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Changes in keratin expression during fetal and postnatal development of intestinal epithelial cells

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We have investigated keratin expression in fetal, newborn and adult rat intestines by immunofluorescence staining, immunoblotting of two-dimensional gels and Northern blot analysis of total cellular RNAs. Keratin-type intermediate filaments, composed predominantly of keratin no. 19, were observed already in the undifferentiated stratified epithelium present at 15–16 days of gestation. The marked maturation and differentiation of the epithelium taking place at 18–19 days of gestation was characterized by the appearance of the differentiation-specific keratin no. 21 and by a significant increase in the relative amount of keratin no. 8. The keratin pattern typical of adult villus cells became established at the time of birth, and was marked by a considerable increase in the complexity of the keratin-related polypeptides detected on two-dimensional gels, indicative of extensive post-translational modification of all keratins. Starting at 20 days of gestation there was a major increase in the relative abundance of mRNAs coding for keratin nos. 8, 19 and 21; in contrast, the relative amount of keratin no. 18 mRNA reached a peak shortly after birth and declined to very low levels in adult intestine. These results demonstrated marked changes in keratin expression and post-translational processing taking place at key stages of intestinal development. The appearance of keratin no. 21 in coincidence with the formation of an adult-type brush border and terminal web would be consistent with it having an important role in the organization of the intermediate filament network in the apical cytoplasm of the differentiated intestinal cells.

INTRODUCTION

During the last week of a 21–22-day gestational period, the fetal rat intestine is characterized by marked cell growth, reorganization and maturation; the appearance of digestive enzymes and transport functions associated with the luminal or brush border membrane of the enterocytes [1–5], and the morphological and ultrastructural changes in both epithelial and mesenchymal components [6–10], have been well documented. As early as the 13th day of gestation, light- and electron-microscopic studies have demonstrated the presence of an undifferentiated single-layered epithelium surrounding a tiny lumen [7–10]. The first stage of maturation, associated with rapid cell proliferation, is characterized by the formation of a stratified epithelium, up to 10 cells thick, present between 15 and 18 days of gestation [7,8]. During this period, the cells facing the lumen are covered with a few microvilli of variable length, and junctional complexes are rare. The transition from stratified to single epithelium occurring at 18–19 days of gestation is accompanied by formation of the villi, and represents a major step in the maturation of the intestinal mucosa. At this time most digestive enzymes first become detectable, and the luminal aspect of the epithelial cells becomes covered with well-formed microvilli [1–5,11].

Assembly of the brush border cytoskeleton and of the apical terminal web are therefore relatively late events in fetal intestinal maturation [12], and have been studied predominantly in chicks [13,14] and mice [15,16]. They have been described as gradual and complex processes, characterized in the chick by the apical redistribution of actin-binding proteins occurring a few days before the appearance of adult-type microvilli [13,14] on the luminal surface of the enterocytes. In both chick and mouse embryos, villin and fimbrin become concentrated in the apical cytoplasm asynchronously [13–15], but in all cases the terminal web cytoskeleton is assembled later in development [15,17], at a time when most digestive enzymes also begin to be expressed [1–5].

The developmentally regulated assembly of the brush border and terminal web in fetal intestinal cells bears many similarities to analogous processes taking place during crypt cell differentiation in adult animals [18,19]. Thus it represents a complementary *in vivo* model system to investigate the possible roles of different cytoskeletal proteins in these processes. We have previously demonstrated marked changes in keratin expression taking place during cell differentiation in adult rat intestine [20], and have identified and characterized a new type I keratin (keratin no. 21) which is restricted to functional non-proliferative enterocytes [21]. Although keratins are normally found in pairs, composed of one of each of the acidic (type I) and basic (type II) family members, this appeared not to be true in the proliferative crypt cells, where keratin no. 19 was prevalent [20]. In the present study we have investigated and compared the keratin patterns of fetal, newborn and adult rat intestines, to correlate the appearance of known morphological, ultrastructural and enzymic markers of intestinal maturation and differentiation with specific changes in keratin expression, and to obtain further evidence for the role of these keratins in the intestinal cells.

EXPERIMENTAL

Materials

Sprague–Dawley rats (CD strain) of either sex, weighing 100–170 g, were obtained from Charles River Breeding Laboratories Inc. (Wilmington, MA, U.S.A.) and bred in our

Abbreviations used: DTT, dithiothreitol; FITC, fluorescein isothiocyanate; NEPHGE, non-equilibrium pH gradient electrophoresis; PBS, phosphate-buffered saline (0.01 M-sodium phosphate buffer, pH 7.2, 0.154 M-NaCl); PMSF, phenylmethanesulphonyl fluoride; SSC, standard saline citrate (0.15 M-NaCl/15 mM-sodium citrate, pH 7.0).

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animal facilities. Tris, Hepes, phenylmethane-sulphonyl fluoride (PMSF), aprotinin, leupeptin and antipain were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); affinity-purified goat anti-mouse IgG (H+L) [F(ab') fragment, fluorescein isothiocyanate (FITC)-conjugated] was from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.); [^{14}C]formaldehyde (1% aqueous solution; 40–60 mCi/mmol) and ^{14}C-labelled M_r markers (carbonic anhydrase, BSA, phosphorylase *b*, globulins, myosin) were from New England Nuclear (Boston, MA, U.S.A.); and acrylamide, bisacrylamide, nitrocellulose blotting membranes were from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Antibodies

Preparation and characterization of the mouse monoclonal antibodies RK1-RK7 to rat intestinal keratins has been previously reported [20]. In this study we have used, in most cases, antibodies RK4 (specific for keratin no. 8), RK5 (specific for keratin no. 21) and RK7 (specific for keratin no. 19), alone or in combination. The monoclonal antibodies to rat lactase (YBB2/61), sucrase–isomaltase (BBC1/35). maltase (BB8/2), aminopeptidase N (BB4/33), dipeptidyl peptidase IV (CLB4/40) and alkaline phosphatase (BB5/16) have also been previously described [22,23]. All of these antibodies are of the IgG class, and were used as hybridoma-conditioned media or as immunoglobulins purified from ascites fluids [22].

cDNA probes

Cloning and characterization of cDNAs coding for rat keratin nos. 8, 19 and 21 has been previously reported [21]. The mouse Endo B (mouse keratin 18 homologue) cDNA [24] was obtained as an insert in a pUC9 plasmid from Dr. Robert G. Oshima (La Jolla Cancer Research Foundation, La Jolla, CA, U.S.A.). A *Eco*RI restriction fragment of approx. 1000 bp [24] was isolated and used as a probe for keratin no. 18. A *Hin*dIII restriction fragment (1700 bp) from exon 2 of the *Drosophila* actin 5C gene (E. Keller, Cornell University) was amplified in pUC18 and subsequently used as a control probe for RNA hybridization analyses. All cDNA probes were isolated from vector DNA by appropriate restriction endonuclease digestion and agarose gel resolution prior to nick translation [25] in the presence of [^{32}P]dCTP.

Tissues used in this study

Rat fetuses were collected from timed-pregnant rats on days 16, 18 and 20 post-conception; whole intestines were removed from the fetuses under a dissecting microscope and rinsed in ice-cold 0.9% NaCl. Small intestines were also collected from newborn (days 2 and 11 post-partum) and adult (40 days of age or older) rats, rinsed intraluminally twice with ice-cold saline, inverted and then used for preparation of mucosal scrapings, the starting material for subsequent purification of cytoskeletal fractions, purified brush border membranes and total cellular RNA.

Purification of intestinal cell cytoskeletal fractions and brush border membranes

All operations were performed on ice, and centrifugations were at 4°C. Intestinal samples were suspended in 20 mM-Tris/HCl, pH 7.4, 1 mM-PMSF, aprotinin (50 μg/ml), leupeptin (25 μg/ml) and antipain (25 μg/ml) (buffer A). After centrifugation at 2700 *g* for 5 min, the supernatants were discarded and the pellets were resuspended in 15 ml of buffer A by homogenizing in a Potter–Elvehjem homogenizer (30 strokes). Following centrifugation (4300 *g* for 10 min), the supernatants were used for purification of brush border membranes by the method of Kessler *et al.* [26], and the pellets were processed for preparation of cytoskeletal fractions (high-salt-extracted three times [27]) as follows. The pellets were suspended in 40 ml of buffer A, rehomogenized (20 strokes), left for 30 min on ice and centrifuged at 11000 *g* for 10 min (Sorvall centrifuge; SS-34 rotor). Pellets were homogenized once more as described above, centrifuged, suspended in 40 ml of buffer B (20 mM-Tris/HCl, pH 7.4, 1% Triton X-100, and the same protease inhibitors mixture as in buffer A), rehomogenized with 20 strokes in the Dounce homogenizer, and left for 30 min on ice. Pellets obtained by centrifugation (11000 *g*, 10 min) were homogenized twice more in buffer B, as described above. Intestinal cytoskeletons were finally suspended in buffer C [75 mM-KCl, 5 mM-$MgCl_2$, 1 mM-EGTA, 10 mM-imidazole, 0.2 mM-PMSF, 0.1 mM-dithiothreitol (DTT), 1 mM-ϵ-aminocaproic acid, pH 7.3]. For high-salt extraction, cytoskeletal preparations were then mixed with 3 vol. of buffer D (2.0 M-KCl, 0.2 M-NaCl, 1% Triton X-100, 0.2 mM-PMSF, 1 mM-ϵ-aminocaproic acid, 0.1 mM-DTT, 10 mM-Tris/HCl, pH 7.4) and incubated for 30 min at 4°C. The remaining cytoskeletal components were pelleted by centrifugation at 50000 *g* for 20 min, and the pellets obtained were extracted twice more in 1.0 M-KCl, 0.1 M-NaCl, 0.5% Triton X-100, 0.2 mM-PMSF and 10 mM-Tris/HCl, pH 7.4. High-salt-extracted cytoskeletal preparations were washed three times with phosphate-buffered saline (PBS) containing 0.2 mM-PMSF and 0.1 mM-DTT, and finally stored at −20°C in PBS.

Purification and analysis of brush border enzymes

Brush border membrane proteins were labelled with [^{14}C]formaldehyde and purified with monoclonal antibodies bound to Sepharose 4B beads as previously described [28]. The specifically bound antigens were separated by SDS/PAGE under reducing conditions and were detected by fluorography [28].

Immunofluorescence staining

Portions of small intestine obtained from fetal, newborn and adult rats were rinsed with 0.155 M-NaCl, cut into small (0.5–1 cm) fragments, embedded in optimum cutting temperature (O.C.T.) compound, and quickly frozen in liquid nitrogen. Sections 4–6 μm thick were cut using a Histostat Cryostat (AO Scientific Instruments, Buffalo, NY, U.S.A.), spread on glass slides, and allowed to dry at room temperature for at least 1 h. Staining was by the double antibody fluorescence technique as previously described [28].

Gel electrophoresis

Brush border membrane proteins and cytoskeletal proteins were analysed by slab gel electrophoresis in 5–12% acrylamide gels containing 0.1% SDS as previously described [22]. At the end of the electrophoresis, gels were stained for protein by the silver staining method of Blum *et al.* [29]; alternatively, proteins were transferred to nitrocellulose membranes for immunoblotting (see below). Two-dimensional slab gel electrophoresis was performed using non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension as in [30], with some modifications. The samples were thawed, freeze-dried, and then directly solubilized in lysis buffer; samples were run in the presence of 8 M urea and 2% LKB Ampholines (Pharmacia–LKB, Piscataway, NJ, U.S.A.) (0.4% of pH 3.5–10 + 1.6% of pH 5–8 Ampholines were used in most cases). Gels were run at 400 V in 4% acrylamide tubular gels for 4 h.

Immunoblotting

Proteins from SDS/polyacrylamide gels or two-dimensional gels were equilibrated (4 × 200 ml over a 1 h period) in 50 mM-Tris/HCl, pH 7.4, containing 20% glycerol, and then transferred

to nitrocellulose membranes as described in [31], but using a carbonate blot buffer (10 mM-$NaHCO_3$, 3 mM-Na_2CO_3, pH 9.9, in 20% methanol) in a Bio-Rad (Rockville Centre, NY, U.S.A.) Trans-Blot Cell. Transfer was at 60 V for 90 min. Membranes were blocked overnight in PBS containing 3% BSA and 0.05% NaN_3, and then incubated with monoclonal antibodies (straight hybridoma-conditioned media or ascites fluids diluted 1:100 in PBS containing 0.2% BSA) or antisera (diluted 1:50 in PBS) for 2 h at room temperature. After washing in PBS, the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Promega Biotec, Madison, WI, U.S.A.) diluted 1:7500 in PBS containing 0.2% BSA, further washed in PBS, followed by a 5 min wash in 5 mM-$MgCl_2$/100 mM-NaCl/20 mM-Tris/HCl, pH 9.5 (alkaline phosphatase substrate buffer), and finally incubated in the same buffer with freshly prepared Nitroblue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate substrate for alkaline phosphatase detection (Gibco–BRL Life Technologies, Gaithesburg, MD, U.S.A.). Finally the blots were rinsed with water, photographed and air-dried.

Purification and analysis of intestinal RNAs by Northern blotting

Total RNA was extracted from fetal, newborn and adult rat intestinal tissue as described by Chirgwin *et al.* [32]. For hybridization analyses, RNA was denatured with formaldehyde (6.6%) and resolved by electrophoresis on 1.2% agarose/formaldehyde gels [33]. Electroblotting of RNA agarose gels on to Gene-Screen Plus membranes was performed as recommended by the supplier (Du Pont Co., NEN Products, Boston, MA, U.S.A.). Following transfer, the RNA was irreversibly cross-linked to the membrane by u.v. irradiation (Stratalinker, Stratagene) [34]. Prehybridizations (3 h) and hybridizations (18 h) were performed at 42°C in 50% formamide/1% SDS/1 M-NaCl/10% dextran sulphate, and contained 100 mg of denatured salmon sperm DNA/ml. Labelled probes were denatured by boiling and were added directly to prehybridization solution to achieve final concentrations of approx. 5–10 ng of probe DNA/ml (10^7–10^8 d.p.m./μg). Following hybridization, the membranes were washed at various stringencies (0.1 × SSC to 2 × SSC, 1% SDS) at 60°C for 1 h, blotted dry and exposed to Kodak XAR-5 film with an intensifying screen for 4–12 h at −70°C. Radiolabelled ([^{32}P]ATP) RNA standards (Gibco) were prepared and analysed as above for mRNA size estimation following autoradiography.

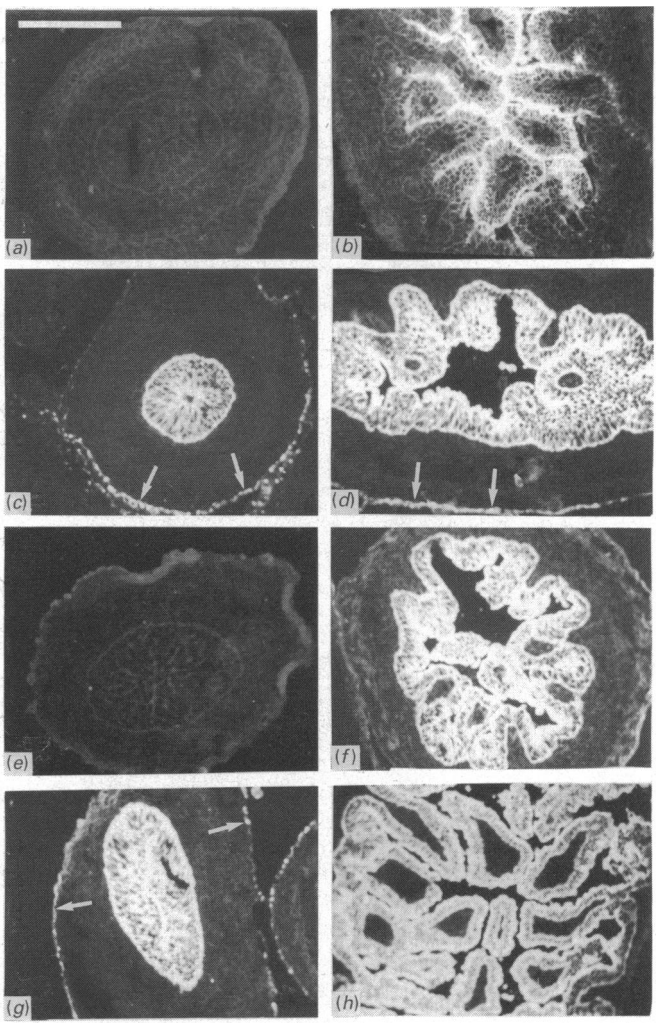

Fig. 1. Immunofluorescence staining of fetal rat intestine

Tissue was obtained from fetuses at 16 (*a*, *c*, *e* and *g*) and 20 (*b*, *d*, *f*, *h*) days of gestation, Monoclonal antibodies were to lactase (YBB1/57; *a*, *b*) and keratin nos. 8 (RK4; *c*, *d*), 21 (RK5; *e*, *f*) and 19 (RK7; *g*, *h*). Frozen sections, fixed with 1% formaldehyde, were stained by the double-antibody immunofluorescence technique. Bars = 50 μm.

RESULTS

Localization of intestinal cytokeratins during embryogenesis

Expression and distribution of keratin nos. 8, 19 and 21 was determined, using antibodies RK4, RK7 and RK5 [20] respectively, by immunocytochemical staining of frozen sections of intestine obtained from fetuses ranging from 13 days of gestation to birth. In these studies an antibody to lactase, the first digestive enzyme to appear in rat intestine at the time of brush border formation [5], was used to evaluate the state of differentiation and maturation of the specimens used. Representative results, obtained with intestines from rats at 16 and 20 days of gestation, are presented in Fig. 1. The stratified epithelium present between 15 and 18–19 days of gestation was stained intensely only with antibodies RK4 (Fig. 1*c*) and RK7 (Fig. 1*g*); these antibodies also reacted with the outer serosal layer of the intestine (see arrows in Figs. 1*c*, 1*d* and 1*g*). A patchy staining for lactase and keratin no. 21 was first observed on some epithelial cells at 18 days of gestation, and extended to the entire single-layered epithelium 1–2 days before birth (see Figs. 1*b* and 1*f*), when formation of the villi was nearly complete. At this time, the luminal aspect of the epithelial cells was also stained by antibodies to aminopeptidase N, maltase and dipeptidyl peptidase IV (results not shown). The outer serosal cells, while still positive with antibodies RK4 and RK7, were at all times negative with RK5. It should be noted that, in the rat, crypts are first formed shortly after birth [7–9,11,35), when both keratin no. 21 and all digestive enzymes (with the exclusion of dipeptidyl peptidase IV; see [23]) become confined to the differentiated intestinal cells (results not shown).

Biochemical analysis of keratins expressed in fetal, newborn and adult intestines

Cytoskeletal fractions were resolved by two-dimensional slab gel electrophoresis (NEPHGE followed by SDS/PAGE) and gels were either processed for silver staining or analysed by immunoblotting with a mixture of all antibodies (RK1–RK7) [20], or with antibodies RK4, RK5 and RK7 used separately.

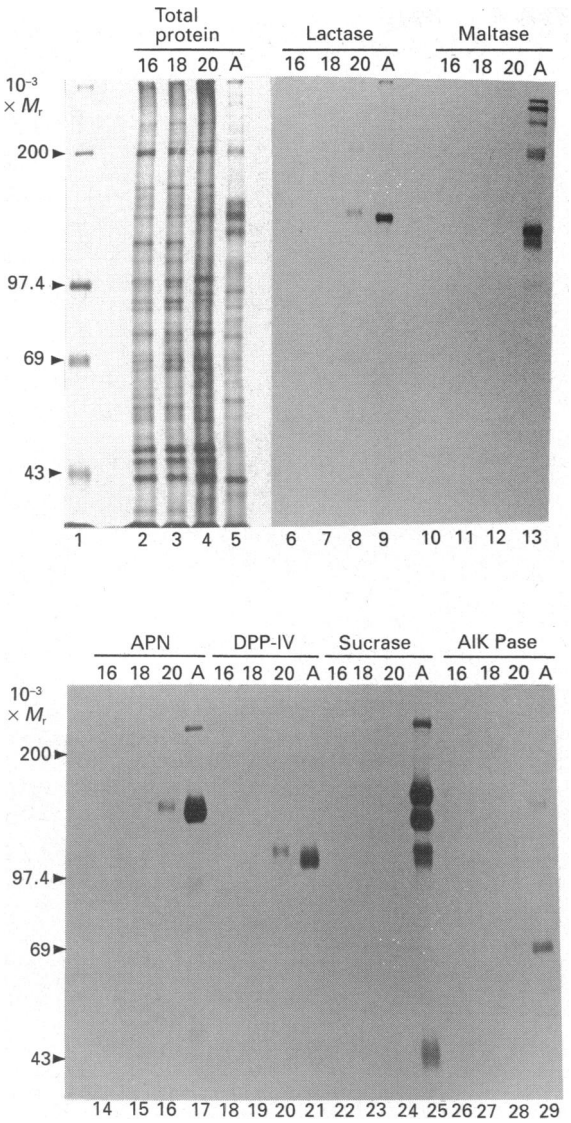

Fig. 2. Expression of digestive enzymes during pre- and post-natal development of the rat small intestine

Brush border membranes, purified from the same tissue samples from which cytoskeletal fractions were also obtained, were labelled by reductive alkylation with [^{14}C]formaldehyde, solubilized with 1% Triton X-100, and sequentially incubated with monoclonal antibodies [22] YBB2/61, specific for lactase; BB8/2, specific for maltase; BB4/33, specific for aminopeptidase N (APN); CLB4/40 [23], specific for dipeptidyl peptidase IV (DPP-IV); BBC1/35, specific for sucrase–isomaltase; BB5/16, specific for alkaline phosphatase (Alk Pase). Bound antigens were released from the antibodies–beads conjugates and analysed by SDS/PAGE followed by fluorography, as previously described [28]. Samples from fetal rats at 16, 18 and 20 days of gestation, and from adult (A) rats, are included in the Figure as indicated above the lanes. Lanes 2–5 contain total protein patterns of purified brush border membranes, stained with Coomassie Blue.

The stage of development of the intestinal specimens used in these studies was confirmed by analysis of the pattern of digestive enzymes expressed. Brush border membranes were purified from the same tissue homogenates from which cytoskeletal proteins were obtained and were labelled with [^{14}C]formaldehyde prior to solubilization and immunoprecipitation of representative, stage-specific, enzymes (lactase, maltase, aminopeptidase N, dipeptidyl peptidase IV, sucrase–isomaltase and alkaline phosphatase). Enzyme patterns were analysed by SDS/PAGE followed by fluorography of the dried gels. Representative results are presented in Fig. 2. All of the above enzymes were absent until 18–19 days of gestation; lactase (lane 8), aminopeptidase N (lane 16) and dipeptidyl peptidase IV (lane 20) were consistently apparent at 20 days of gestation; maltase and alkaline phosphatase were first detectable immediately before birth (21–22 days of gestation; results not shown); sucrase appeared at the time of weaning (20–22 days after birth). In Fig. 2, adult forms (lanes 9, 13, 17, 21, 25 and 29) of the enzymes are included in all cases for comparison. Overall, these results are in good agreement with those obtained by the immunofluorescent staining of frozen tissue sections described above, and with prevoius reports [1–3,5], but also allowed a semi-quantitative evaluation of antigens present at each stage of development.

The patterns of immunologically detectable keratins present in intestinal specimens at key stages of fetal development (16, 18 and 20 days of gestation), at 2 and 11 days after birth and in adult intestine are illustrated in Figs. 3 and 4. At 16 days of gestation, only keratin no. 19 could be clearly identified both on silver-stained gels (results not shown) and by immunoblotting (Fig. 3*a*); a small amount of keratin no. 8 was also detected by immunoblotting in some samples. At a stage of gestation marking the beginning of the process of brush border morphogenesis and intestinal cell differentiation (18–19 days), a small amount of keratin no. 21 was first detected as two isoelectric variants of M_r 48000 (Fig. 3*b*); a progressive increase in the relative amount of keratin no. 8 was also observed. A more marked change in the keratin pattern was observed at 20 days of gestation, when formation of the intestinal villi and expression of several brush border enzymes (see above) were clearly established over the entire length of the small intestine. The relative amounts of keratins 8 and 21, compared with that of keratin 19, increased markedly (Figs. 3*c* and 4*e*). Coincident with these changes, the complexity of keratin patterns on both the silver-stained gels (Fig. 4*e*) and immunoblots (Figs. 3*c* and 4*f*) increased dramatically. These results are suggestive of extensive post-translational processing of keratins, generating several isoelectric variants and/or proteolytic degradation products, starting at 20 days of gestation for keratin no. 8-related polypeptides (recognized by antibody RK4; see Fig. 4*g*), and shortly after birth for keratin no. 19 (recognized by antibody RK7; see Figs. 3*d* and 4*l*).

Later stages of intestinal development were characterized by a further increase in the complexity of the two-dimensional keratin patterns (Figs. 3 and 4) and the appearance of the M_r-46000 variant of keratin no. 21, which in adult intestine was much more abundant than the M_r-48000 form (Fig. 4*i*). It should be noted that in all of the above comparisons the relative abundance of the different keratin-related polypeptides could be best determined by silver staining of gels, a procedure which gives a linear estimate of protein concentrations in the range 50 pg/mm^2 to 2 ng/mm^2 [36]. Immunoblotting often revealed as intense spots apparently minor components, undetectable on gels stained for protein. For example, while keratin no. 19, even in adult cytoskeletal preparations, was present predominantly as two major isoelectric variants in silver-stained gels (Fig. 4*i*), staining of nitrocellulose blots with antibody RK7 produced a far more complex pattern, including many minor immunoreactive polypeptides (Fig. 4*l*).

Detection and analysis of intestinal keratin mRNAs at different stages of development

To evaluate the presence and relative abundance of keratin mRNAs, total cellular RNA preparations were resolved by

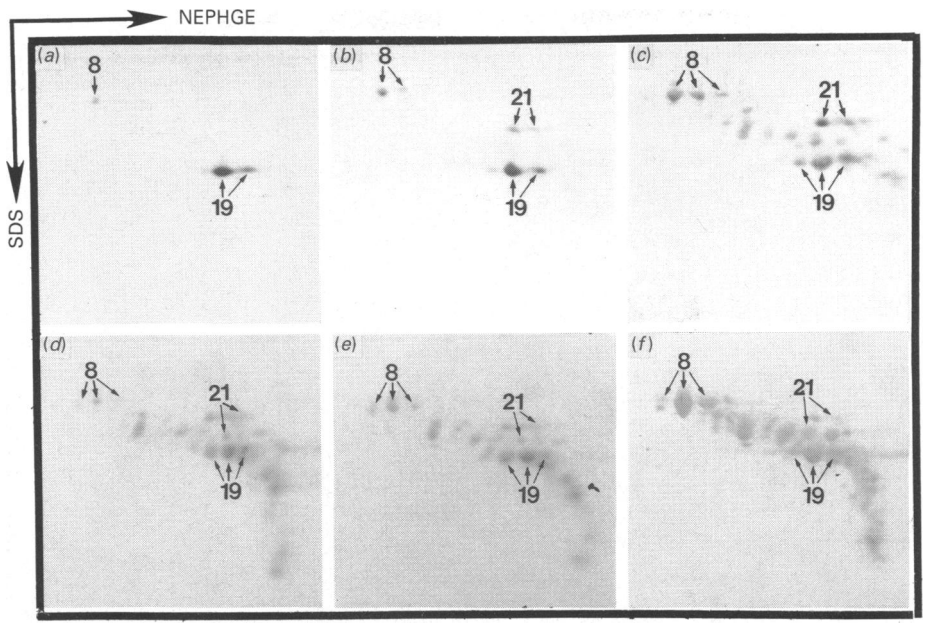

Fig. 3. Keratin-related polypeptides in intestinal cells from fetal, newborn and adult rats analysed by two-dimensional slab gel electrophoresis and immunoblotting

Water-insoluble cytoskeletal proteins, high-salt-extracted three times, were separated by NEPHGE in the first dimension, followed by SDS/PAGE (10% acrylamide gel) under reducing conditions (50 mM-DTT), and then transferred to nitrocellulose membranes and stained with a mixture of RK1–RK7 antibodies. Fetal samples were taken after (*a*) 16, (*b*) 18 and (*c*) 20 days of gestation; newborn samples were taken at (*d*) 2 and (*e*) 11 days after birth. (*f*) Adult samples. Arrows indicate the main isoelectric variants of keratin nos. 8, 19 and 21, as identified on parallel samples stained individually with antibodies RK4, RK7 and RK5 respectively, and on silver-stained gels. In all panels, the more basic polypeptides are towards the left.

agarose gel electrophoresis and transferred to Gene-Screen Plus membranes. Replicate hybridizations performed with nick-translated cDNA probes specific for actin or keratin nos. 8 (pRK4), 21 (pRK5) or 19 (pRK7) are presented in Fig. 5. Weak but distinct signals corresponding to mRNAs coding for keratin nos. 8 and 19 could be detected in intestinal specimens obtained at 16 days of gestation. Their relative intensities were, however, in contrast with the results obtained by two-dimensional electrophretic analysis of cytoskeletal preparations obtained from the same tissue, in which keratin no. 19 was present in much larger amounts than keratin no. 8 (Fig. 3*a*). No signal corresponding to keratin no. 21 mRNA could be detected at this stage of development, in accordance with the immunoblotting experiments. At later stages of intestinal maturation, the relative abundance of all keratin mRNAs (compared with actin mRNA) appeared to increase markedly, eventually reaching a peak in adult animals. It was particularly surprising to note that, while keratin no. 21 appeared to represent a relatively minor keratin component in both silver-stained gels (Fig. 4*i*) and immunoblots, its mRNA signal was at least as intense as those corresponding to keratin nos. 8 and 19 (Fig. 5) in total cellular RNA preparations from adult intestine.

The availability of a cDNA probe for keratin no. 18 (which could not be positively identified on immunoblots because of the lack of a monospecific antibody to the rat homologue) also allowed us to determine the presence and relative abundance of its mRNA in intestinal tissues at all stages of development. Replicate blots obtained with cDNA probes for keratin nos. 18 and 8 are compared in Fig. 6. A signal corresponding to keratin no. 18 mRNA was detected in fetal intestine already at 16 days of gestation but, in contrast with the results obtained for all other keratins, its intensity (after increasing markedly until the time of birth), was very low in adult small intestine.

DISCUSSION

The results obtained in this study indicate that the changes in intestinal keratin expression observed during late embryonic development are similar, in many respects, to those seen during crypt-to-villus differentiation in adult rat [20]. Starting at the earliest time of gestation examined (13 days; results not shown), and up until the period of differentiation characterized by the appearance of most digestive enzymes and formation of the intestinal villi (18–19 days of gestation), gel electrophoretic and immunoblotting analyses revealed that the rat homologue of human keratin no. 19 is the major intermediate filament component of the intestinal epithelial cells (Fig. 3*a*), a pattern quite similar to that observed in adult crypt cells [20]. Only a very small amount of keratin no. 8 could be observed by immunoblotting (Fig. 3*a*). Although we could not directly identify keratin 18 on immunoblots, no major polypeptide spot was observed at its expected position [27,37] on silver-stained two-dimensional gels, or after incubation with a mixture of all antibodies (RK1-RK7). This observation is surprising in view of the Northern blot analysis demonstrating that, at this stage of intestinal maturation, steady-state levels of mRNAs coding for keratin nos. 8 and 18 were comparable with, if not higher than, that of keratin no. 19 (Figs. 5 and 6). Our findings also contrast with previous observations demonstrating that keratins nos. 8 and 18 are the main tonofilament components of early embryos [38]. In most tissues and cells examined these keratins, when present, have been observed in near-stoichiometric amounts [39–41].

The prevalence of keratin no. 19 in early embryonic intestinal cells (and in adult crypt cells) is difficult to explain. Most previous studies have demonstrated that unpaired type I or type II keratins are not expressed in significant amounts, in spite of the presence of the corresponding mRNAs, because they fail to

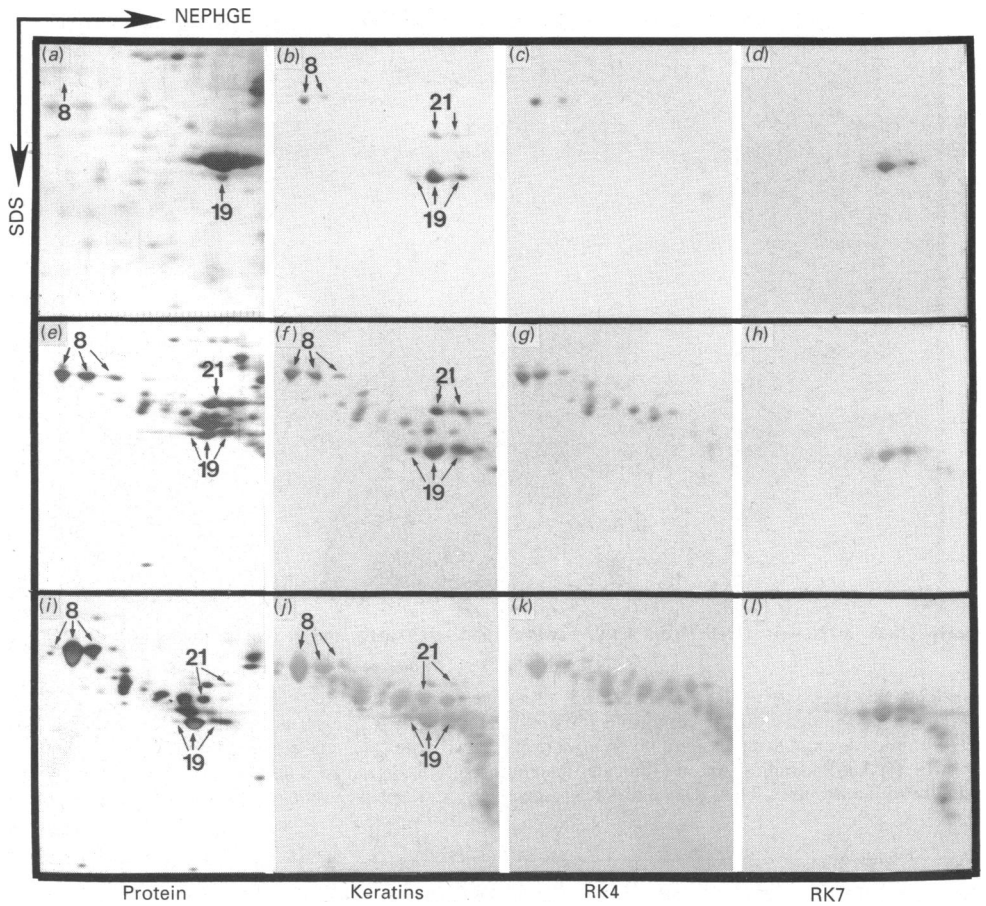

Fig. 4. Progressive increase in the complexity of the patterns of keratin-related polypeptides present in intestinal cells starting late in gestation

Water-insoluble cytoskeletal proteins, high-salt-extracted three times, were obtained from fetal [18 (*a–d*) and 20 (*e–h*) days of gestation] and adult rats (*i–l*), and resolved by two-dimensional slab gel electrophoresis as described in the legend to Fig. 3. (*a*, *e*, *i*) Silver-stained gels; (*b*, *f*, *j*) immunoblots obtained with a mixture of antibodies RK1–RK7; (*c*, *g*, *k*) membranes stained with antibody RK4 (specific for keratin no. 8); (*d*, *h*, *l*) membranes stained with antibody RK7 alone (specific for keratin no. 19). Arrows point to the positions of the main isoelectric variants of keratin nos. 8, 19 and 21. In all panels, the more basic polypeptides are towards the left.

polymerize and are rapidly degraded within a few hours of synthesis [42–45] by very efficient proteolytic enzymes [45]. However, keratin no. 19 is known to have a wide cell and tissue distribution in both simple and stratified epithelia, and its sequence is more related to those of other keratins of stratifying epithelia than to that of keratin no. 18, which is typical of simple epithelia [46]. Keratin 19 has been often identified by gel electrophoretic analysis as being among the most abundant keratin components in several non-epidermal stratified epithelia [41,47], but it has been rarely observed as the major type I keratin *in vivo*, with one dissenting report on the oral epithelium [48].

Proper organization of a keratin cytoplasmic network appears to require a correct heterotypic type I/type II keratin pairing for polymerization [49–51]. Terminal-tail-truncated keratins alone were found to form only short, kinked, structures [49]. Keratin no. 19 differs from all other intermediate filament proteins because of the absence of a typical tail domain, replaced in this case by a continuation of the α-helical character through a short stretch of 13 amino acids [52]. It has been suggested that its primary role may be to correct type I/type II imbalances [53], potentially resulting in the formation of short non-filamentous structures [50,54]. *In vivo*, keratin no. 19 can pair with keratin no. 8 to form intermediate filaments [38], but has never been found to form such polymers in cells or tissues devoid of keratin no. 18 [48]. While *in vitro* studies have been unable to demonstrate qualitative differences between filaments formed with keratin pairs 8/18 and 8/19 [51], within the cytoplasmic environment of living cells the network formed by keratins 8 and 18 was morphologically superior to the more irregular and discontinuous one formed by keratins 8 and 19 [49]. Such differences may be vitally important in cells characterized by continuous and rapid growth, such as early fetal intestinal epithelial cells and adult crypt cells.

The very limited expression of keratin no. 8 (and possibly no. 18) at a time of development when the intestinal epithelium is stratified would also be compatible with the fact that they have never been observed by gel-electrophretic analysis among the cytoskeletal proteins extracted from stratified epithelia [38]. By immunohistochemical staining of tissues, these keratins have, however, been shown to occur together with other cytokeratins in specialized cell types of complex epithelia, such as in the basal layers of some human stratified epithelia [38,46].

Brush border morphogenesis and formation of a well-organized terminal web at 18–19 days of gestation was accompanied by marked changes in the pattern of keratins observed by two-dimensional slab gel electrophoresis and immunoblotting (Figs. 3 and 4), including the appearance of keratin no. 21, and a marked increase in the relative abundance of keratin no. 8. At

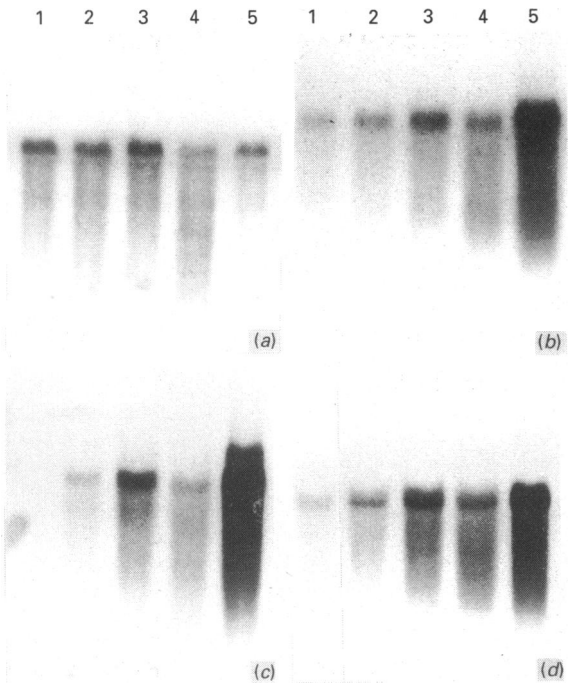

Fig. 5. RNA transfer hybridization of total RNA extracted from fetal, newborn and adult rat small intestine

Lanes: 1, 16 days of gestation; 2, 18 days of gestation; 3, 20 days of gestation; 4, 2 days after birth; 5, adult. In all cases, RNA (5 μg/lane) was denatured and resolved on 1.2% agarose/formaldehyde gels, then electrophoretically transferred to Gene-Screen Plus membranes and u.v.-cross-linked. Replicate lanes were hybridized to nick-translated cDNA probes: a *Hin*dIII restriction fragment (1700 bp) from exon 2 of the *Drosophila* actin 5C gene (*a*), pKRK4 (*b*), pRK5 (*c*) or pRK7 (*d*). After hybridization, membranes were washed with a buffer containing either 0.1 × SSC (pRK4, pRK5 and pRK7 probes) or 1.0 × SSC (actin probe), in 1% SDS at 60°C (3 × 30 min).

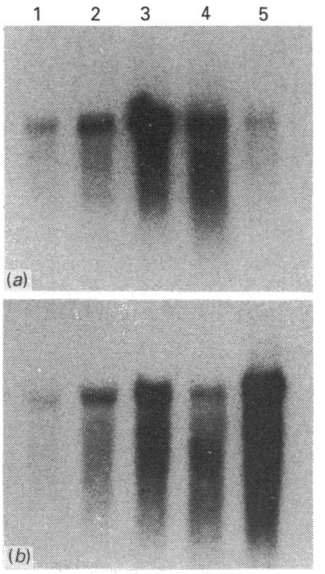

Fig. 6. RNA transfer hybridization of total RNA extracted from fetal, newborn and adult rat small intestine

Lanes: 1, 16 days of gestation; 2, 18 days of gestation; 3, 20 days of gestation; 4, 2 days after birth; 5, adult. In all cases, RNA (5 μg/lane) was denatured and resolved on 1.2% agarose/formaldehyde gels, then electrophoretically transferred to Gene-Screen Plus membranes and u.v.-crosslined. Replicate lanes were hybridized to nick-translated cDNA probes for mouse Endo B (keratin no. 18; *a*), or pKRK4 (keratin no. 8; *b*). After hybridization, membranes were washed with a buffer containing either 0.1 × SSC (pRK4 probe) or 1.0 × SSC (mouse Endo B probe) in 1% SDS at 60°C (3 × 30 min).

the same time, Northern blot analysis also revealed a progressive increase in the steady-state levels of all keratin mRNAs (Fig. 5), including that of keratin no. 18 (Fig. 6). This suggests that during the last 2–3 days of fetal life intermediate filaments containing keratin no. 21 and those composed of the keratin 8/18 pair may participate in the organization of the apical cytoplasmic network, or play some other significant role(s) in the intestinal epithelial cells. At the same time in the rat, and at comparable stages of development in chicks and mice, other important cytoskeletal proteins (such as villin, brush border myosin I, spectrin, caldesmon and TW 260/240) have been found to appear, or to undergo significant changes in cellular distribution [13–16,55,56].

At the time of birth, or shortly thereafter, the pattern of keratins expressed by the intestinal epithelial cells became similar, or identical, to that observed in adult animals. The most significant alterations were: (*a*) a progressive increase in the amount of keratin no. 21 and its predominant conversion to a M_r-46000 form; (*b*) a marked decline in the steady-state level of keratin no. 18 mRNA (Fig. 6), concomitant with an increase in those mRNAs corresponding to keratin nos. 8, 19 and 21 (particularly noticeable in RNA preparations from adult intestine) (Fig. 5); and (*c*) a considerable increase in the number of keratin-related polypeptides, particularly those detected by antibodies RK4 and RK7 on two-dimensional immunoblots (Fig. 4). These results suggest that, after birth, intermediate filaments formed by the keratin pair 8/18 become progressively less important than those including keratin no. 8 in combination with keratin no. 21.

In newborn and adult differentiated enterocytes, relatively large amounts of keratin mRNAs may be required to preserve cellular architecture and terminal web organization in the presence of a rapid turnover of brush border cytoskeletal proteins [57,58]. Such processes may extend to keratin-related polypeptides and be responsible, at least in part, for the increased complexity of the two-dimensional immunoblots. The phosphorylation of selected keratins may also occur, generating several isoelectric variants, a process that may be part of a cascade of events leading to initiation of DNA synthesis and subsequent cell division [59,60]. This may be particularly important in the intestinal epithelial cells, which are characterized by one of the shortest cell cycle times known *in vivo* [4].

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