Molecular Characterization and Expression of the Stratification-related Cytokeratins 4 and 15

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Abstract. A number of human cytokeratins are expressed during the development of stratified epithelia from one-layered polar epithelia and continue to be expressed in several adult epithelial tissues. For studies of the regulation of the synthesis of stratification-related cytokeratins in internal tissues, we have prepared cDNA and genomic clones encoding cytokeratin 4, as a representative of the basic (type II) cytokeratin subfamily and cytokeratin 15, as representative of the acidic (type I) subfamily, and determined their nucleotide sequences. The specific expression of mRNAs encoding these two polypeptides in certain stratified tissues and cultured cell lines is demonstrated by Northern blot hybridization. Hybridization in situ with antisense riboprobes and/or synthetic oligonucleotides shows the presence of cytokeratin 15 mRNA in all layers of esophagus, whereas cytokeratin 4 mRNA tends to be suprabasally enriched, although to degrees varying in different regions. We conclude that the expression of the genes encoding these stratification-related cytokeratins starts already in the basal cell layer and does not depend on vertical differentiation and detachment from the basal lamina. Our results also show that simple epithelial and stratification-related cytokeratins can be coexpressed in basal cell layers of certain stratified epithelia such as esophagus. Implications of these findings for epithelial differentiation and the formation of squamous cell carcinomas are discussed.

EPITHELIAL differentiation is usually characterized by the formation of intermediate-sized filaments (IFs)[1] of the cytokeratin type (20, 28, 85, 86). The early embryonal epithelia, i.e., ecto- and endoderm, are simple polar epithelia and possess IFs of the most simple polypeptide composition, i.e., one representative of the acidic (type I) subfamily, i.e., cytokeratin 18, and one representative of the more basic (type II) cytokeratin subfamily, i.e., cytokeratin 8 (17, 42, 43, 48, 55, 70). When during the development of certain organs the organization of one-layered polar epithelia changes and transforms to stratified epithelia, the synthesis of other cytokeratins, i.e., cytokeratins 1-6 and 9-17, is induced; these appear to be related to the stratification process (for early embryonal epithelia see references 3, 13, 55, 68, 92). Among the earliest stratification-related cytokeratin polypeptides are cytokeratin 4, a type II polypeptide, and the type I cytokeratin 15, which are expressed during the embryonic development of all the diverse stratified epithelia studied so far, including epidermis (68). Cytokeratin 4 seems to disappear in later stages of epidermal maturation, whereas cytokeratin 15 expression is continued in adult epidermis where it persists as a minor component (11, 30, 65, 68, 73). While cytokeratin 4 is one of the most abundant cytokeratins in several adult nonepidermal stratified epithelia such as oral and lingual mucosa, laryngeal and pharyngeal epithelia, epiglottis, esophagus, exocervix, and vagina and in squamous cell carcinomas derived from these tissues (4, 5, 31, 35, 65-69, 72, 73, 94), cytokeratin 15 is usually detected in these epithelia only as a minor component.

Recent immunocytochemical studies using monoclonal antibodies specific for the stratification-related cytokeratins 4 and 13 have localized these proteins to the suprabasal layers of various stratified epithelia (91), suggestive of a correlation of these two cytokeratins with the vertical differentiation process. In this respect, information about cytokeratin 15 is totally lacking as so far no antibody specific for this protein has been described. In order to learn more about the regulation of the selective appearance of stratification-related cytokeratins in different tissues as well as in different layers of the same epithelial tissue it was obviously necessary to have the adequate nucleic acid probes. So far human DNA clones are available only for some cytokeratins expressed in epidermis (e.g., 36, 37, 61, 82, 88) and certain simple epithelia (34, 56, 71, 80). In the present study, we describe the cloning of genes encoding cytokeratins 4 and 15 and their amino acid sequences, and show their specific expression in various squamous epithelial tissues and cultured cell lines.

1. *Abbreviations used in this paper*: IF, intermediate-sized filament; pfu, plaque-forming unit.

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Materials and Methods

Tissue Preparation

Tissue samples were obtained during surgery for various indications (Surgery Clinics, Women's Hospital, and Dermatology Department, Mannheim, and University of Heidelberg-Mannheim Medical School). Small pieces of epithelium were frozen in liquid nitrogen or in isopentane precooled in liquid nitrogen to −130°C within 15 min after surgical removal. For RNA extraction epithelial cell layers were either quickly peeled off with forceps or scraped off with a scalpel. The collected epithelial material was either frozen in liquid nitrogen or immediately homogenized in 4 M guanidinium isothiocyanate buffer (in 0.1 M Tris-HCl, pH 7.5, 10 mM dithiothreitol, (DTT), and 5 mM EDTA).

Library Screening and DNA Sequencing

A cDNA library in λgt10 constructed from poly(A)$^+$-RNA of the vulvar carcinoma cell line A-431 was kindly provided by Dr. A. Ullrich (Genentech, South San Francisco, CA; cf. 90). A genomic library in European Molecular Biology Laboratory (EMBL)-3 phage constructed from partially digested human blood was kindly obtained from Dr. R. Cortese (EMBL, Heidelberg, Federal Republic of Germany (FRG); cf. 7). Screening and DNA extraction procedures were performed essentially as described (56). As nick-translated screening probe we used a mixture of combined cDNA inserts excised from various bovine type I and II cytokeratin cDNA clones, including pKBIa, pKBIb/c, pKBIII, pKBIV, pKBVI, pKBVII, pKB8^1, and pKB19^1 (2, 45–47, 60). 120,000 phages were plated from the amplified cDNA library. The *Eco*RI inserts of the purified phage DNAs were subcloned into the pTZ18 R vector (Pharmacia, Uppsala, Sweden). Both strands of the clones pKH4^1 (encoding cytokeratin 4) and pKH15^1 (encoding cytokeratin 15) were sequenced according to the standard protocol of Maxam and Gilbert (62). The nick-translated 3'-specific *Xho*I fragment of clone pKH15^1 was subsequently used to screen 1.2×10^6 phages of the genomic library. Fragments of one of the three selected genomic clones (λKH15^2) were purified, subcloned into the transcription vectors Bluescribe and Bluescript (Stratagene, San Diego, CA), and sequenced.

Construction of a 3'-specific Subclone and In Vitro Expression of a Reconstructed Complete cDNA Clone for Cytokeratin 15

Two polynucleotides of 75 residues, taken from both strands of the 3'-noncoding region (residues 1606–1680; see Results), were synthesized and purified as described (57), attached to *Eco*RI linkers and cloned into the transcription vector Bluescript (clone pKH15^2). For the construction of a complete hybrid cDNA clone encoding cytokeratin 15, the *Kpn*I/*Kpn*I fragment of 479 nucleotides of the genomic phage clone λKH15^2 which contains a large part of the first exon was ligated to the unique *Kpn*I site of the cDNA clone pKH15^1. The *Bam*HI/*Eco*RI insert of this clone was further subcloned into Bluescript (clone pKH15^3). The transcript of pKH15^3 obtained with T7 RNA-polymerase was translated in vitro (cf. 59) and the translational product was analyzed by coelectrophoresis with cytoskeletal proteins from A-431 cells or tissues (cf. 53, 60, 65).

RNA Preparation and Northern Blot Analysis

Total RNA and poly(A)$^+$-RNA were extracted from cultured human cells of the vulvar epidermoid carcinoma-derived cell line A-431, including clonal sublines expressing cytokeratin 4 as well as clones with high relative contents of either cytokeratin 13 (clone E_3) or cytokeratin 15 (clone E_6; for different sublines, see also reference 65), the breast carcinoma cell line MCF-7, the pharyngeal carcinoma-derived cell line Detroit-562, and the bladder carcinoma-derived cell line RT-112 (65, 66, 73). For comparison, RNA from SV40-transformed human fibroblasts (27) was used. Culture dishes were rinsed three times with PBS. After carefully decanting the buffer, the cells were directly suspended in the 4 M guanidinium isothiocyanate buffer (see above), scraped off, and homogenized with an Ultra Turrax blender (Janke and Kunkel KG, Staufen, FRG). RNA was extracted from homogenates of cultured cells and from tissue samples essentially as described (56). Poly(A)$^+$-RNA was bound to an oligo-dT cellulose matrix and eluted as described by Kreis et al. (53). Purified RNA was electrophoretically separated on 1.2% agarose gels containing formaldehyde, blotted onto Gene Screen Plus and hybridized with RNA polymerase in vitro transcripts (56).

Hybrid Selection and Translation In Vitro

Poly(A)$^+$-RNA from A-431 cells (clones E_3 and E_6) or total RNA from Detroit-562 cells were hybridized to filter-bound subclones and selected mRNAs were translated in vitro using [^{35}S]methionine as label (cf. 46, 60). In experiments with the short subclone pKH15^2, the hybridization temperature was lowered to 32°C and the bound RNA was successively removed by washes at increasing temperature. Two-dimensional gel electrophoresis was performed as coelectrophoresis of the in vitro translation products with an excess of unlabeled reference proteins and cytoskeletal proteins from A-431 cells or from esophageal tissue to allow the identification of the translational products (cf. 46, 58, 60, 65).

Hybridization In Situ

Upon linearization of pKH4^1 with *Hind*III and pKH15^1 with *Bgl*I riboprobes were obtained that could be radioactively labeled by in vitro transcription with T7 RNA polymerase (56). The 75-mer polynucleotide complementary to residues 1606–1680 (see above) was 5' end-labeled with [γ-^{32}P]ATP using polynucleotide kinase and purified (cf. 57). The protocol for hybridization in situ was as described (9, 56). Posthybridization treatment with RNase A was omitted when synthetic oligonucleotide probes were used.

Immunofluorescence Microscopy

4–5 μm cryostat sections of various tissues, including esophagus, epidermis, endo- and exocervix, vagina, tongue, lung, liver, and colon were processed as described (cf. 1). Monoclonal antibody 6B10, specific for cytokeratin 4 (91) was kindly provided by Dr. G. van Muijen (University of Leiden, Netherlands).

Results

Immunocytochemical Localization of Stratification-related Cytokeratins in Internal Epithelia

Most internal squamous stratified epithelia are characterized by abundant amounts of cytokeratins 4 and 13, together with usually lesser amounts of cytokeratins 5, 6, 14, and 15 (cf. 73). In immunofluorescence microscopy, monoclonal antibodies specific for cytokeratins 4 and 13 stain, in the epithelia studied so far, all suprabasal cell layers rather uniformly but leave the basal cell layer unstained. For example, Fig. 1, *a* and *b* shows the reactivity of the cytokeratin 4-specific monoclonal antibody 6B10 on esophagus, and similar pictures were also obtained with other stratified tissues (not shown) such as exocervix (cf. 22), vagina, oral, and lingual mucosa (e.g., 72). Remarkably, cytokeratin 4-specific immunostaining is not restricted to the stratified epithelium but positive reaction can also be seen in certain cells or cell clusters in the ducts of mucous and serous glands of the esophageal submucosa (Fig. 1, *c* and *d*; cf. 40). As antibodies specific for cytokeratins 5, 6, 14, or 15 are not available the tissue distribution of these cytokeratins in internal epithelia is not known.

Isolation of cDNA Clones Encoding Human Cytokeratins

A λgt10 cDNA library of the cell line A-431 was initially chosen because these cells coexpress a total of up to eleven different cytokeratins (65, 73), including cytokeratins 5, 6, 13–15, and at least in some sublines, small amounts of cytokeratin 4 as well (unpublished data; 91). 1.2×10^5 plaque-forming units (pfu) of the amplified library were screened with a mixed probe containing the nick-translated inserts of

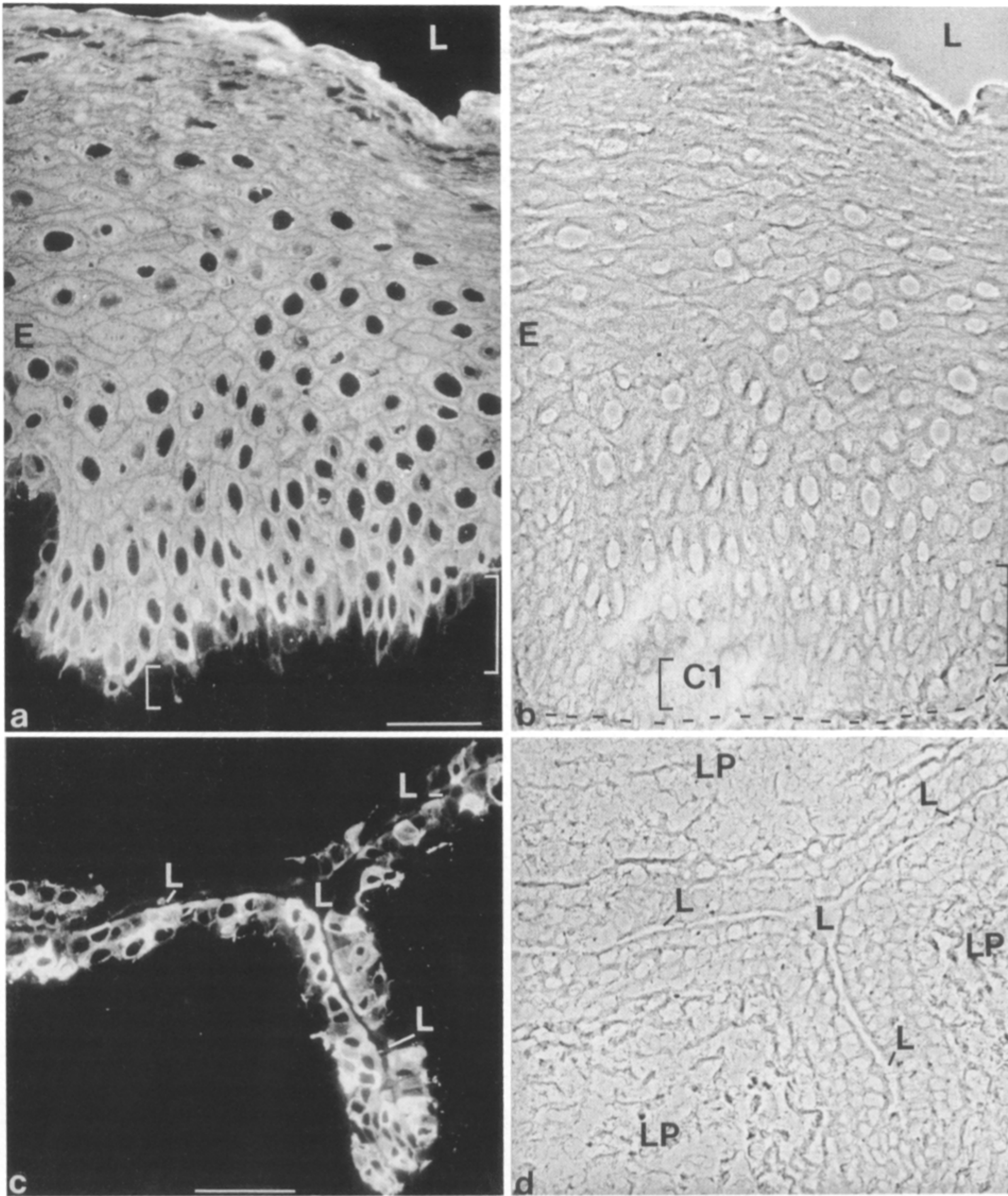

Figure 1. Immunofluorescence microscopy of frozen sections of human esophagus and its submucosal glands after reaction with monoclonal antibody 6B10 specific for cytokeratin 4. The same field is shown in epifluorescence (*a*, *c*) and phase-contrast (*b*, *d*) optics. *L*, lumen; *E*, epithelium; *LP*, lamina propria. (*a*, *b*) Intense immunofluorescence of suprabasal cell layers, whereas the two to three basalmost cell layers, forming the basal compartment C1 (*brackets*; the *broken line* in *b* denotes the basal lamina), are unstained. (*c*, *d*) Heterogeneous reaction of certain groups of cells in the duct epithelium of an esophageal gland. Most of the positively stained cells are adluminal. Bars, 50 μm.

diverse bovine cDNA clones (see Materials and Methods) under low stringency conditions. 19 phages were plaque-purified and 11 of them were confirmed as containing cytokeratin-positive clones by Southern blot hybridization. Hybridization with clones pKH8[1] and pKH18[2] under stringent conditions, in addition to restriction enzyme mapping, identified three phage clones as positive for cytokeratin 8 and four clones positive for cytokeratin 18 (cf. 56 and 71). The inserts of

three of the remaining clones (λKH4[1], λKH4[2], and λKH15[1]) were integrated into the transcription plasmid pTZ 18R.

Identification of a cDNA Clone Encoding Human Cytokeratin 4

After hybridization with RNA from A-431 cells, the subclone pKH4[1] selected a mRNA which yielded, on release and in vitro translation, a barely detectable product which did not comigrate with any of the major cytokeratins present in A-431 cells (data not shown). However, a hybrid selection-translation experiment with RNA from Detroit-562 cells revealed a distinct translational product which comigrated with authentic cytokeratin 4 (Fig. 2, *a* and *b*).

When RNA extracted from diverse tissues and cultured cell lines was probed for mRNA encoding cytokeratin 4 in Northern blot hybridization experiments, using specific riboprobes derived from clone pKH4[1] (for details, see Fig. 3), all those tissues and cells in which this cytokeratin had been found by immunocytochemistry and/or gel electrophoresis were also positive in this test (Fig. 2, *c–e*). In contrast, epithelial cell cultures as well as simple (colon) and squamous stratified (epidermis) epithelial tissues, in which this cytokeratin has not been detected at the protein level, were negative in Northern blot hybridization with this probe (Fig. 2, *c–e*). These results also show that, under our stringency conditions of hybridization, cross-hybridization with mRNAs for other cytokeratin members of the same subfamily did not occur, thus allowing the specific detection of cytokeratin 4 mRNA (cf. 46; see, however, 32, 49).

The by far highest concentration of mRNA for cytokeratin 4 was found in esophageal RNA (Fig. 2 *c*, lanes *4* and *4'*; Fig. 2 *d*, lane *10*). When cultured cells were probed for cytokeratin, two of the carcinoma cell lines derived from stratified tissues, i.e., pharyngeal carcinoma line Detroit-562 and the bladder carcinoma-derived line RT-112, showed mRNA signals of moderate intensity (Fig. 3 *c*, lanes *2'* and *3'*, and *3 d*, lane *7*), whereas only a weak signal was obtained with RNA from A-431 cells from which the cDNA clone was isolated (Fig. 2 *d*, lanes *8* and *9*). This seems to be in agreement with biochemical findings of only miniscule amounts of cytokeratin 4 in this cell line (cf. 65, 91).

Sequence Characteristics of Human Cytokeratin 4

Sequencing shows that the 1760-bp insert of clone pKH4[1] offers an open reading frame encoding 408 amino acids (Fig. 3) and a 3'-noncoding portion of 536 bp which contains a ca-

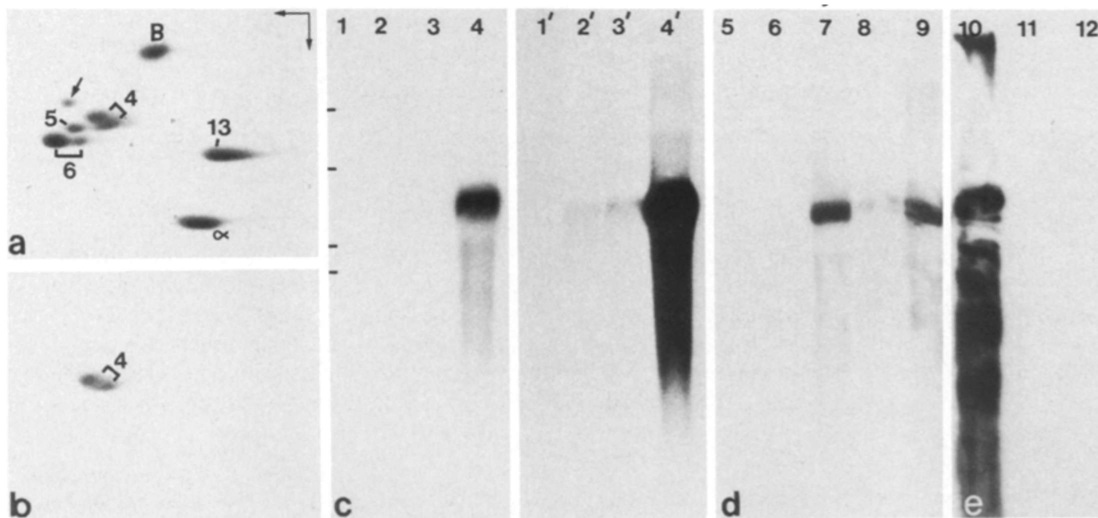

Figure 2. Identification and characterization of clone pKH4[1] as coding for human cytokeratin 4 by hybrid selection and translation (*a*, *b*) or Northern blot hybridization analysis (*c–e*). (*a*, *b*) Hybrid-selection experiment in which total RNA from cultured human Detroit-562 cells have been exposed to clone pKH4[1], showing the in vitro translated product of the specifically bound mRNA to comigrate with authentic cytokeratin 4 of a cytoskeletal preparation of esophagus. (*a*) Coomassie Blue-stained polypeptides separated by two-dimensional gel electrophoresis (in *a*, the direction of nonequilibrium pH-gradient electrophoresis in first dimension is from right to left and the second dimension SDS-PAGE is from top to bottom, as indicated by arrows in the upper right corner) include the major esophageal cytokeratins 4, 5, 6, and 13, reference proteins (bovine serum albumin, *B*, and α-actin, α) and an endogenous component of the rabbit reticulocyte lysate system used for in vitro translation (*arrow*). (*b*) Autoradiograph of the gel shown in *a*, revealing the [^{35}S]methionine-labeled cytokeratin 4, and a slightly more acidic degradation product, as the only labeled polypeptides. (*c–e*) Autoradiographs of different Northern blots hybridized with antisense riboprobes for the 3'-noncoding sequence of clone pKH4[1] (see Materials and Methods). 20 μg of total RNA was loaded on the gel in *c*, whereas 10 μg of total RNA was applied in *d* and *e*, with the exception of lanes *8* (5 μg of poly(A)$^+$-RNA) and *9* (50 μg of poly(A)$^+$-RNA). RNA was from SV-40-transformed fibroblasts (lanes *1*, *1'*, and *5*), the pharynx carcinoma-derived cell line Detroit-562 (lanes 2, *2'*, and *7*), the bladder carcinoma-derived cell line RT-112 (lanes *3* and *3'*), human esophagus (lanes *4*, *4'*, and *10*; in the overexposed lanes, reactivity with degraded RNA is more prominent), the breast carcinoma cell line MCF-7 (lane *6*), the vulvar epidermoid carcinoma-derived cell line A-431 (lanes *8* and *9*), human colon mucosa (lane *11*), and adult epidermis (lanes *12*). Prolonged autoradiographic exposure of lanes *1–4* is shown in lanes *1'–4'*. Bars on the left margin indicate the positions of (from top to bottom) the 28 S, 23 S, 18 S, and 16 S rRNAs coelectrophoresed as markers. The size of the mRNA encoding cytokeratin 4 was estimated to be ~2.2 kb. We cannot satisfactorily explain the different signal intensities in lanes 2, *2'*, and *7*; perhaps they are due to different RNA transfer efficiencies.

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   1 TCACTCAACCAGAGCTTGCTCACCCCCCTCCACGTGGAGATTGACCCTGAGATCCAGAAAGTCCGGACGGAAGAGCGCGAACAGATCAAG
   1  S  L  N  Q  S  L  L  T  P  L  H  V  E  I  D  P  E  I  Q  K  V  R  T  E  E  R  E  Q  I  K

  91 CTCCTCAACAACAAGTTTGCCTCCTTCATCGACAAGGTGCAGTTCTTAGAGCAACAGAATAAGGTCCTGGAGACCAAATGGAACCTGCTC
  31  L  L  N  N  K  F  A  S  F  I  D  K  V  Q  F  L  E  Q  Q  N  K  V  L  E  T  K  W  N  L  L

 181 CAGCAGCAGACGACCACCACCTCCAGCAAAAACCTTGAGCCCCTCTTTGAGACCTACCTCAGTGTCCTGAGGAAGCAGCTAGATACCTTG
  61  Q  Q  Q  T  T  T  T  S  S  K  N  L  E  P  L  F  E  T  Y  L  S  V  L  R  K  Q  L  D  T  L

 271 GGCAATGACAAAGGGCGCCTGCAGTCTGAGCTGAAGACCATGCAGGACAGCGTGGAGGACTTCAAGACTAAGTATGAAGAGGAGATCAAC
  91  G  N  D  K  G  R  L  Q  S  E  L  K  T  M  Q  D  S  V  E  D  F  K  T  K  Y  E  E  E  I  N

 361 AAACGCACAGCAGCCGAGAATGACTTTGTGGTCCTAAAGAAGGACGTGGATGCTGCCTACCTGAACAAGGTGGAGTTGGAGGCCAAGGTG
 121  K  R  T  A  A  E  N  D  F  V  V  L  K  K  D  V  D  A  A  Y  L  N  K  V  E  L  E  A  K  V

 451 GACAGTCTTAATGACGAGATCAACTTCCTGAAGGTCCTCTATGATGCGGAGCTGTCCCAGATGCAGACCCATGTCAGCGACACGTCCGTG
 151  D  S  L  N  D  E  I  N  F  L  K  V  L  Y  D  A  E  L  S  Q  M  Q  T  H  V  S  D  T  S  V

 541 GTCCTTTCCATGGACAACAACCGCAACCTGGACCTGGACAGCATTATTGCCGAGGTCCGTGCCCAGTACGAGGAGATTGCCCAGAGGAGC
 181  V  L  S  M  D  N  N  R  N  L  D  L  D  S  I  I  A  E  V  R  A  Q  Y  E  E  I  A  Q  R  S

 631 AAGGCTGAGGCTGAAGCCCTGTACCAGACCAAGGTCCAGCAGCTCCAGATCTCGGTTGACCAACATGGTGACAACCTGAAGAACACCAAG
 211  K  A  E  A  E  A  L  Y  Q  T  K  V  Q  Q  L  Q  I  S  V  D  Q  H  G  D  N  L  K  N  T  K

 721 AGTGAAATTGCAGAGCTCAACAGGATGATCCAGAGGCTGCGGGCAGAGATCGAGAACATCAAGAAGCAGTGCCAGACTCTTCAGGTATCC
 241  S  E  I  A  E  L  N  R  M  I  Q  R  L  R  A  E  I  E  N  I  K  K  Q  C  Q  T  L  Q  V  S

 811 GTGGCTGATGCAGAGCAGCGAGGTGAGAATGCCCTTAAAGATGCCCACAGCAAGCGCGTAGAGCTGGAGGCTGCCCTGCAGCAGGCCAAG
 271  V  A  D  A  E  Q  R  G  E  N  A  L  K  D  A  H  S  K  R  V  E  L  E  A  A  L  Q  Q  A  K

 901 GAGGAGCTGGCACGAATGCTGCGTGAGTACCAGGAGCTCATGAGTGTGAAGCTGGCCTTGGACATCGAGATCGCCACCTACCGCAAACTG
 301  E  E  L  A  R  M  L  R  E  Y  Q  E  L  M  S  V  K  L  A  L  D  I  E  I  A  T  Y  R  K  L

 991 CTGGAGGGCGAGGAGTACAGAATGTCTGGAGAATGCCAGAGTGCCGTGAGCATCTCTGTGGTCAGCGGTAGCACCAGCACTGGAGGCATC
 331  L  E  G  E↑ E  Y  R  M  S  G  E  C  Q  S  A  V  S  I  S  V  V  S  G  S  T  S  T  G  G  I

1081 AGCGGAGGATTAGGAAGTGGCTCCGGGTTTGGCCTGAGTAGTGGCTTTGGCTCCGGCTCTGGAAGTGGCTTTGGGTTTGGTGGCAGTGTC
 361  S  G  G  L  G  S  G  S  G  F  G  L  S  S  G  F  G  S  G  S  G  S  G  F  G  F  G  G  S  V

1171 TCTGGCAGTTCCAGCAGCAAGATCATCTCTACCACCACCCTGAACAAGAGACGATAGAGGAGACGAGGTCCCTGCAGCTCACTGTGTCCA
 391  S  G  S  S  S  S  K  I  I  S  T  T  T  L  N  K  R  R  *

1261 GCTGGGCCCAGCACTGGTGTCTCTGTGCTTCCTTCACTTCACCTCCATCCTCTGTCTCTGGGGCTCATCTTACTAGTATCCCCTCCACTA

1351 TCCCATGGGCTCTCTCTGCCCCAGGATGATCTTCTGTGCTGGGACAGGGACTCTGCCTCTTGGAGTTTGGTAGCTACTTCTTGATTTGGG

1441 CCTGGTGACCCACCTGGAATGGGAAGGATGTCAGCTGACCTCTCACCTCCCATGGGCAGAGAAGAAAATGACCAGGAGTGTCATCTCCAG

1531 AATTATTGGGGTCACATATGTCCCTTCCCAGTCCAATGCCATCTCCCACTAGATCCTGTATTATCCATCTACATCAGAACCAAACTACTT

1621 CTCCAACACCCGGCAGCACTTGGCCCTGCAAGCTTAGGATGAGAACCACTTAGTGTCCCATTCTACTCCTCTCATTCCCTCTTATCCATC

1711 TGCAGGTGAATCTTCAATAAAATGCTTTTGTCATTCAAAAAAAAAAAAAA
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Figure 3. Nucleotide sequence of clone pKH4[1] and the deduced partial amino acid sequence of human cytokeratin 4 (one letter code). The end of the α-helical coiled-coil domains is demarcated by an arrow. Asterisk shows the stop codon. The polyadenylation signal 15 bp upstream from the poly(A)-tail is underlined. The *Hind*III site used for truncation prior to in vitro transcription for preparing the riboprobe is overlined.

nonic polyadenylation site and, 15 bp further downstream, an uninterrupted stretch of 14 adenosine residues, apparently the residue of the poly(A)-region. Sequence comparison with other IF proteins identified the encoded protein as a typical member of the basic (type II) subfamily of cytokeratins (cf. 37). Conformation prediction analyses (for programs applied see 2, 79) revealed the typical organization into central α-helical regions flanked by non-α-helical head and tail domains (cf. Fig. 4). The three highly conserved helical regions (1*a*, 1*b*, and 2) are characterized by a typical heptad pattern, which is interrupted by short spacer sequences, indicating that these regions form stable coiled-coil complexes with complementary cytokeratins (for reviews see 36, 37, 84, 93). The sequence demarcating the abrupt transition from

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              H                      ┌→ C1a                           ←┐          ┌→ C1b
H 4   SLNQSLLTPLHVEIDPEIQKVRTEEREQIKLLNNKFASFIDKVQFLEQQNKVLETKWNLLQQQTTTTSSKNLEPLFETYLSVLRKQLDTL
      15******** *********5** ****** ************ *********************** * *2* ****5 5*** ****
MK57  TINQSLLTPLQVEIDPEIQKIRTAEREQIKTLNNKFASFIDKVRFLEQQNKVLETKWNLLQQQTTTTSPKSLDPFFETYINALRKNLDTL

  ——→ C1b                                                                  ←┐
H 4   GNDKGRLQSELKTMQDSVEDFKTKYEEEINKRTAAENDFVVLKKDVDAAYLNKVELEAKVDSLNDEINFLKVLYDAELSQMQTHVSDTSV
       *********** ************************************5 *******52** ***** 3***2*** ***********
MK57  SNDKGRLQSELKMMQDSVEDFKTKYEEEINKRTAAENDFVVLKKDVDAAYMIKVELEAKMESLKDEINFTRVLYEAELAQMQTHVSDTSV

                    ┌→ C2
H 4   VLSMDNNRNLDLDSIIAEVRAQYEEIAQRSKAEAEALYQTKVQQLQISVDQHGDNLKNTKSEIAELNRMIQRLRAEIENIKKQCQTLQVS
      ************* **********2** 3****5*  ** ******5*5***** ** ** ** ******************* ** *5*
MK57  VLSMDNNRNLDLDGIIAEVRAQYEDIARKSKAEVESWYQIKVQQLQMSADQHGDSLKTTKNEISELNRMIQRLRAEIENIKKQSQTPQAS

  ——→ C2                                                          ←┐                 T
H 4   VADAEQRGENALKDAHSKRVELEAALQQAKEELARMLREYQELMSVKLALDIEIATYRKLLEGEEYRMSGECQSAVSISVVSGSTSTGGI
      ********* ***** ***5*** *** ***2***5**2** ** ******5************* ****** ******** **
MK57  VADAEQRGELALKDAYSKRAELETALQKAKEDLARLLRDYQALMNVKLALDVEIATYRKLLEGEECRMSGECKSAVSISVVGGSQH---W

                         T
H 4   SGGLGSGSGFGLSSGFGSGSGSGFGFGGSVSGSSSSKIIS--TTTLNKRR
        *** ****        ************ 5 * * *** *  * *    *
MK57  RSGLGLGSGFCS----GSGSGSGFGFGGGIYGGSGSKITSSATITKRSPR
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Figure 4. Comparison of the amino acid sequence of human cytokeratin 4 (*H 4*) with that of the murine M_r 57,000 cytokeratin (*MK57*; 51). *Asterisks* show identical residues, numbers denote conservative exchanges: 1 (for S and T), 2 for acidic amino acids (D and E), 3 for basic residues (H, R, K), and 5 for hydrophobic residues (M, I, L, V, A). The coiled-coil rod domain is divided into α-helical coils C1a, C1b, and C2 which are flanked by an incomplete head domain (*H*) and the tail region (*T*). Note the high degree of homology (~85%) of amino acid sequences of the polypeptides which is not restricted to the rod domain but also extends to the head and the tail regions.

coil 2 into the tail domain is the typical "consensus sequence" TYR(X)LLEG present in all IF proteins (32, 36, 37, 73, 84, 93). Comparisons with other protein sequences identified the murine M_r 57,000 (mol wt 56,429) cytokeratin, expressed predominantly in tongue and forestomach (51), as the protein most closely related to pKH4^1 (Fig. 4). The amino acid identity of ~80% between human cytokeratin 4 and this murine cytokeratin was considerably higher than that with human cytokeratin 6 (65%; 37, 88), indicating that the mouse M_r 57,000 cytokeratin may be the equivalent to human cytokeratin 4. None of the various bovine type II cytokeratin sequences determined in our laboratory (cf. 45, 47, 58) showed a similarly close relationship to human cytokeratin 4.

The tail region of human cytokeratin 4 is exceptionally rich in hydroxyamino acids (40%) but contains less glycine residues and glycine-rich oligopeptide repeats than several epidermal type II cytokeratins (37, 45, 82). The conserved sequence motif of the basic heptapeptide DGK$^{L}_{V}$VS$^{T}_{E}$ present in many IF proteins (19, 29, 44, 45, 56, 58, 73, 80; see also below) is absent in both human cytokeratin 4 and its murine equivalent. The carboxyterminus is very basic, as in several other type II cytokeratins (e.g., 45).

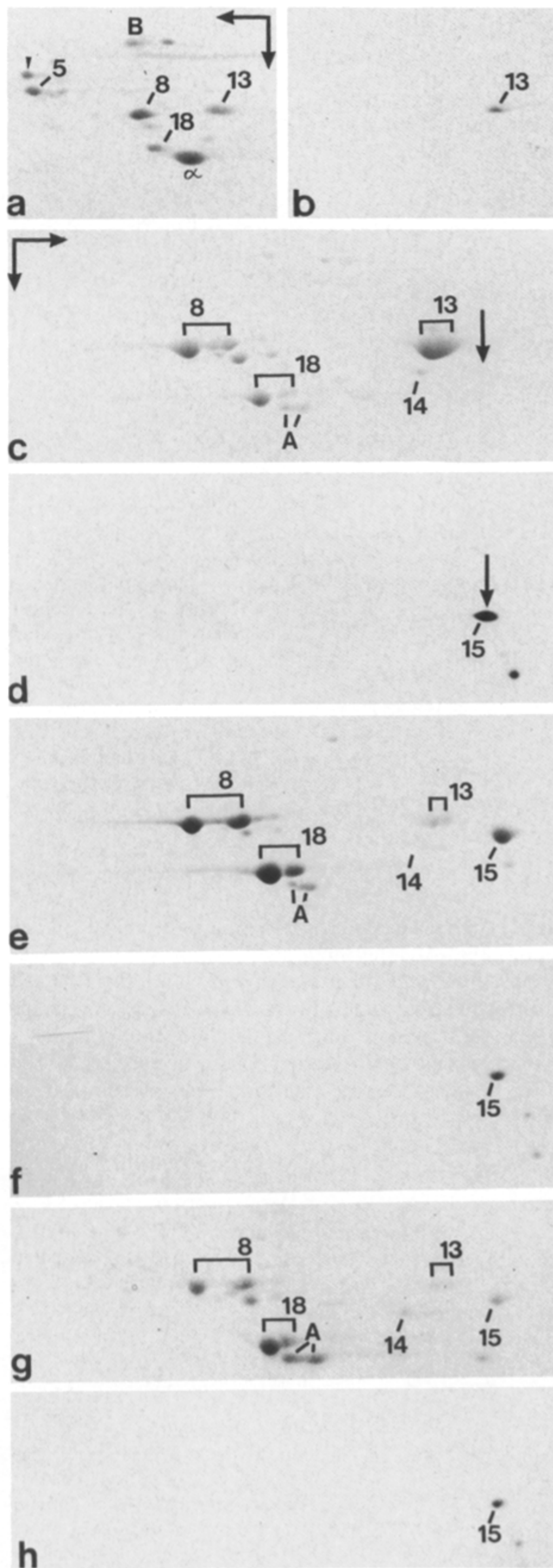


Figure 5. Identification of cDNA clones coding for human cytokeratin 15. (*a, b*) Hybrid selection-translation of poly(A)$^+$-RNA from cells of the A-431 subline E_3 as obtained after hybridization to the cDNA clone pKH15^1. (*a*) Coomassie Blue-stained cytoskeletal proteins of A-431 cells (line E_3) separated by two-dimensional gel electrophoresis (*arrows* as in Fig. 2 *a*), together with proteins of the translational assay, coelectrophoresed with reference proteins (as in Fig. 2 *a*). The major cytokeratins 5, 8, 13, and 18 are denoted; the arrow indicates the major endogenous component of the reticulocyte lysate system. (*b*) Autoradiograph corresponding to *a*. The major [^{35}S]methionine-labeled product of the in vitro translation of mRNA selected by pKH15^1 comigrates with authentic cytokeratin 13. (*c–f*) Two-dimensional gel electrophoresis (*horizontal arrow*, direction of isoelectric focusing used in first dimension; *downward arrow*, direction of second dimension SDS-PAGE) of the polypeptides synthesized in vitro by transcription of the reconstructed complete cDNA clone pKH15^3 and subsequent translation of the RNA obtained with cytoskeletal proteins from A-431 cells of line E_3 (*c, d*) or E_6 (*e, f*). (*c*) Coomassie Blue staining showing the major cytokeratins 8, 13, 14, and 18 and residual β- and γ-actin (*A*) of A-431 cells. The arrow denotes the position of cytokeratin 15 which is only a miniscule cytokeratin in this cell line. (*d*) Autoradiograph of the gel shown in *c*, showing that the polypeptide synthesized from clone pKH15^3 in vitro comigrates with cytokeratin 15 and not with cytokeratin 13. (*e*) Coomassie Blue staining of cytoskeletal proteins of A-431 subline E_6 rich in cytokeratin 15 and poor in cytokeratin 13. (*f*) Autoradiograph of the gel shown in *e*, showing the comigration of the polypeptide synthesized from clone pKH15^3 in vitro with cytokeratin 15 and not with cytokeratin 13. (*g, h*) Hybrid selection-translation of poly(A)$^+$-RNA from cloned A-431 cells (line E_6) hybridized to the 3′-end-specific cDNA clone pKH15^2 (see Results). (*g*) Coomassie Blue-stained polypeptides (as in *e*). (*h*) Autoradiography corresponding to *g*, showing the product of the mRNA from A-431 cells (clone 6) specifically selected by the 3′-specific subclone pKH15^2, the cytoskeletal proteins of clone 6 of A-431 (same denotations as in *e*) together with endogenous components of the reticulocyte lysate system as detected by Coomassie Blue staining. (*h*) Corresponding autoradiograph to *g*. The [^{35}S]methionine-labeled product of the in vitro translation comigrates with authentic cytokeratin 15 and not with cytokeratin 13.

Identification of a cDNA and a Genomic Clone Encoding Human Cytokeratin 15

When the cDNA clone pKH15[1] and the reconstructed cDNA clone pKH15[3] were used in hybrid selection–translation experiments with RNA from A-431 cells of clone E_3 it specifically selected a mRNA that was translated in vitro into a polypeptide comigrating with authentic cytokeratin 13 (e.g., Fig. 5, *a* and *b*). However, results obtained in Northern blot hybridizations, notably with epidermal mRNA, and in mRNA hybrid-release experiments with certain clonal A-431 sublines containing lower amounts of cytokeratin 13 but large amounts of cytokeratin 15 such as clone E_6 were incompatible with the interpretation of a cytokeratin 13-encoding cDNA (data not shown; see also below). For an unequivocal identification of these cDNA clones we therefore used clone pKH15[3] for in vitro synthesis of the corresponding mRNA and polypeptide which was then found to comigrate, in gel electrophoresis, with cytokeratin 15 and not with cytokeratin 13 (Fig. 5, *c–f*, presents results from two different A-431 sublines, E_3 and E_6). This indicated that the protein encoded by this clone is in fact cytokeratin 15 and that cytokeratins 13 and 15 are very closely related. To examine this interpretation we synthesized a polynucleotide of 75 residues located in the 3′-noncoding region, i.e., the region showing the highest sequence divergence within the type I cytokeratin multigene family (46, 47, 74, 79). This probe (pKH15[2]) allowed the distinction between cytokeratins 13 and 15 as demonstrated in the hybrid selection experiment shown in Fig. 5, *g* and *h*.

Northern blot experiments identified a cytokeratin 15 mRNA of ∼1.9 kb in A-431 cells (Fig. 6, lanes *1* and *7*) and in certain stratified epithelial tissues, including epidermis (Fig. 6, lane *3*) and esophagus (Fig. 6, lane *8*). Only a very weak signal was obtained in cultured cells of the line Detroit 562 (Fig. 6, lane *6*) whereas simple epithelia and various cell lines derived therefrom were negative (e.g., Fig. 6, lanes *2*, *5*, and *9*).

Sequence Characteristics of Human Cytokeratin 15

The ∼1.3-kb insert of the cDNA clone pKH15[1] extends from within coil 1a to the 3′-end, including a short poly-A stretch. A 3′-specific, nick-translated fragment of ∼450 nucleotides of this clone was used to screen a human genomic library. The isolated phage clone λKH15[2] contained the complete gene encoding human cytokeratin 15. Nucleic acid sequencing proved the identity of the cDNA and the genomic clone. The nucleic acid sequence of human cytokeratin 15 as deduced from cDNA clone pKH15[1] and the 479-bp *Kpn*I fragment of the genomic clone λKH15[2] encompasses ∼1.7 kb (Fig. 7). S1 nuclease mapping (data not shown) indicated that the transcription started at the *Kpn*I site at the start of the sequence presented in Fig. 7, most probably at position 4, thus defining a short 58-nucleotide-long, 5′-nontranslated region. The cDNA terminates 23 bp downstream of a typical polyadenylation signal with a short poly-(A) stretch.

The coding region of cytokeratin 15 defines a polypeptide of 456 amino acids, amounting to a total mol wt of 49,170 including the initial methonine which is probably lost after translation. This value is in agreement with that estimated from SDS-PAGE analyses (M_r 50,000; cf. 11, 24, 65, 96). The 96-amino acid-long head domain displays features common to various cytokeratins (cf. 2) such as an amino-terminal cluster of hydroxyamino acids, the motif $S^{A}_{V}SS^{A}_{S}RF^{V}_{S}S$ as well as several glycine-rich oligopeptide repeats (Figs. 7 and 8). The very high glycine content (44.3%) in this region is particularly noteworthy.

The structure predicted for the α-helical rod domain is in agreement with the basic IF conformation (for reviews, see 31, 82, 93). The three coiled-coil domains defined by the heptad repeats are interrupted by two spacer sequences, and the "consensus sequence" TYR(X)LLEG is recognized at the end of this domain.

The tail region contains only a short stretch rich in glycine and serine. In contrast to cytokeratin 4 a heptapeptide motif DGQVVSS similar to the hallmark sequence $DGK^{L}_{V}VS^{T}_{E}$ mentioned above, which shows a striking resemblance to the core motif of calcium-binding proteins of the "EF-finger" type (19), is located close to the carboxy terminus.

Comparison of the amino acid sequence of cytokeratin 15 with those of other proteins identified this polypeptide as a member of the acidic (type I) subfamily of cytokeratins (e.g., 36, 83). It shows a particularly close relationship (Fig. 8) to the human cytokeratin 14 (76% identity in the rod region; 62% overall identity; 36, 61), and murine M_r 47,000 cytokeratin (85% identical positions in the rod domain; 51) and the amphibian cytokeratin A_I of the XK81 gene subfamily (44, 63). Surprisingly, it also shows a high similarity (74% identical positions in the rod) with the simple epithelial type I cytokeratin 19 from cow (2).

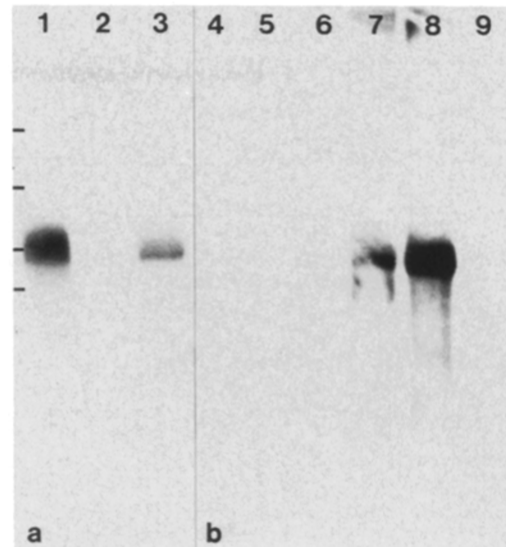


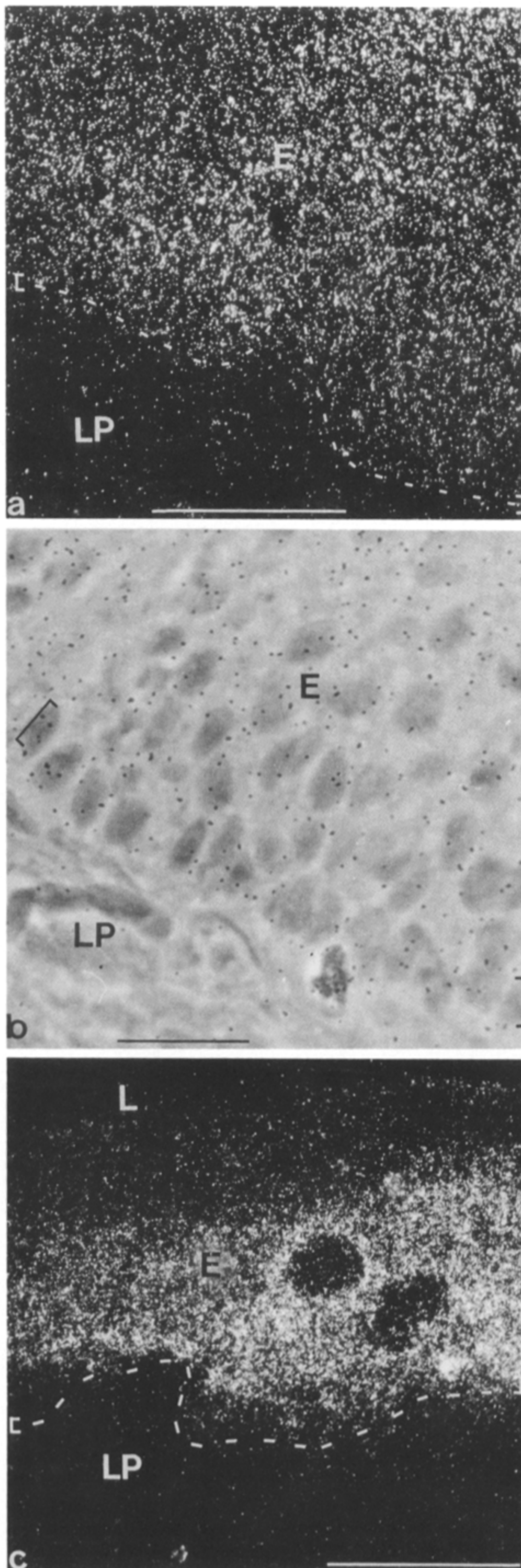
Figure 6. Autoradiographs of Northern blot hybridization experiments using riboprobes derived from the *Bgl*I-truncated clone pKH15[1]. 10 μg of total RNA was applied, except for lanes *2* and *3* (50 μg of total RNA) and lanes *1* and *7* which contain 2 and 5 μg of poly(A)+-RNA, respectively. RNA was extracted from human A-431 cells (lanes *1* and *7*), liver (lane *2*), epidermis (lane *3*), SV-40-transformed fibroblasts (lane *4*), MCF-7 cells (lane *5*), Detroit-562 cells (lane *6*), esophagus (lane *8*), and colon (lane *9*). Note intense reaction with a ∼1.9-kb RNA in A-431 cells, epidermis, and esophagus. A weak but clearly detectable hybridization signal was also seen in Detroit-562 cells after prolonged exposure (not shown). Bars indicate rRNAs as in Fig. 2.

```
   1 GGTACCTCCTGCCAGCACCTCTTGGGTTTGCTGAGAACTCACGGGCTCCAGCTACCTGGCCATGACCACCACATTTCTGCAAACTTCTTC
   1                                                                   M  T  T  T  F  L  Q  T  S  S

  91 CTCCACCTTTGGGGGTGGCTCAACCCGAGGGGGTTCCCTCCTGGCTGGGGGAGGTGGCTTTGGTGGGGGGAGTCTCTCTGGGGGAGGTGG
  11  S  T  F  G  G  G  S  T  R  G  G  S  L  L  A  G  G  G  G  F  G  G  G  S  L  S  G  G  G  G

 181 AAGCCGAAGTATCTCAGCTTCTTCTGCTAGGTTTGTCTCTTCAGGGTCAGGAGGAGGATATGGGGGTGGCATGAGGGTCTGTGGCTTTGG
  41  S  R  S  I  S  A  S  S  A  R  F  V  S  S  G  S  G  G  G  Y  G  G  G  M  R  V  C  G  F  G

 271 TGGAGGGGCTGGTAGTGTTTTCGGTGGAGGCTTTGGAGGGGGCGTTGGTGGGGGTTTTGGTGGTGGCTTTGGTGGTGGCGATGGTGGTCT
  71  G  G  A  G  S  V  F  G  G  G  F  G  G  G  V  G  G  G  F  G  G  G  F  G  G  G  D  G  G  L
                                                                              ▼
 361 CCTCTCTGGCAATGAGAAAATTACCATGCAGAACCTCAATGACCGCCTGGCCTCCTACCTGGACAAGGTACGTGCCCTGGAGGAGGCCAA
 101  L  S  G  N  E  K  I  T  M  Q  N  L  N  D  R  L  A  S  Y  L  D  K  V  R  A  L  E  E  A  N

 451 TGCTGACCTGGAGGTGAAGATCCATGACTGGTACCAGAAGCAGACCCCAGCCAGCCCAGAATGCGACTACAGCCAATACTTCAAGACCAT
 131  A  D  L  E  V  K  I  H  D  W  Y  Q  K  Q  T  P  A  S  P  E  C  D  Y  S  Q  Y  F  K  T  I

 541 TGAAGAGCTCCGGGACAAGATCATGGCCACCACCATCGACAACTCCCGGGTCATCCTGGAGATCGACAATGCCAGGCTGGCTGCGGACGA
 161  E  E  L  R  D  K  I  M  A  T  T  I  D  N  S  R  V  I  L  E  I  D  N  A  R  L  A  A  D  D

 631 CTTCAGGCTCAAGTATGAGAATGAGCTGGCCCTGCGCCAGGGCGTTGAGGCTGACATCAACGGCTTGCGCCGAGTCCTGGATGAGCTGAC
 191  F  R  L  K  Y  E  N  E  L  A  L  R  Q  G  V  E  A  D  I  N  G  L  R  R  V  L  D  E  L  T

 721 CCTGGCCAGGACTGACCTGGAGATGCAGATCGAGGGCCTGAATGAGGAGCTAGCCTACCTGAAGAAGAACCACGAAGAGGAGATGAAGGA
 221  L  A  R  T  D  L  E  M  Q  I  E  G  L  N  E  E  L  A  Y  L  K  K  N  H  E  E  E  M  K  E

 811 GTTCAGCAGCCAGCTGGCCGGCCAGGTCAATGTGGAGATGGACGCAGCACCGGGTGTGGACCTGACCCGTGTGCTGGCAGAGATGAGGGA
 251  F  S  S  Q  L  A  G  Q  V  N  V  E  M  D  A  A  P  G  V  D  L  T  R  V  L  A  E  M  R  E

 901 GCAGTACGAGGCCATGGCGGAGAAGAACCGCCGGGATGTCGAGGCCTGGTTCTTCAGCAAGACTGAGGAGCTGAACAAAGAGGTGGCCTC
 281  Q  Y  E  A  M  A  E  K  N  R  R  D  V  E  A  W  F  F  S  K  T  E  E  L  N  K  E  V  A  S

 991 CAACACAGAAATGATCCAGACCAGCAAGACGGAGATCACAGACCTGAGACGCACGATGCAGGAGCTGGAGATCGAGCTGCAGTCCCAGCT
 311  N  T  E  M  I  Q  T  S  K  T  E  I  T  D  L  R  R  T  M  Q  E  L  E  I  E  L  Q  S  Q  L

1081 CAGCATGAAAGCTGGGCTGGAGAACTCACTGGCCGAGACAGAGTGCCGCTATGCCACGCAGCTGCAGCAGATCCAGGGGCTCATTGGTGG
 341  S  M  K  A  G  L  E  N  S  L  A  E  T  E  C  R  Y  A  T  Q  L  Q  Q  I  Q  G  L  I  G  G

1171 CCTGGAGGCCCAGCTGAGTGAGCTCCGATGCGAGATGGAGGCTCAGAACCAGGAGTACAAGATGCTGCTTGACATAAAGACACGGCTGGA
 371  L  E  A  Q  L  S  E  L  R  C  E  M  E  A  Q  N  Q  E  Y  K  M  L  L  D  I  K  T  R  L  E

1261 GCAGGAGATCGCTACTTACCGCAGCCTGCTCGAGGGCCAGGATGCCAAGATGGCTGGCATTGGCATCAGGGAAGCCTCTTCAGGAGGTGG
 401  Q  E  I  A  T  Y  R  S  L  L  E  G  Q ↓D  A  K  M  A  G  I  G  I  R  E  A  S  S  G  G  G

1351 TGGTAGCAGCAGCAATTTCCACATCAATGTAGAAGAGTCAGTGGATGGACAGGTGGTTTCTTCCCACAAGAGAGAAATCTAAGTGTCTAT
 431  G  S  S  S  N  F  H  I  N  V  E  E  S  V  D  G  Q  V  V  S  S  H  K  R  E  I  *

1441 TGCAGGAGAAACGTCCCTTGCCACTCCCCACTCTCATCAGGCCAAGTGGAGGACTGGCCAGAGGGCCTGCACATGCAAACTCCAGTCCCT

1531 GCCTTCAGAGAGCTGAAAAGGGTCCCTCGGTCTTTTATTTCAGGGCTTTGCATGCGCTCTATTCCCCCTCTGCCTCTCCCCACCTTCTTT

1621 GGAGCAAGGAGATGCAGCTGTATTGTGTAACAAGCTCATTTGTACAGTGTCTGTTCATGTAATAAAGAATTACTTTTCCTTTTGCAAATA

1711 AAAAAAAAAAAAA
```

Figure 7. Nucleotide sequence and deduced amino acid sequence of human cytokeratin 15. The sequence 5′ of the triangle was derived from the *Kpn*I/*Kpn*I fragment of the genomic clone λKH15[2] whereas the sequence 3′ of the triangle was determined from the cDNA clone pKH15[1], which was confirmed by sequencing the genomic clone. Asterisk denotes the stop codon. The end of the α-helical coiled-coil rod domain is demarcated by an arrow, the canonical polyadenylation site is underlined. The *Bgl*I site used for truncation prior to in vitro transcription is overlined. The broken line denotes the region represented by the synthetic polynucleotide cloned in pKH15[2].

```
H 14   MTTCSRQFTSSSSMKGSCGIGGGIGAGSSRISSVLAGGSCRAPNTYGGGLSVSSSRF-SSGGAYGLGGGYGGGFSSSSSSFGSGFGGGYG
H 15   MTTTFLQ-TSSSTFGGGSTRGGSLLAGGGGFGGGSLSGGGSRS-----ISASSARFVSSGS----GGGYGGGMRVCG--FGGGAGSVFG
XK 81  ---------------------------------------------MTSYRSSSASYYSGSSSKCGFGSRSLAGSNSYGGSSFGAGFSS

        H              ┌→ C1a                               ←┐                     ┌→ C1b
H 14   GGLGAGLGGGFGGGFAGG-DGLLVGSEKVTMQNLNDRLASYLDKVRALEEANADLEVKIRDWYQRQRPA----EIKDYSPYFKTIEDLRN
H 15   GGFGGGVGGGFGGGFGGGDGGLLSGNEKITMQNLNDRLASYLDKVRALEEANADLEVKIHDWYQKQTPA---SPECDYSQYFKTIEELRD
XK 81  GVGSGFSSSGGNFAMAEAASSSFGGNEKHAMQNLNDRLASYLEKVRALEATNSDLEGKIRNWYDKQSDAGIGAGSKDYSKYFEIIAELRN

  ——→ C1b                                                                              ←┐
H 14   KILTATVDNANVLLQIDNARLAADDFRTKYETELNLRMSVEADINGLRRVLDELTLARADLEMQIESLKEELAYLKKNHEEEMNALRGQV
H 15   KIMATTIDNSRVILEIDNARLAADDFRLKYENELALRQGVEADINGLRRVLDELTLARTDLEMQIEGLNEELAYLKKNHEEEMKEFSSQL
XK 81  KIRAATIDNATVTLQIDNARLAADDFRLKFENELALRQSVEGDSNGLRRVLDELILARGDFEIQIESLTEELAYLKKNHEEEMSHAKSQS

                       ┌→ C2
H 14   GGDVNVEMDAAPGVDLSRILNEMRDQYEKMAEKNRKDAEEWFFTKTEELNREVATNSELVQSGKSEISELRRTMQNLEIELQSQLSMKAS
H 15   AGQVNVEMDAAPGVDLTRVLAEMREQYEAMAEKNRRDVEAWFFSKTEELNKEVASNTEMIQTSKTEITDLRRTMQELEIELQSQLSMKAG
XK 81  AGKVSVEMDAALGVDLTSILNNMRADYEILAEKNRRDAELWFNQKSGELKKEISVGVEQVQASKSEITELKRSLQSLEIELQSQLAMKQS

  ——→ C2                                                                   ←┐          T
H 14   LENSLEETKGRYCMQLAQIQEMIGSVEEQLAQLRCEMEQQNQEYKILLDVKTRLEQEIATYRRLLEGEDAHLSSSQFSSGSQSSRDVTSS
H 15   LENSLAETECRYATQLQQIQGLIGGLEAQLSELRCEMEAQNQEYKMLLDIKTRLEQEIATYRSLLEGQDAKMAGIGIREASSGGGG--SS
XK 81  VEGNLNELQGFYSSQLQQIQNTIGSLEEQLLQIRSDMEHQNTEYKLLLDIKTRLEMEIQTYRRLLEGEIGQVTTVANTSSVFSKTESSST

                  T
H 14   SRQIRTK----VMDVHDGKVVSTHEQVLRTKN
H 15   SNFHIN-----VEESVDGQVVSSHK---REI-
XK 81  STTRTRMVKTIVEEVVDGKVVSS-----RVE-
```

Figure 8. Comparison of the amino acid sequence of human cytokeratin 15 (*H 15*) with those of human cytokeratin 14 (*H 14*; 61) and the *Xenopus laevis* cytokeratin XK81 A_1 (XK81; 44, 63). Residues which are identical to human cytokeratin 15 are printed in bold letters. The positions of the α-helical coils in rod domains are designated C1a, C1b, and C2 (*arrows*). *H*, head region, *T*, tail domain. Sequence conservation of the three proteins is high in the rod domain but restricted to certain oligopeptide motifs in the tail and head region.



Distribution of mRNAs for Cytokeratins 4 and 15 as Determined by In Situ Hybridization

To examine the expression of the genes encoding cytokeratins 4 and 15 in complex stratified tissues, we prepared radioactively labeled antisense RNA probes and used them for hybridization in situ. For example, we show the results obtained for esophagus. Intense reactions were seen for both mRNAs (Figs. 9 and 10), in particular when compared to the much weaker hybridization signals obtained with the probes for the simple epithelial type cytokeratins 8 and 18 (cf. 9). In autoradiographs using the cytokeratin 4 mRNA probe the label was spread over most of the epithelial cell layers (Fig. 9, *a* and *b*) but showed, in some regions, a reduction over the basal cell layer (Fig. 9 *c*). Interestingly, in some epithelial regions the silver grain density was also drastically reduced in the upper, i.e., adluminal strata (Fig. 9 *c*).

The antisense riboprobe for cytokeratin 15 mRNA always yielded uniform hybridization in all cell layers of all samples examined (Fig. 10, *a* and *b*), indicative of the presence of this mRNA in the entire epithelium, including the basal cell layer. The same signal distribution was seen after hybridization with the synthetic polynucleotide probe representing the 3'-noncoding region (Fig. 10 *c*). Hybridization reactions were also seen over ductal epithelia traversing the lamina propria, particularly for cytokeratin 15 mRNA (Fig. 10 *b*, *arrow*).

Similar results were obtained for other stratified epithelia such as exocervix (data not shown). Neither for cytokeratin 4 nor for cytokeratin 15 mRNA have we noticed regional intraepithelial heterogeneities of the kind recently described for murine tongue mucosa (76).

Discussion

In this study we have introduced and characterized two DNA clones encoding cytokeratins 4 and 15 which are both typical for certain differentiation programs of stratified epithelia. Notably, cytokeratin 4, which usually occurs in heterotypic "pair" complexes with cytokeratin 13 (11, 23, 38, 87), has been described as a hallmark of a developmental line for certain squamous nonepidermal epithelia ("esophageal type of differentiation" *sensu* 11, 12, 87).

The general molecular features of these two polypeptides are similar to those of other type II (polypeptide 4) and I (polypeptide 15) cytokeratins. Interestingly, both cytokeratins present a tail region with a comparatively low glycine content, unlike many other cytokeratins from squamous epi-

Figure 9. Microscopic autoradiographs of frozen sections of human esophagus, showing the differential distribution of cytokeratin 4 mRNA after hybridization with ^{3}H-labeled transcripts of the *Hind*III-linearized clone pKH4^{1}. Symbols used are the same as in Fig. 1. (*a*, *b*) A region of the upper esophagus, showing a silver grain distribution that is nearly even over most of the epithelium (*E*), with somewhat lower density over the basal cell layer (*brackets*; *broken line* denotes basal lamina). (*a*) Survey autoradiograph (dark field illumination); (*b*) higher magnification (bright field photograph). (*c*) Dark field illumination picture of a section from lower esophagus (in the transition zone to the stomach), showing distinctly lower concentrations of hybridization signals in the basal cell layers and in the upper cell layers (*L*, lumen). Note the absence of labeling over protrusions of the lamina propria (*LP*). Bars: (*a* and *c*) 250 μm; (*b*) 25 μm.

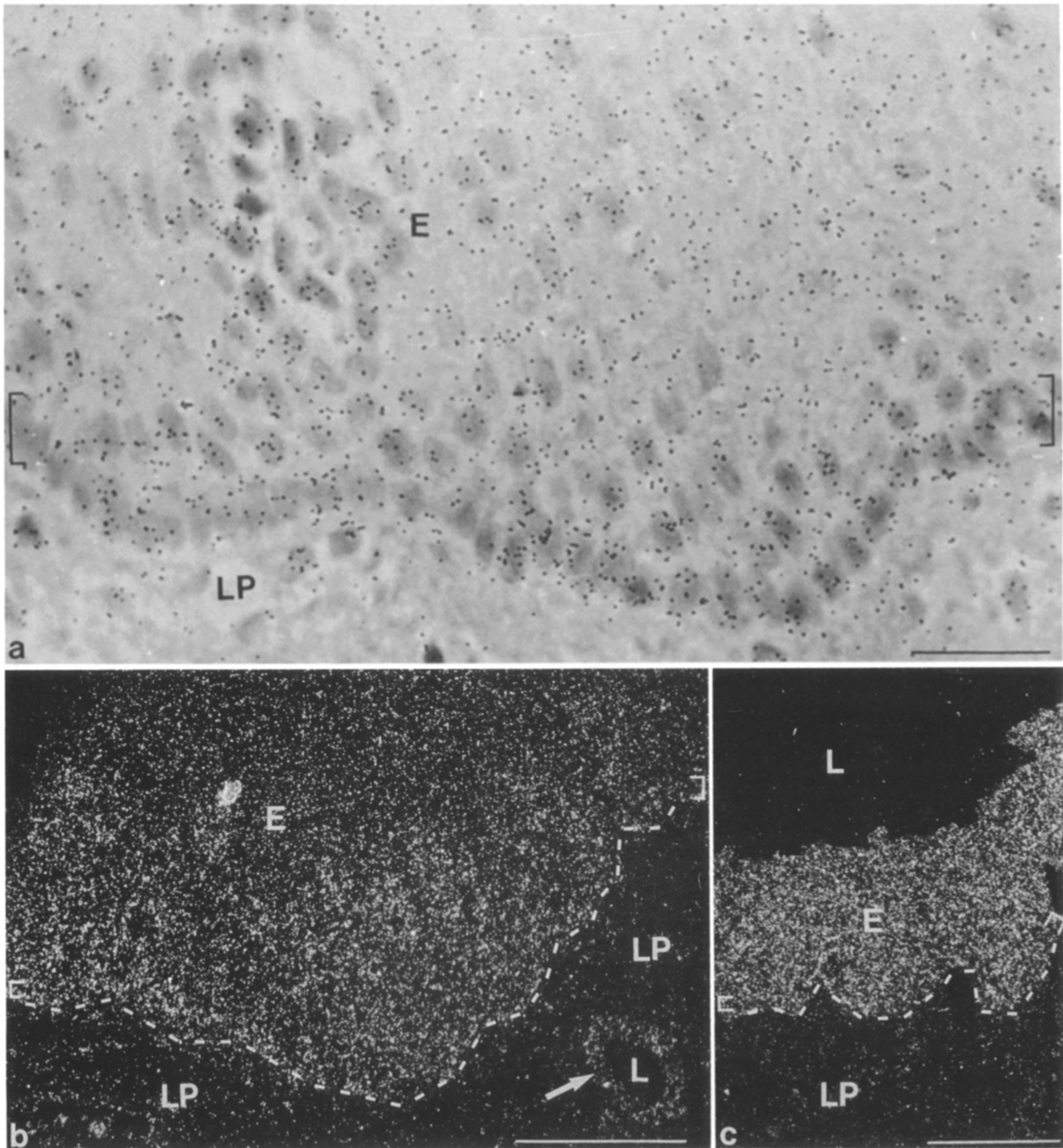


Figure 10. Microscopic autoradiograph of frozen sections of human esophagus, illustrating the homogeneous distribution of cytokeratin 15 mRNA after hybridization with cRNA probes derived from the *BglI*-truncated clone pKH15' (*a*, *b*) and the 5'-end-labeled, 3'-specific polynucleotide (*c*). Symbols are as in Fig. 9. (*a*) Bright field micrograph showing the rather evenly spread label obtained after hybridization with the [α-^{32}P]UTP-labeled cRNA probe for cytokeratin 15. (*b*) Dark field survey autoradiograph of a section hybridized with antisense RNA of pKH15'. Note the even signal distribution over the entire stratified epithelium and in the glandular ducts (*arrow* in *b*). (*c*) Dark field illumination of a section of the lower esophagus after hybridization with the [γ-^{32}P]ATP-labeled polynucleotide complementary to residues 1606–1680 (for details, see Results). Bars: (*a*) 25 μm; (*b* and *c*) 250 μm.

thelia such as various epidermal cytokeratins of human, bovine, murine and amphibian origin (37, 39, 45, 47, 54, 79, 82, 83, 88). The probes specific for the 3'-noncoding region of both cytokeratins, which react specifically with only one mRNA species in the various cells and tissues examined, should be valuable in studying gene expression programs related to the formation of stratified epithelia, including squamous metaplasia of simple epithelia, as well as in the

characterization of various types of squamous cell carcinomas which may be distinguished by the presence or absence of cytokeratin 4 and/or cytokeratin 15 (for gel electrophoretic and immunocytochemical analyses see references 4, 5, 8, 31, 35, 64–67, 91).

The immunocytochemical results of the present study, as well as previous reports on the distribution of cytokeratin 4 in stratified tissues (22, 72, 91; see there also for similar data with cytokeratin 13 antibodies), have shown reduced or even absent reactions in the basal cell layer. This is reminiscent of the negative reactions of basal cell layers of epidermis obtained with antibodies to certain epidermal components, (10, 41, 47, 55, 81, 87, 95). This has usually been interpreted to mean that the synthesis of these proteins is initiated only during or after commitment to vertical, i.e., suprabasal differentiation and is related to the transition of the specific cell from the basal to the suprabasal compartment (cf. 30). However, our in situ hybridizations show that mRNA for both stratification-related cytokeratins can be synthesized in all layers of esophagus, including the basal layer. Clearly, the gene encoding cytokeratin 15 is actively transcribed and cytokeratin 15 mRNA accumulated already in the basal cell layer. For cytokeratin 4, mRNA can also be seen in basal cells of some esophageal regions whereas other regions present only with low levels of mRNA in the basal compartment, which seems to correspond to the absence of immunocytochemically detectable amounts of this protein in basal layers of certain epithelia (this study; 22, 91). The reasons for the observed regional heterogeneities of cytokeratin 4 expression in the basal cell layer as well as in adluminal cell layers are not known.

These results, together with those obtained for cytokeratins 8, 14, and 18 (9), also indicate that at least in certain stratified epithelia commitment to suprabasal translocation and vertical cell differentiation can already commence in the basal cell layer and that the genes for both kinds of cytokeratins, the simple epithelial ones and the stratification-related ones, can be coexpressed, at least in certain regions. This also demonstrates that proliferation and expression of suprabasal marker proteins do not necessarily exclude each other. These conclusions also receive support from observations of the onset of synthesis of cytokeratin 4 in cell clusters of certain glands and glandular ducts (this study; 91), in individual cells of various simple and complex epithelia such as endocervix (22) and bronchial epithelium (91), which may reflect early changes toward squamous metaplasia, and in certain layers of fetal human epidermis (92). Noteworthy in this context are also observations that, in certain cells of squamous and transitional cell carcinomas, the simple epithelial cytokeratins 8 and 18 are often coexpressed with stratification-related ones such as cytokeratins 4–6 and 13–17, although often only focally (cf. 22, 91). It will be important to examine, by in situ hybridization, whether such cellular heterogeneities are only due to immunocytochemical phenomena such as epitope masking (for examples with IF proteins, see 14, 16, 21, 26, 95), or whether it is also evident at the mRNA level.

The observation of coexpression of simple epithelial cytokeratins and cytokeratins 4 and 15 in basal cells of certain stratified tissues also raise the question of the nature of the cytokeratin complexes present in these cells. It is well conceivable that these polypeptides, when present at low concentrations, as this is probably the case in the basal cells, can form heterotypic complexes with each other as well as with other complementary partners, as it has been shown by recombination of purified cytokeratins in vitro (18, 38) and by integration of mRNAs or cloned cytokeratin genes into cultured epithelial cells (25, 33). The possible combination of cytokeratin 4 with type I cytokeratins other than cytokeratin 13 is also supported by reports of cells in which only cytokeratin 4 but not cytokeratin 13 has been detected such as amnion epithelium (75), certain cells of cornea and pancreatic ducts (91), some squamous cell carcinomas (5, 35, 65), and fetal epidermis (92). Vice versa, a number of cultured cell lines and squamous cell carcinomas synthesize considerable amounts of cytokeratin 13 without detectable cytokeratin 4 (1, 67, 77, 78). This raises the question of the coordinate regulation of "pairs" of cytokeratins in some cell types as opposed to the noncoordinated synthesis of these polypeptides in other cells. The new cDNA probes described in this paper will hopefully contribute to the identification of the level of regulation at which such coordinations and disproportionate syntheses take place, also with respect to changes of expression due the environment (15, 50, 89).

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