for microdissection as described (58, 59), and strips of tissue containing the basalmost 3–4 layers were separated from the upper layers. Cytoskeletal material was prepared therefrom and its protein composition analyzed by two-dimensional gel electrophoresis (1, 58–60).

Antibodies and Immunohistochemistry

The following murine monoclonal antibodies were used in this and the accompanying (51) study: (*a*) antibody K_8 8.13, broadly cross-reactive with type II cytokeratins as well as with cytokeratin 18 (type I; 32; available from Bio-Makor, Rehovot, Israel); (*b*) antibody lu-5 reacting with various type I and type II cytokeratins (25; available from Boehringer, Mannheim, FRG); (*c*) antibody PKK1 reported to react with several epithelial cytokeratins (92; from Labsystems, Helsinki, Finland); (*d*) antibody TROMA 1 specific for cytokeratin 8 (44; kindly provided by Dr. R. Kemler, Max Planck-Institute for Immunology, Freiburg i.Br., FRG); (*e*) antibody K_s 8.1.42, selectively reacting with cytokeratin 8 (from Progen Biotechnics, Heidelberg, FRG); (*f*) antibody M20 reacting with cytokeratin 8 (90; kindly provided by Dr. G. van Muijen); (*g*) antibody 6B10, specific for cytokeratin 4 (88; kindly provided by Dr. G. van Muijen); (*h*) antibody 1C7, specific for cytokeratin 13 (88); (*i*) antibody K_s 13.1 preferentially reactive with cytokeratin 13 (57; from Progen, Heidelberg, and Boehringer, Mannheim); (*j*) antibody CK-2 specific for cytokeratin 18 (12, 13; from Boehringer, Mannheim, FRG); (*k*) antibody RGE-53 specific for cytokeratin 18 (69; from Paesel, Frankfurt, FRG); (*l*) antibody K_s 18.27 (IgG1) which is also specific for cytokeratin 18, as determined by immunoblotting and immunocytochemistry (from Progen, Heidelberg); (*m*) antibody K_s 18.18 specifically reacting with certain heterotypic complexes of cytokeratin 18 (26); (*n*) antibody K_s 18.174 (57; from Progen, Heidelberg); (*o*) human cytokeratin 19-specific antibody A53-1B/A2 (42; K_s 19.2, from Progen, Heidelberg); (*p*) antibody K_s 19.2 specific for cytokeratin 19 of human and some other species (from Progen, Heidelberg).

Immunofluorescence microscopy, using secondary antibodies coupled to Texas-Red or rhodamine compounds or fluorescein, was as previously described (cf. 1, 61).

Results

In our previous gel electrophoretic analyses of cytoskeletal proteins from microdissected stratified epithelia (including epidermis from various body sites, gingiva, lingual, and oral mucosa, pharyngeal epithelium, esophagus, exocervix, vagina, and penile mucosa; 2, 58–60, 64, 65; see also references 5, 6, 9, 35, 84, 86), the only simple epithelial cytokeratin frequently, though not always, detected in appreciable amounts was cytokeratin 19. In some of these tissues such as esophagus, minor amounts of cytokeratins 8 were also occasionally seen (data not shown). However, given the technical limitations of the microdissection method and the general problem that contributions from the frequent glandular ducts (cf. 40) cannot be excluded, we could not make definitive conclusions as to the presence or absence of cytokeratins 8 and 18 in the stratified epithelial cells. To examine the possibility that the genes for cytokeratins 8 and 18 are expressed in these epithelia, we have therefore applied in situ localization techniques, using cytokeratin polypeptide-specific antibodies and cDNA probes for cytokeratin mRNAs. In the present report, we shall particularly concentrate on esophageal tissue, because this is the non-epidermal stratified epithelium most widely studied biochemically with respect to its cytokeratin pattern in both man (5, 6, 31, 35, 58, 59, 94) and animals (9, 20, 56) which has also been taken as a prototype tissue for a group of non-epidermal stratified epithelia ("esophageal–epithelial type of differentiation"; 9, 10, 83, 84).

Reactivity of Antibodies Specific for Simple Epithelial Type Cytokeratins on Stratified Epithelia

As expected, all antibodies to cytokeratins 8, 18, and 19 reacted intensely with the mucinous and the serous acini of the esophageal glands and their ducts, including the intraepithelial ostia (results not shown; cf. 8), in contrast to the lack of reactivity reported with other cytokeratin antibodies by Hopwood et al. (40). However, results obtained in the stratified epithelium were rather variable between different samples or different regions of the sample as well as between different antibodies reactive with the same cytokeratin polypeptide.

Fig. 1 presents results of immunofluorescence microscopy, using a number of different monoclonal antibodies specific for the three simple epithelial cytokeratins 8, 18, and 19 on human esophageal epithelium. In the samples shown in Fig. 1, antibody PKK1, reported to react with several cytokeratins including components 8, 18 and 19, selectively stained the cells in the basal layer (Fig. 1, *a* and *b*), regionally also with the first and second suprabasal layers although usually with lower intensity. Antibodies to cytokeratin 8 reacted differently. All three of them (TROMA 1, M20 and K_s 8.1.42) reacted strongly with a certain type of sparse cells located within or near to the basal cell layer, in a manner reminiscent of the distribution of the "Merkel cells" of epidermis (cf. 61), which were probably esophageal neuroendocrine cells (data not shown). In addition, antibody K_s 8.1.42 stained, in several but not all samples, many more cells of the basal layer, locally also groups of suprabasal cells (Fig. 1, *c* and *d*). Antibody M20 also stained, in several samples, the basal cell layer and some suprabasal cells, although with somewhat lower intensity (not shown). However, in other samples all three cytokeratin 8 antibodies were negative on the non-Merkel type cells of the basal layers.

In many samples of esophageal tissue, both cytokeratin 19-specific antibodies reacted with the basal cell layer but exhibited marked regional differences: In some regions the staining was restricted to the basal layer (Fig. 1, *e* and *f*), in others a substantial number of cells in the lower suprabasal compartment was also positive (Fig. 1 *g*), confirming recent findings of Bartek et al. (8).

The monoclonal antibodies specific for cytokeratin 18 gave grossly different results, varying both between different samples and diffeent regions of the same sample. Some of them such as antibodies CK-2, RGE-53 and K_s 18.27 were negative on the esophageal cells (data not shown) and reacted only with the individual sparse cells of putative neuroendocrine character, in agreement with previous reports (e.g., 13, 69). The conformation-dependent antibody K_s 18.18, however, reacted, regionally, with the basal layer (Fig. 1, *i* and *j*), in some places also with moderate reactivity in the lower suprabasal compartment (not shown). In contrast, the very sensitive, cytokeratin 18-specific antibody K_s 18.174 stained, in a number of places, some suprabasal cell layers in addition to the basal cells (Fig. 1 *h*). These differences of immunostaining between different antibodies to the same polypeptide and between different samples of esophageal tissue could not

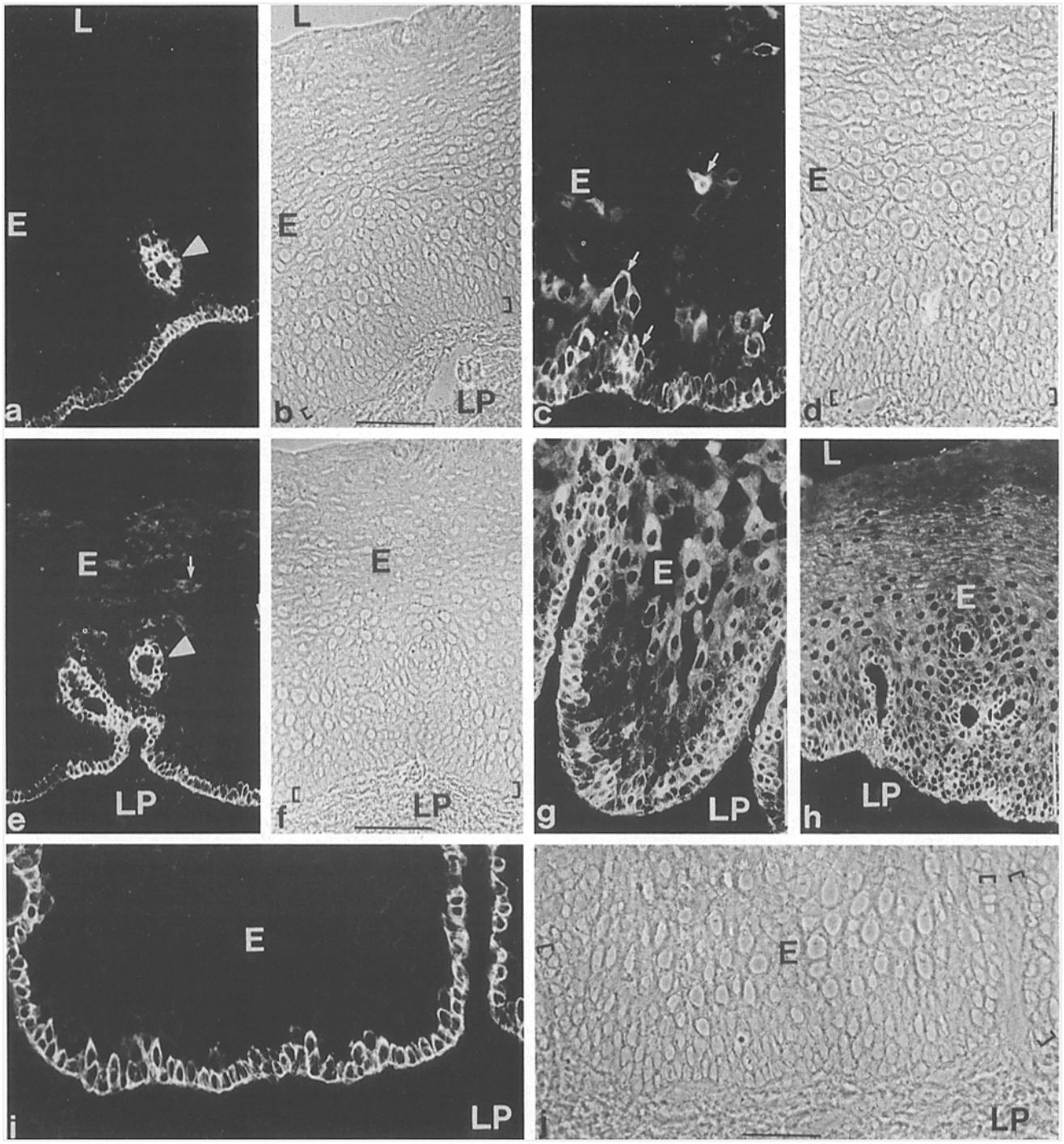


Figure 1. Immunofluorescence microscopy of frozen sections of human esophagus, showing examples of differential reactivity of monoclonal antibodies specific for simple epithelial type cytokeratins. *E*, epithelium; *L*, lumen; *LP*, *Lamina propria*. The arrowheads point to circular structures that are either ostia of glandular ducts or protrusions of the *Lamina propria*. (*a* and *b*) Antibody PKK1, reactive with several cytokeratins including components 8, 18, and 19, staining exclusively the basal cell layer (demarcated by brackets in *b*); (*a*) epifluorescence; (*b*) phase-contrast picture of the same field. (*c* and *d*) Monoclonal antibody K_s 8.1.42 specific for cytokeratin 8 staining the basal cell layer (denoted by brackets in *d*) and some isolated suprabasal cells (*arrows* in *c*). (*c*) Epifluorescence; (*d*) phase-contrast optics. (*e*, *f* and *g*) Cytokeratin 19-specific antibody A53-B/A2 (K_s 19.1) in different areas of esophageal epithelium. The fluorescence is either restricted to basal cells (demarcated by brackets in *f*) with a few weakly stained suprabasal cells (*arrows* in *e*; *e*, epifluorescence; *f*, phase contrast) or extends to numerous suprabasal cells (*g*). (*h*) Cytokeratin 18-specific antibody K_s 18.174 stains, in this sample, basal cell layers as well as several suprabasal cell layers, although with lesser intensity in the latter. (*i* and *j*) Antibody K_s 18.18, also reactive with cytokeratin 18 containing IFs, stains, in this sample, predominantly cells in the basal layer (*i*, epifluorescence; *j*, phase contrast; brackets as in *a*). Bars: (*a–i*) 100 μm; (*j*) 50 μm.

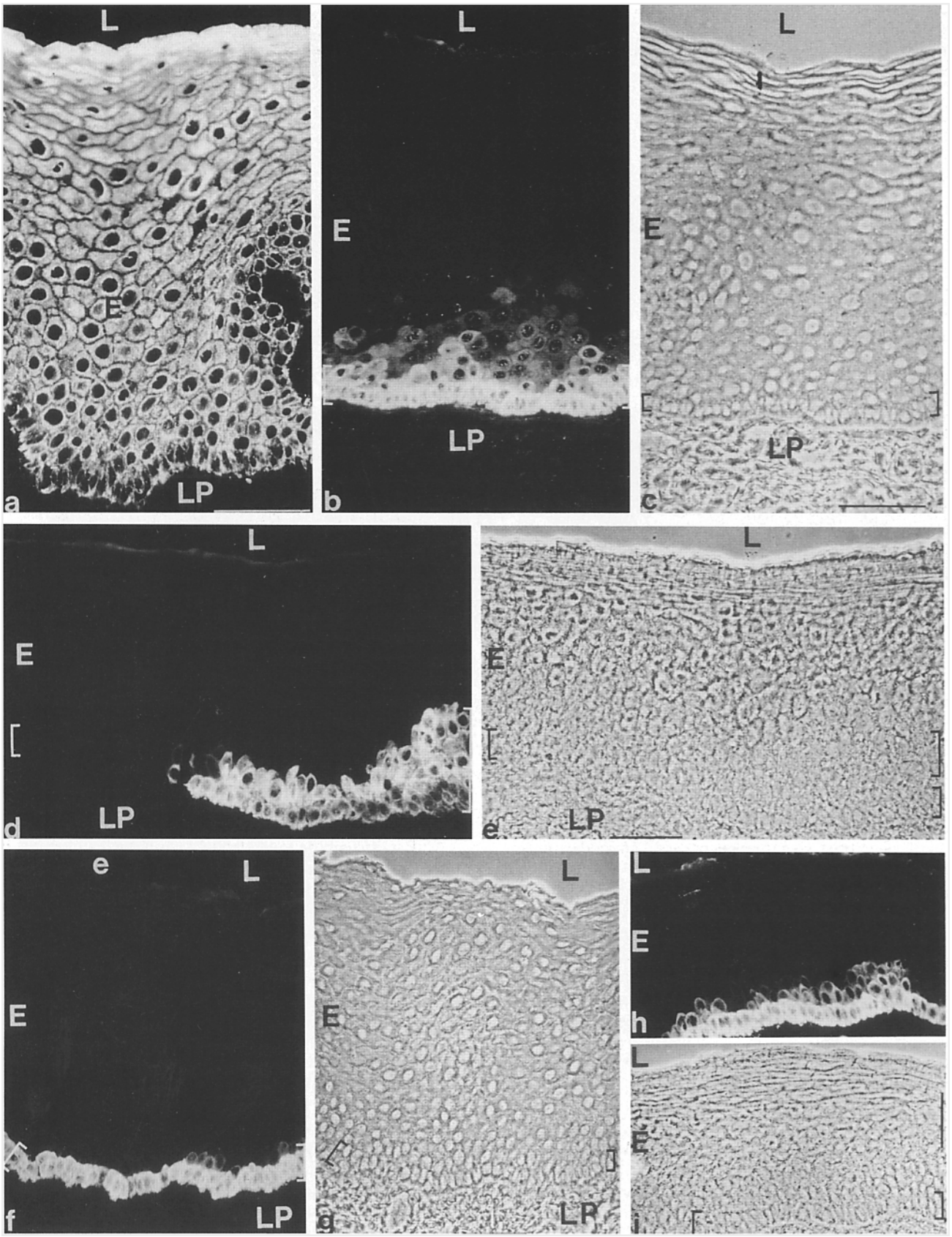


Figure 2. Immunofluorescence microscopy of frozen sections of samples of human vagina, showing different patterns of reactivities with various cytokeratin antibodies (symbols as in Fig. 1). (*a*) Uniform staining throughout all epithelial layers after reaction with broad range cytokeratin antibody K_s 8.13. (*b–e*) Reactions of antibody K_s 8.1.42 against cytokeratin 8, showing a region with strong staining of basal and some suprabasal cells (*b*, epifluorescence; *c*, phase contrast) and a region with an abrupt change from positive to negative reactivity (*d*, epifluorescence; *e*, phase contrast; upper bracket denotes lower suprabasal compartment, lower bracket shows basal cell layer). The significance of this local change of reactivity is indicated by the reactivity of all layers with broad range cytokeratin antibodies such as K_g 8.13 in parallel sections. (*f–i*) Distribution of cytokeratin 19-positive cells as detected by antibody A53-B/A2. The fluorescence is predominantly over the basal cell layer (*f*, epifluorescence; *g*, phase contrast optics), with some local suprabasal positive cell clusters (*h*, epifluorescence; *i*, phase contrast). Bars, 50 μm.

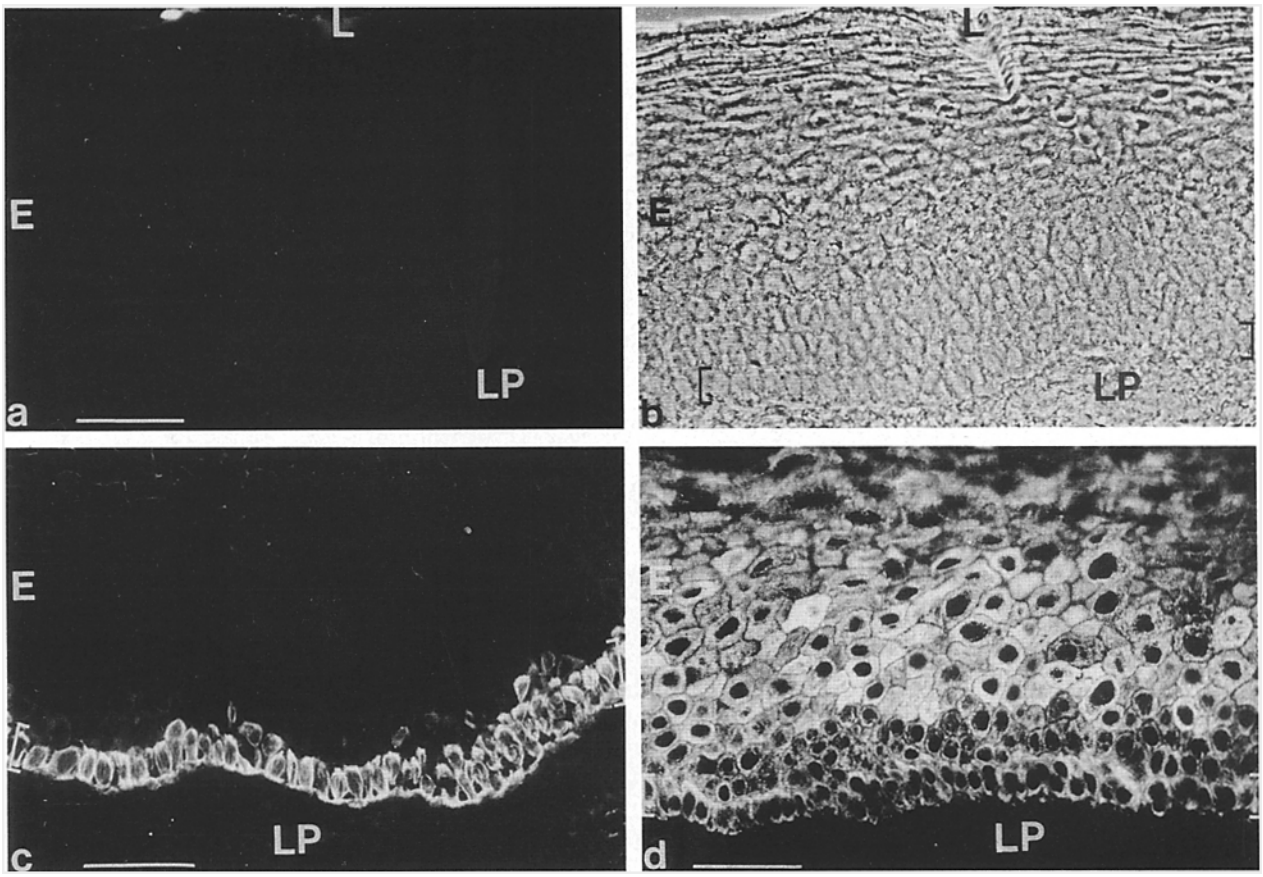


Figure 3. Immunofluorescence microscopy of frozen sections of the same sample of human vagina, showing differential reactivity with various monoclonal antibodies to cytokeratin 18 (symbols as in Fig. 1). (*a* and *b*) Antibody CK-2 shows negative reaction throughout all epithelial cell layers (*a*, epifluorescence; *b*, phase contrast). (*c*) Antibody K_s 18.18 stains selectively the basal cell layer (*bracket*). (*d*) Antibody K_s 18.174 reacts with most cells in all layers of vaginal epithelium. Bars, 50 μm.

be correlated with differences of anatomical topology, e.g., upper vs. lower esophagus.

Such differential reaction patterns with antibodies to simple epithelial cytokeratins were not unique to the esophagus but were also seen in some samples of other stratified squamous epithelia, though not in all. For example, results obtained for a sample of human vaginal epithelium in which differences of immunostaining were particularly apparent, are shown in Figs. 2, *a–i* and 3, *a–d*. While broad range cytokeratin antibodies such as K_G 8.13 reacted uniformly with all epithelial layers, thus demonstrating the immunocytochemical availability of the cytokeratin IFs present in this tissue, some of the antibodies specific for simple epithelial cytokeratin showed very selective staining patterns. For example, in some regions cytokeratin 8 antibody K_s 8.1.42 strongly stained the basal cell layer and some cells of the lower suprabasal compartment (Fig. 2, *b* and *c*), whereas in other regions such as that shown in Fig. 2, *d* and *e*, abrupt changes from strong positive staining of the basal cell layer and the lower suprabasal compartment to complete negativity were seen (Fig. 2, *d* and *e*). Staining with both cytokeratin 19 antibodies showed, in most regions, homogeneous staining of the basal layer but local clusters of positively stained cells in the lower suprabasal compartment (Fig. 2, *f–i*) were sometimes also seen, essentially in agreement with Bartek et al. (8).

As described above for esophagus, different cytokeratin 18 antibodies yielded different results also on vaginal epithelium: In the sample shown in Fig. 3, antibodies CK-2, RGE-53, and K_s 18.27 were all negative (for an example see Fig. 3, *a* and *b*), with the exception of certain dispersed, probably neuroendocrine cells (not shown). Antibody K_s 18.18 stained predominantly the basal cell layer (Fig. 3 *c*) but regions without any significant reaction were also noticed. Antibody K_s 18.174 did react, in the sample shown in Fig. 3 *d*, not only with the basal cell layer but, in many sample places, also with several suprabasal layers, often throughout almost the entire epithelium.

As in esophagus, the immunoreactivities for cytokeratins 8 and 18 in vaginal tissue also varied from sample to sample. Positive immunocytochemical reactions with certain antibodies to cytokeratin 8, 18 and 19 were occasionally also seen in some samples of other stratified epithelia, mostly in basal cell layers, such as the exocervix (see also references 8 and 19), gingiva, oral and lingual mucosa, adult anal epidermis, and fetal epidermis (week 13–20), but not in normal adult interfollicular epidermis (data not shown). Again, in all these tissues the intensity and the distribution of the reaction var-

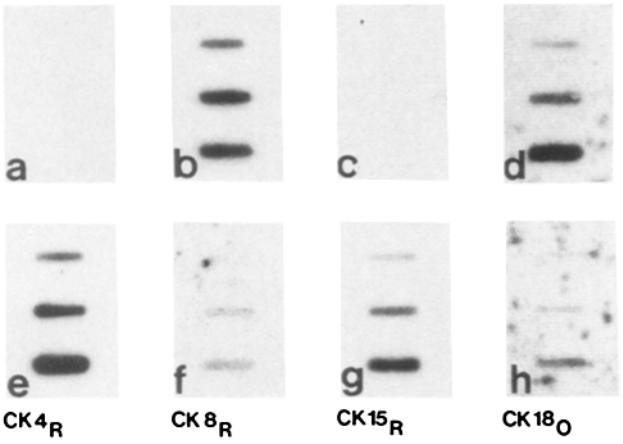


Figure 4. Detection of cytokeratin 8 and 18 mRNA in human esophageal RNA by hybridization ("dot blot") analysis. Fivefold serial dilutions (0.2, 1, and 5 μg, from top to bottom) of total RNA extracted from a simple epithelium (colon, upper panel, *a–d*) and a stratified epithelium, esophagus (lower panel, *e–h*) were hybridized with ^{32}P-labeled cRNA probes for cytokeratin 4 (*a* and *e*), cytokeratin 8 (*b* and *f*), cytokeratin 15 (*c* and *g*) or a ^{32}P-labeled synthetic oligonucleotide specific for cytokeratin 18 (*d* and *h*). Exposure time for the cytokeratin 8 and 18 mRNA-probed filters was 120 h; the filters probed for cytokeratin 4 and 15 mRNAs were exposed for 40 h. Note intense mRNA reactivity for cytokeratins 8 and 18 in colon (*b* and *d*) and weak reaction in esophagus (*f* and *h*) whereas mRNAs for cytokeratins 4 and 15 are only detected in esophageal RNA (*e* and *g*) but not in colon RNA (*a* and *c*).

ied somewhat in different samples and in different regions of the same sample.

Examples of immunocytochemical staining of antibodies to simple epithelial cytokeratins selective for basal cell layers were also observed in stratified epithelia (esophagus, tongue, vagina) of some animal species such as Syrian hamster and cow (our unpublished results).

Antibodies to cytokeratin 7 were negative on all stratified epithelia examined, in agreement with Ramaekers et al. (70).

Detection of mRNA for Cytokeratins 8 and 18 in RNA Extracted from Esophagus

To provide a control for the immunocytochemical results obtained with some of the antibodies specific for simple epithelial cytokeratins, we have probed for the corresponding mRNAs using nucleic acid hybridization on filters. To this we have extracted total RNA from tissue samples of various epithelia, esophagus included, blotted the RNA samples and hybridized them with antisense riboprobes and oligonucleotides (see Materials and Methods). The specificity and high sensitivity of the antisense riboprobes derived from our cDNA clones to cytokeratins 8 and 18 has been demonstrated previously by Northern blot analysis of total RNA extracted from different mammary carcinomas as well as by in situ hybridization to frozen sections of mammary carcinomas (50).

As expected, mRNAs of cytokeratin 8 and 18 were present in positive control tissue samples such as colon in appreciable amounts (Fig. 4, *b* and *d*). However, both mRNAs could also be detected in RNA from esophagus, although in much lesser quantities (Fig. 4, *f* and *h*). Vice versa, riboprobes derived from cDNA clones encoding cytokeratins 4 and 15 (51) gave strong reactions with esophageal RNA (Fig. 4, *e* and *g*) but not with colon RNA (Fig. 4, *a* and *c*), in agreement with previous reports of a lack of these proteins in intestinal and colonic cells (58). While these findings showed that the genes encoding cytokeratins 8 and 18 were expressed somewhere in the esophagus they did not allow to decide whether these mRNAs came from glandular duct elements and/or the sparse neuroendocrine cells or whether they were also present in the cells of the stratified epithelium.

Localization of Cytokeratin 8 and 18 mRNA by Hybridization In Situ

For in situ hybridization, frozen sections of esophageal tissue were fixed in 4% formaldehyde, proteinase K-treated, postfixed and hybridized with antisense cRNAs ("riboprobes") complementary to 3′-regions of mRNAs of cytokeratins 8 and 18 as previously described (cf. 50), except that we increased the stringency of hybridization by raising the temperature to 50°–52°C. Fig. 5 presents two micrographs of different areas of the same esophagus hybridized with the ^{3}H-labeled cRNA probe of cytokeratin 8, showing a markedly positive reaction in the basalmost cell layer of the epithelium. Label in the upper strata of the epithelium was much weaker, and the silver grain density over elements of the *Lamina propria* was not significantly above background. In histological survey pictures of some sections (Fig. 6) a marked enrichment of autoradiographic silver grains can be seen over the basal cell layer and in certain epithelial formations which represent ostia of glandular ducts or the basal cell layer of *Lamina propria* protrusions. Only locally, significant silver grain densities were also seen over small clusters of cells in the lower suprabasal compartment.

In the tissue sample shown in Figs. 5–7 the distribution of cells containing cytokeratin 18 mRNA was quite different from that of cytokeratin 8-expressing cells. In general, cell labeling obtained for cytokeratin 18 mRNA was much weaker than that obtained for cytokeratin 8. Remarkably, however, these weak, though significant signals were not restricted to the basal layer. Fig. 7 *a* shows a low magnification dark field photomicrograph after hybridization with a ^{3}H-labeled cytokeratin 18 cRNA probe and long exposure time in which all cell layers of the epithelium are rather uniformly labeled. In Fig. 7 *b*, a small area of the same esophagus section is shown at higher magnification, using bright field illumination, to facilitate the visualization of the sparse individual silver grains. Only locally did we notice a weak enrichment in the basal cell layer.

As our cDNA probe for cytokeratin 18 contained a stretch of sequence coding for the α-helical part of the polypeptide, due to the absence of suitable restriction sites within the 3′-noncoding portion of the cDNA clone, we could not formally exclude the possibility of cross-hybridization of our cRNA probe with mRNAs encoding other type II cytokeratins, despite the high stringency of hybridization. We have therefore repeated the in situ hybridizations with a 5′-^{32}P-labeled synthetic oligonucleotide complementary to 30 nucleotides within the 3′ non-coding portion of cytokeratin 18 mRNA, which displays no significant homology to any other cytokeratin sequence thus far known. The hybridization signal obtained was similar to that described for the cRNA

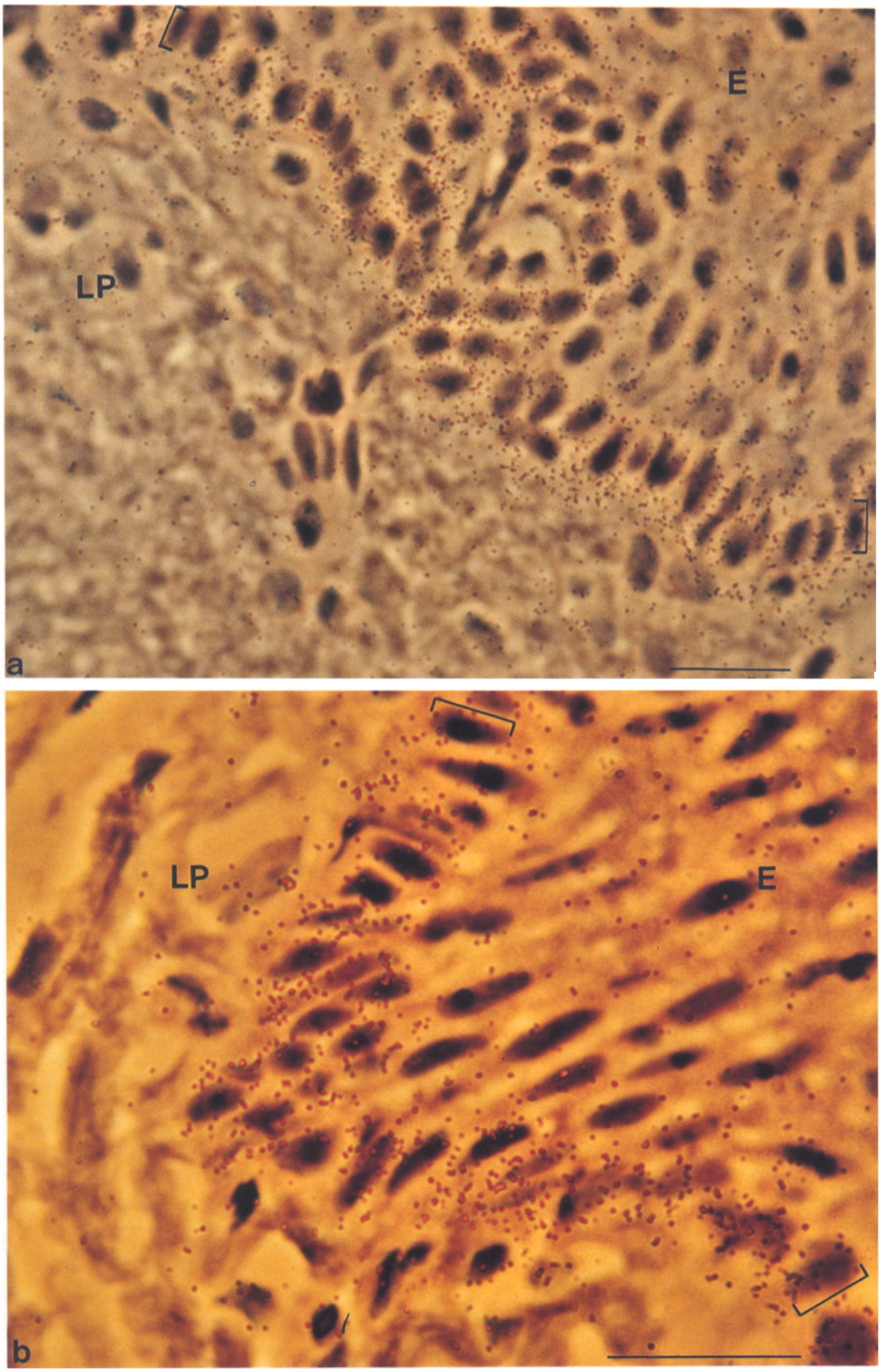


Figure 5. Microscopic autoradiographs of frozen section stained with hematoxylin and eosin, showing enrichment of cytokeratin 8 mRNA in the basal cell layer of human esophagus epithelium (*E*) by in situ hybridization with pKH8[2]. The tritiated cRNA probe of cytokeratin 8 hybridized predominantly to the basal cell layer (*brackets*). In *a*, label is very weak in upper strata; in *b* label is not significantly above background, as seen from comparison with the *Lamina propria* (*LP*) and its components (*a*, survey picture; *b*, higher magnification). Bars, 25 μm.

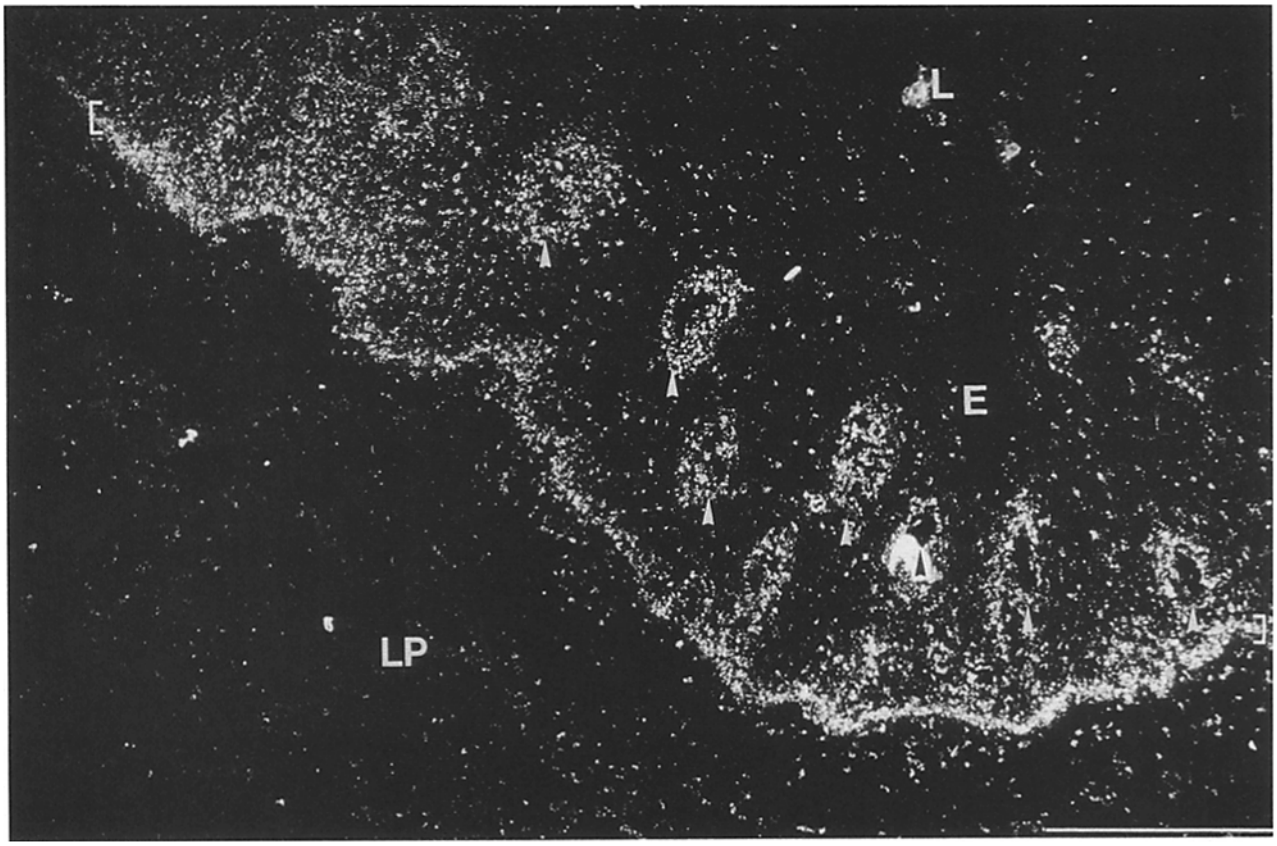


Figure 6. Survey autoradiomicrograph (dark-field illumination) of frozen section of esophagus, showing hybridization with the 3H-labeled cytokeratin 8 cRNA probe. The brackets demarcate the basal cell layer which shows enrichment of label. Triangles point to intensely labeled circular structures, probably protrusions of the *Lamina propria* (symbols as in Figs. 1–3 and 5). Bar, 250 μm.

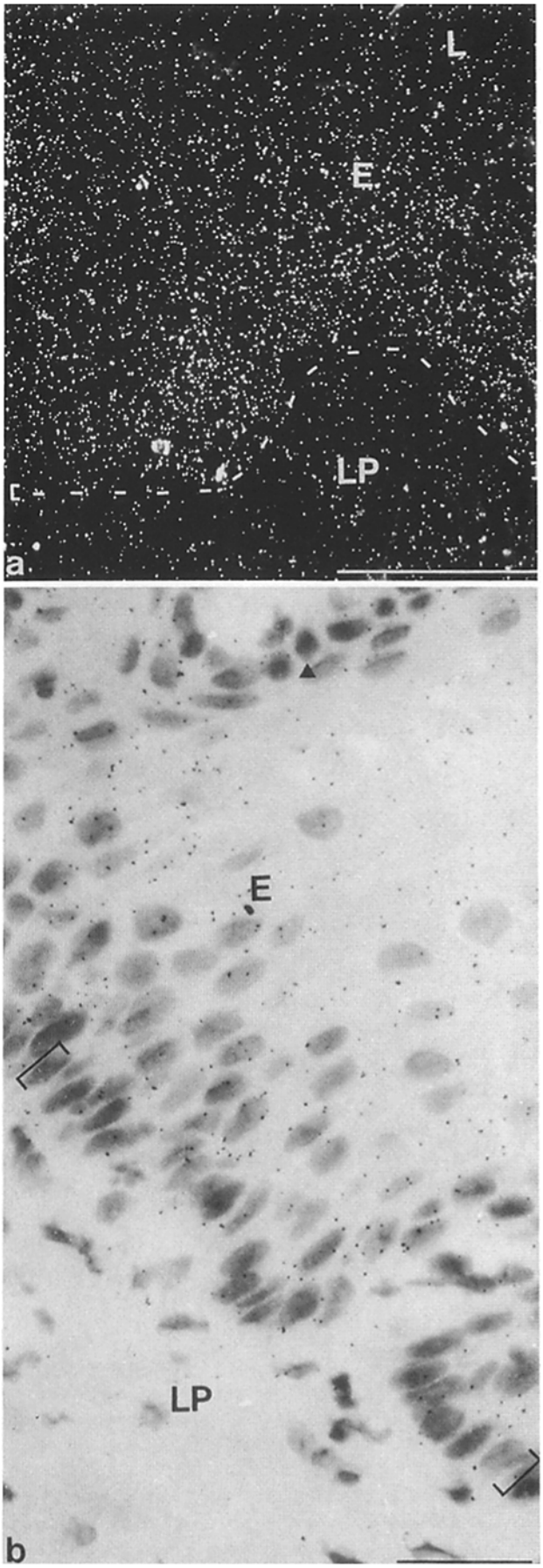
probe, showing rather uniform labeling over the entire epithelium (data not shown).

In epidermis, cytokeratins 5 and 14 have been reported by immunocytochemistry and hybridization in situ to occur in the basal cell layer but also in several suprabasal layers (cf. 87, 95). Since these two cytokeratins are also present in the esophagus, as shown by two-dimensional gel electrophoresis (58), we used a synthetic oligonucleotide (n = 30) complementary to the 3′ non-coding region of cytokeratin 14 mRNA (55) for comparison. On Northern blot hybridization with total RNA extracted from human epidermal tissue, this oligonucleotide probe selectively detected a mRNA band of ~1.6 kb, whereas no hybridization was seen with total RNA from colon (data not shown). On the other hand, the cytokeratin 18-specific oligonucleotide (n = 30, see above) hybridized with a single mRNA band of 1.4 kb from colon but did not hybridize to epidermal RNA (data not shown). In situ hybridization using the cytokeratin 14 oligonucleotide on frozen sections of esophagus yielded a uniform hybridization reaction to all cell layers, including the basal one (Fig. 8 *a*). When the same type of experiment was done for controls on human epidermis (Fig. 8, *b* and *c*), predominant but not exclusive synthesis of cytokeratin 14 mRNA was seen in basal layers, with appreciable amounts still detected in the upper strata, confirming earlier work of others (e.g., 27, 28, 83, 84, 95). We conclude that cytokeratin 14 mRNA expression in esophagus, as in epidermis, is not restricted to the basal layer. We have also not seen significant in situ hybridization for cytokeratin 8 and 18 mRNAs in interfollicular epidermis (data not shown).

Adjacent sections were also examined for the expression of mRNA encoding cytokeratin 4, the most abundant type II cytokeratin of esophageal epithelium (58), and the type I cytokeratin 15 also present in substantial amounts (58; see also Fig. 9). The results, presented elsewhere (51), showed intense labeling for cytokeratin 15 mRNA in all layers, and preferential cytokeratin 4 mRNA labeling of suprabasal cell layers, with additional label of the basal layer in some regions.

Gel Electrophoresis

To enrich cytoskeletal proteins of basal cell layers we have used esophageal tissue with few, if any, glandular ducts and have separated, by microdissection of frozen tissue sections, upper epithelial layers from material including the 3–4 basal layers, with some adjacent *Lamina propria* material. Two-dimensional gel electrophoresis using sensitive silver-staining methods for detection and co-electrophoresis with authentic cytokeratins for identification (Fig. 9, *a* and *b*; see also Materials and Methods) we confirmed the abundance of cytokeratins 4–6 and 13, together with moderate amounts of cytokeratins 14–17, as described previously (58, 59, 68). In addition, we found, in heavily loaded gels, considerable amounts of cytokeratin 19 and small amounts of cytokeratin



8 (Fig. 9 *b*). We noted only faint staining in the position of cytokeratin 18 which, however, was too weak and too close to the major spot of residual actin to be considered as evidence of the existence of cytokeratin 18 in these analyses. The gel electrophoretic results made evident that the amounts of cytokeratins 8 and 18 in this tissue are very low.

Discussion

The results of this study show that the expression of genes encoding cytokeratins of the simple epithelial type, i.e., polypeptides 8, 18 and 19, is not necessarily restricted to simple epithelia but can also occur in stratified epithelia. While the total amounts of cytokeratin 19 found in various nonepidermal stratified epithelia, esophagus included, are usually sufficient to be identified among the abundant cytokeratins characteristic for stratified epithelia in gel electrophoretic analyses (e.g., 58–60), the concentrations of cytokeratin 8, and even more so of cytokeratin 18, are very low, if detectable at all. Obviously, we cannot exclude that the minor amounts of some simple epithelial cytokeratins occasionally seen in gel electrophoretic analyses are contributions from cells other than the stratified esophageal cells such as neuroendocrine and glandular elements. The same reservations apply to reports of the occurrence of small amounts of cytokeratin 18 mRNA in human exocervix (74) as well as of cytokeratin 8 mRNA in human (this study) and bovine (53) esophagus. However, our in situ localization results with specific antibodies and mRNA probes prove that indeed some cytokeratin 8 and 18 can occur in cells of stratified tissue.

We conclude that the synthesis of cytokeratins 8, 18, and 19 is, in principle, compatible with the formation and differentiation of a stratified epithelium and that the disappearance of simple epithelial cytokeratins is not a necessary prerequisite for the development and maintenance of a stratified epithelium. Rather, our observations indicate that these simple epithelial cytokeratins are frequently, but not always, expressed in many nonepidermal stratified epithelia where cytokeratins 8 and 19 often, but not always, are enriched in the basal cell layer(s). The concept of a continual expression of simple epithelial cytokeratins in stratified epithelia is also in agreement with the gel electrophoretical and immunocytochemical findings of cytokeratins 8, 18, and 19 in several developing stratified epithelia, including esophagus, of human fetuses of week 16–20 (54, 71). Whether the relatively small amounts of these simple epithelial cytokeratins in certain

Figure 7. Microscopic autoradiographs of frozen sections of human esophagus, showing expression of cytokeratin 18 mRNA in basal and suprabasal cell layers (symbols as in previous figures) after hybridization in situ with pKH18[3] (see Materials and Methods). (*a*) Low magnification survey picture (dark-field photomicrograph), showing weak labeling over the entire epithelium from basal cells (indicated by brackets; dashed line demarcates basal lamina) to the lumen. The section was exposed to Kodak NTB2 emulsion for 50 d (silver grain densities over lumen, (*L*) and *Lamina propria*, (*LP*) represent background). (*b*) High magnification bright field micrograph taken from the same esophagus section, showing the weak, uniform hybridization signal over the basal cell layer (*brackets*) and several suprabasal layers. Bars: (*a*) 250 μm; (*b*) 25 μm.

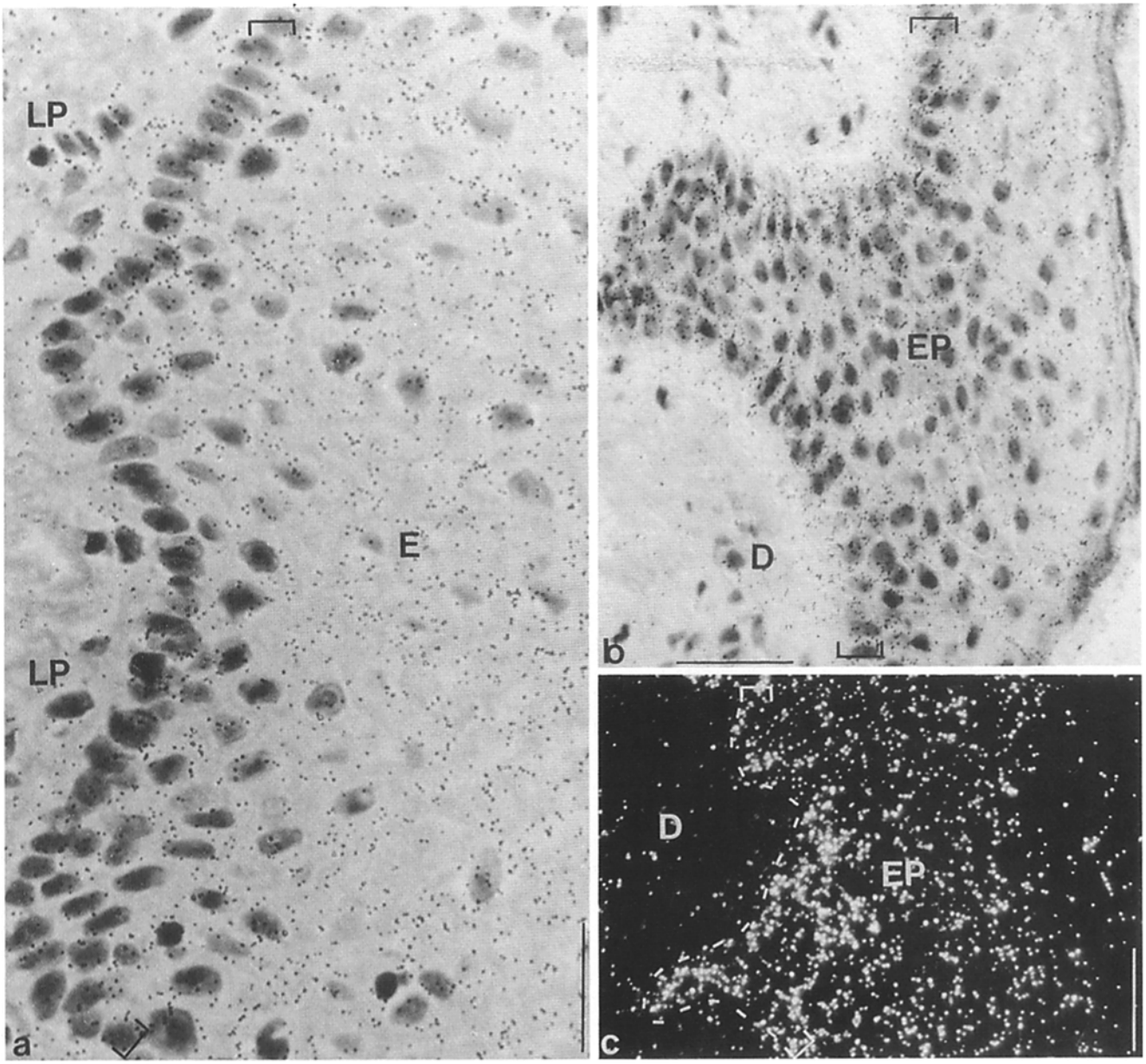


Figure 8. Autoradiomicrograph of frozen sections of human esophagus (*a*) and epidermis (*b* and *c*) hybridized with a γ-^{32}P-dATP-labeled synthetic oligonucleotide specific for human cytokeratin 14. (*E*) epithelium; (*LP*) *Lamina propria*; (*D*) dermis; (*EP*) epidermis. (*a*) Silver grain distribution over basal and suprabasal cell layers of esophagus (*bracket*, basal layer). (*b* and *c*) Enrichment of cytokeratin 14 mRNA in the basal compartment (*brackets* in *b*; broken line in *c* shows basal lamina) of epidermis but not that significant label also extends into the suprabasal layers. Proteinase K treatment was omitted in this specific experiment. (*b*) Bright field; (*c*) dark-field illumination. Bars, 50 μm.

stratified epithelia serve special functions, for example, in the basal cell layers, remains to be seen.

Taking together our results on the distribution of mRNAs encoding cytokeratins 4, 8, 14, 15, 18, and 19 (this study and reference 51) in esophagus, we further conclude that the genes encoding simple epithelial cytokeratins can be co-expressed with other cytokeratins such as 4, 13, 14, and 15 which are characteristic of stratified epithelia (for in situ hybridization of mRNAs encoding stratification-related cytokeratins in other tissues see also references 72 and 87). This indicates that the principles regulating the expression of the genes encoding simple and stratified epithelial cytokeratins are not necessarily mutually exclusive but may allow, at least in certain cells, that both groups of cytokeratins are co-expressed.

Our demonstration that different monoclonal antibodies specific for the same cytokeratin polypeptide may react differently on the same tissue questions the general validity of judgments of the absence of a given protein based on single epitopes. We have no experimental evidence to explain why, in the same tissue block, certain antibodies to cytokeratin 8 stain only the sparse individual, probably neuroendocrine cells whereas others such as antibody K_s 8.1.42 stain primarily the basal cell layer(s), or why some cytokeratin 18-

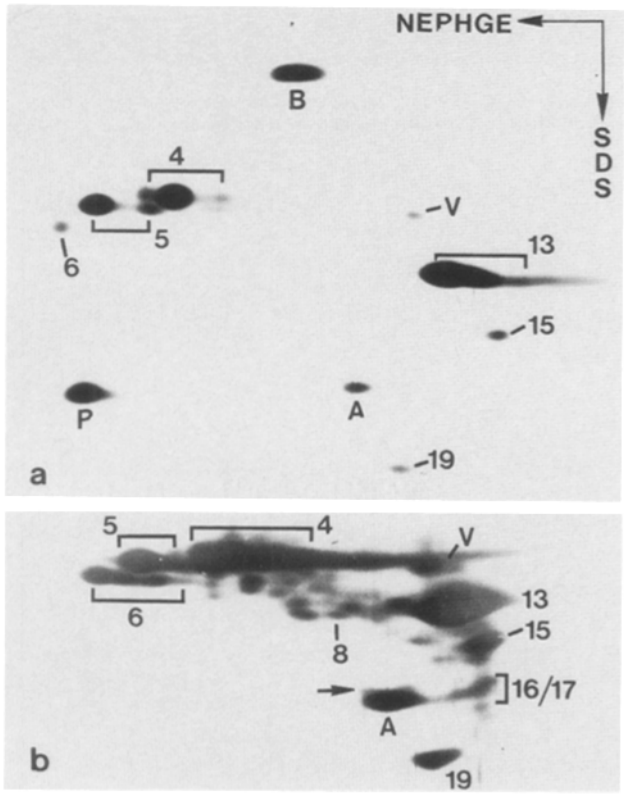


Figure 9. Two-dimensional gel electrophoresis of cytoskeletal proteins (*NEPHGE*, direction of nonequilibrium pH gradient electrophoresis in first dimension; [*SDS*] SDS-PAGE in second dimension) of microdissected basal cell layers from frozen sections of human esophagus as seen after silver staining. (*a*) Moderate protein load (*B*), BSA; (*P*) 3-phospho-glycerokinase from yeast; (*A*) actin, were used as reference proteins). (*b*) Heavy loading. Identified cytokeratins are numbered; the arrow denotes the position of cytokeratin 18 at the upper margin of the actin spot which is hardly detectable. (*V*) vimentin from contaminating nonepithelial cells.

antibodies are negative in all strata, whereas others stain predominantly the basal layer(s) alone or together with some suprabasal layers. At least in certain samples, as shown above, the latter, more extended reaction corresponds to the in situ hybridization result, underlining its credibility. The most likely explanation for such different immunocytochemical results with different monoclonal antibodies to the same proteins seems to be differential masking of the specific epitopes by conformational folding or by interaction with other cellular components. Cell type-selective masking of IF protein epitopes in relation to processes of cell differentiation (e.g., 11, 48, 95), cell cycle (e.g., 18, 23) and cell metabolism (15, 38) is apparently a rather widespread phenomenon and therefore should always be considered as a possible explanation for unexpected negative findings. Interestingly, antibody K_c 18.174 is not only very sensitive in immunoblotting but also differs from the other cytokeratin 18-antibodies in that its epitope is not located in the α-helical rod (our unpublished results). Future detailed mapping of the epitope(s) of the various antibodies should help in the elucidation of the changes responsible for the selective negativity of some epitopes in some cells and cell layers.

Another unexpected and puzzling finding of our study is the frequency of regional differences of both the immunocytochemical and in situ hybridization reactions in the same sample as well as between samples from different donors. At present we do not know whether these regional differences represent "stable" localized differentiations or reflect local responses of the tissue to proliferative stimuli, local inflammation, or other environmental influences. Positive effects of certain environmental factors such as the concentration of vitamin A on the synthesis of simple epithelial cytokeratins have been demonstrated in cell cultures (e.g., 29, 33, 43, 97).

The enrichment of cytokeratin 8 mRNA and protein, together with sizable amounts of cytokeratin 19, in the basal cell layer, is particularly interesting in relation to the demonstrated high proliferative potential and the special immunoreactivities of this layer in various epithelia, epidermis included (e.g., 14, 41, 46, 47, 66, 78, 89-91, 95). Clearly, it is this basal region in which most cell divisions take place, and it is also the proliferatively more active basal layers which probably give rise to the formation of squamous cell carcinomas. Therefore, it is conceivable that the occurrence of cytokeratins 8, 18, and 19 in certain squamous cell carcinomas, albeit in low amounts and variable (19, 58-60), results from the selective proliferation of cells of the basal compartment of these tissues during malignant growth. Likewise, the widespread occurrence of cytokeratins 8, 18, and 19 in cultured cells derived from squamous stratified epithelia and carcinomas (for references see Introduction) might reflect the maintained expression of these proteins in cells derived from the proliferative basal compartment which are selected during growth in vitro. Of course, we cannot exclude the alternative possibility, i.e., the induction or increase of synthesis of cytokeratins 8, 18, and 19 during transformation and tumor growth, and we are aware of reports showing the appearance of these cytokeratins upon transformation of epidermal keratinocytes by simian virus SV40 (63, 85).

Cytokeratins 8 and 18 appear, in many simple epithelia, as a "pair" forming the tetrameric heterotypic subunits of two chains each (67). Our finding of a different distribution pattern of the concentrations of mRNAs encoding these two polypeptides in the various layers of esophageal epithelium shows that the synthesis of these two cytokeratins is not necessarily coupled but may be regulated independently. Clearly, cytokeratin 8 can pair with other type I cytokeratins such as No. 19 (for examples see intestinal epithelium and cerain cultured cell lines such as MCF-7) to form IFs. Future studies in other epithelia and in animal tissues will be needed to examine the general validity of our findings with human esophagus.

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