

Type IV Collagen mRNA Accumulates in the Mesenchymal Compartment at Early Stages of Murine Developing Intestine

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Abstract. The expression of type IV collagen mRNA during mouse intestinal morphogenesis was examined by in situ hybridization using a cDNA probe corresponding to mRNA for $\alpha 1$ (IV) chain. Type IV collagen mRNA is detected in the embryonic mesenchymal cells at early stages of development (12 d of gestation). A segregation of mesenchymal cells expressing high levels of type IV collagen mRNA in close vicinity of the epithelium occurs just before villus formation. During villus outgrowth, type IV collagen mRNA, still confined to mesenchyme-derived tissues, is progressively restricted to the mucosal connective tissue (the lamina propria) and to a lesser extent to the muscular layers. In the adult, the amount of messenger is quite low as compared to the level found in the developing intestine and the in situ hybridization signal,

is indistinguishable from the background, is uniform throughout the whole intestinal wall. At all developmental stages no detectable specific hybridization signal is virtually observed over the epithelium cell layer.

These results show that high amounts of the type IV collagen messenger are detected during phases of intensive morphogenetic events. Furthermore, they reinforce the notion already gained previously (Simon-Assmann et al. 1988) that the mesenchymal compartment is the principal endogenous source of type IV collagen. They also indicate that the continuous migration of epithelial cells along the basement membrane of intestinal villi in the mature organ is not accompanied by a significant remodeling of the collagen IV network.

INTESTINAL epithelial cells differentiate from the embryonic endoderm, while the connective tissue and the muscular layers are derived from a loosely arranged population of mesenchymal cells. Several experimental studies have shown that epithelial/mesenchymal tissue interactions play a fundamental role in the morphogenesis of the gut and in the differentiation of endodermal cells into enterocytes (Keding et al., 1986; Haffen et al., 1987). Most of these experiments have been carried out using embryonic tissue recombinations between epithelial and mesenchymal components derived from different organs and even different animal species. A possible role of extracellular matrix in these cell interactions has been suggested by the observation of changes in the distribution and localization of various extracellular molecules occurring during development (Simon-Assmann et al., 1986; Aufderheide and Ekblom, 1988). Concerning basement membrane proteins (type IV collagen, laminin, nidogen), we could show that these molecules, in addition of being displayed at the epithelial/mesenchymal interface, were found around cells scattered within the mesenchyme and which segregated in the region surrounding the basement membrane before villus formation (Simon-Assmann et al., 1986).

The cells responsible for the production and deposition of basement membrane components are becoming reliably

identified. Combined use of experiments yielding chimeric chick/rat intestines and of immunocytochemistry with species-specific antibodies provided information on the somewhat disputed origin of the basement membrane. Such experimental models allowed us to demonstrate the cooperation between endodermal and mesenchymal cells as to the formation of the intestinal basement membrane. Indeed, while heparan sulfate proteoglycan molecules located in the basement membrane are produced exclusively by the epithelial cells (Simon-Assmann et al., 1989), the mesenchymal compartment was proposed to be the endogenous source of type IV collagen (Simon-Assmann et al., 1988). In parallel, Senior et al. (1988) showed by in situ hybridization that laminin is produced by mesenchymally-derived lamina propria cells. All together these observations strengthen the fact that the widely accepted belief of an exclusive epithelial origin of basement membrane components has to be revised.

To supplement our previous immunocytochemical study, we have attempted to gain clear-cut information about the tissue distribution of type IV collagen mRNA in the intestine. For this purpose, we have performed in situ hybridization experiments to localize collagen transcripts throughout embryonic and postnatal development. This technique has already been applied successfully to visualize several types of collagen mRNAs in chick embryo (Hayashi et al., 1986;

Devlin et al., 1988; Nah et al., 1988; Solursh and Jensen, 1988; Swalla et al., 1988) and in human fetal tissues (Sandberg and Vuorio, 1987; Sandberg et al., 1988). The results reported here confirm that this basement membrane molecule is produced by the mesenchyme-derived cells; furthermore, they show that accumulation of type IV collagen mRNA is prominent during the phases of intensive morphogenesis.

Materials and Methods

Northern Blot Hybridizations

Cytoplasmic RNA was extracted from IOPS/OFl mouse (Iffa-Credo Saint Germain, France) intestines using the method described by Auffray and Rougeon (1980). It was further separated by electrophoresis using 1% agarose/17% formaldehyde gels in morpholine propane sulfonic buffer (Lehrach et al., 1977). After blotting onto nitrocellulose (Thomas, 1980), the filters were hybridized with the labeled probes, washed at 60°C in a solution containing 0.2 × SSC, 0.1% SDS (1 × SSC: 150 mM NaCl, 15 mM Na-citrate, pH 7), and exposed to x-ray film. Radioactive bands were scanned in a dual-wavelength flying-spot scanning densitometer (Shimadzu Scientific Instruments, Inc., Columbia, MD).

For the detection of the $\alpha 1$ (IV) collagen mRNA, we used a plasmid derived from the clone pFAC, kindly provided by Oberbäumer et al. (1985). It contains a 1-kb-long Bam HI/Sau 3AI cDNA fragment, inserted into the Bam HI restriction site of the vector pSP65. This cDNA fragment was purified by electroelution after digestion of the plasmid with restriction enzymes Eco RI and Hind III, followed by preparative agarose gel electrophoresis. We also used, as control probe, the recombinant plasmid p4G9 which contains a fragment of ~1.2 kb cDNA corresponding to α actin, inserted into the Pst I restriction site of vector pBR322 (gift of Dr. R. Heilig). The DNAs were ³²P-labeled according to Feinberg and Vogelstein (1983), using the Multiprime labeling Kit (Amersham Corp., Arlington Heights, IL). The specific activity was up to 1 × 10¹⁰ cpm/μg of DNA.

In Situ Hybridization

The technique used in this work is mainly based on the work by Hafen et al. (1983), with slight modifications.

Preparation of Tissue Sections

Intestinal segments from IOPS/OFl mice (Iffa-Credo) were taken at various developmental stages; they were immediately embedded in Tissue-Tek compound, frozen in Freon-cooled in liquid nitrogen and stored at -70°C until use. Transverse sections (8-μm-thick) were cut at -25°C, collected on glass microscope slides pretreated with TESPA (or 3-aminopropyl-triethoxysilan, Fluka) and with paraformaldehyde (Berger, 1986) to allow sufficient retention of the sections in the subsequent steps of the procedure. Slides were then placed on a slide warmer at 50°C for 1-2 min, and allowed to dry at room temperature for 1-2 h. The sections were fixed at room temperature in PBS containing 4% paraformaldehyde for 20 min, dehydrated through increasing concentrations of ethanol, and stored at -20°C in dry boxes until use.

Preparation of cDNA Probes

The electroeluted cDNA fragment for the $\alpha 1$ chain of type IV collagen was labeled for in situ hybridization using a conventional nick translation protocol (Hafen et al., 1983) in the presence of various concentrations of DNase (1.7-8.3 μg/ml). Specific activity of the resulting ³H-labeled DNA probes was ~1 × 10⁷ cpm/μg. The sizes of the labeled DNA were checked by electrophoresis onto 20% urea-polyacrylamide gels. Samples between 40- and 100-bp-long were retained to perform in situ hybridization.

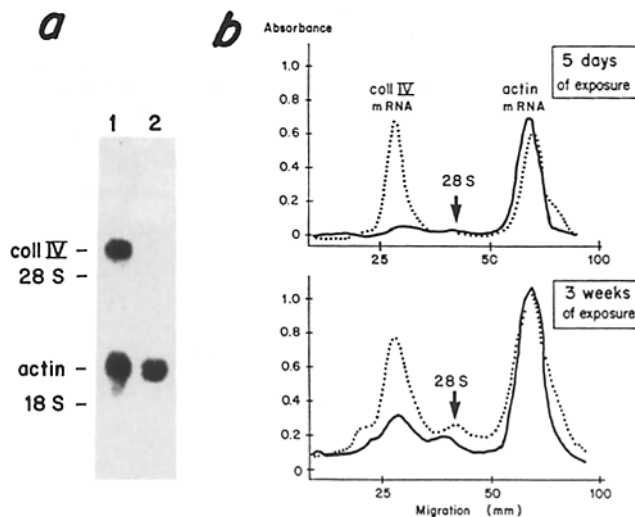


Figure 1. Northern blot analysis of collagen type IV mRNA: (a) ~20 μg of RNA extracted from intestines of 2-d-old suckling (lane 1) and adult (lane 2) mice were separated by electrophoresis, transferred onto nitrocellulose filters, and hybridized with cDNA probes for type IV collagen and actin messengers. The migration of the 28S and 18S ribosomal RNA is indicated. (b) Densitometric profiles of the autoradiograms shown in a exposed for 5 d (above) and overexposed for 3 wk (below). Scans of the 2-d-old suckling (· · · · ·) and of the adult (—) mice were overlaid allowing direct comparison of the autoradiograms. The migration of the 28S rRNA is indicated.

Hybridization Treatment

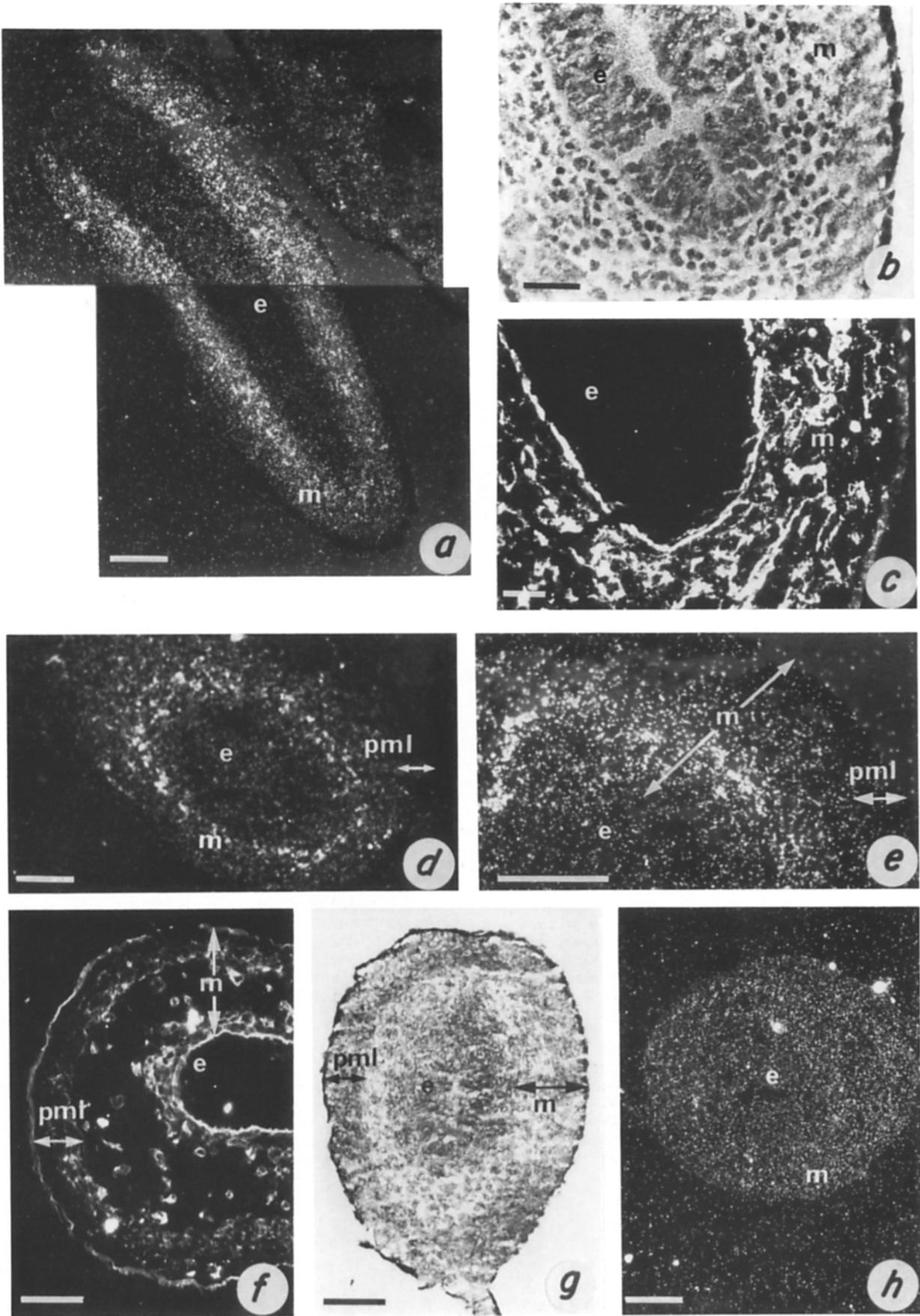
Proteolytic pretreatment of sections was performed as described by Hafen et al. (1983). The slides were then acetylated in a freshly prepared mixture of 0.25% acetic anhydride in triethanolamine buffer for 10 min (Hayashi et al., 1978), washed, and dehydrated.

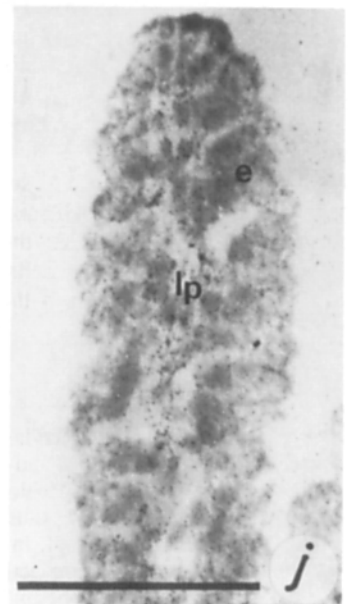
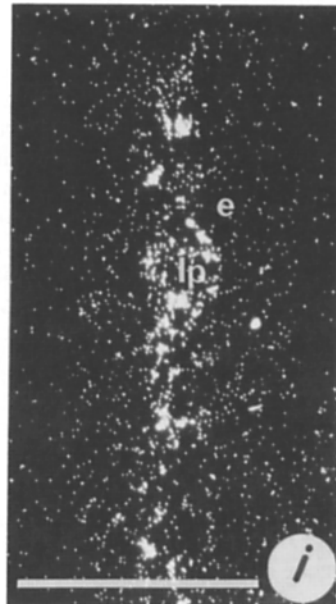
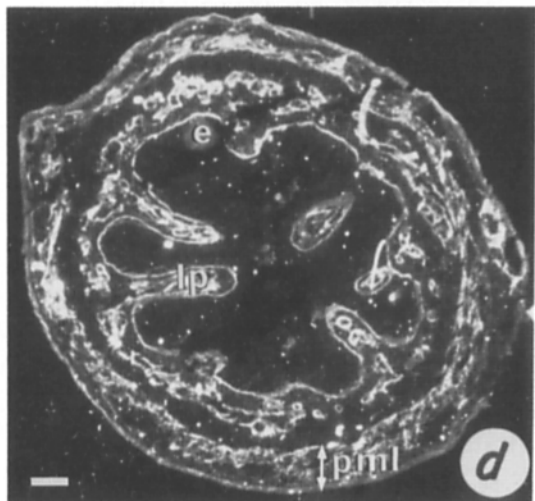
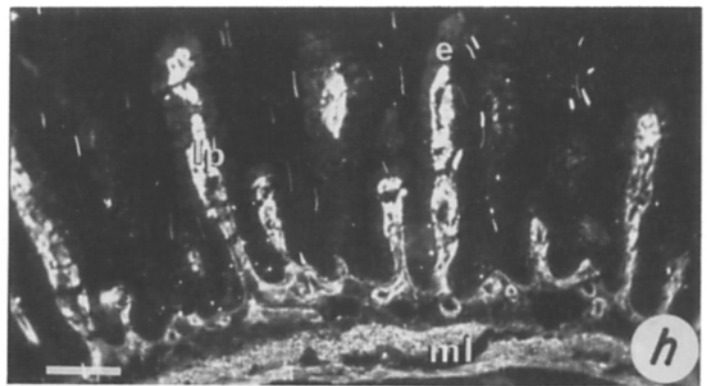
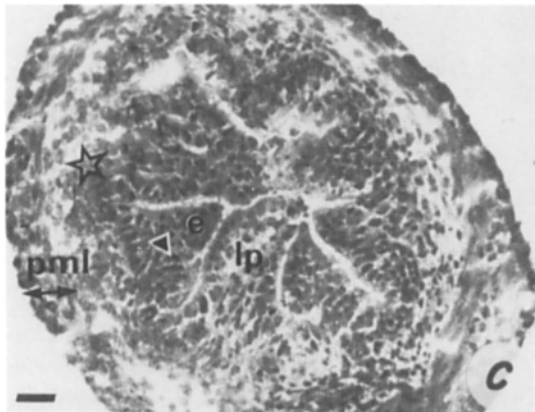
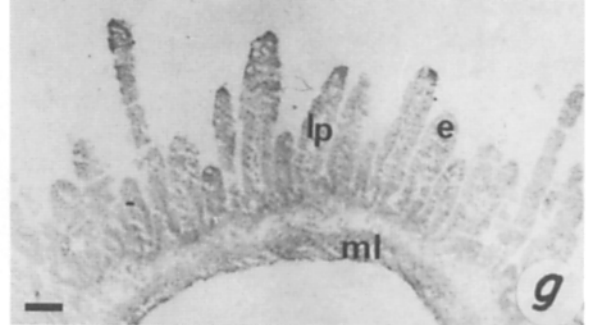
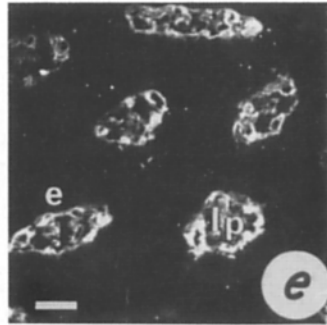
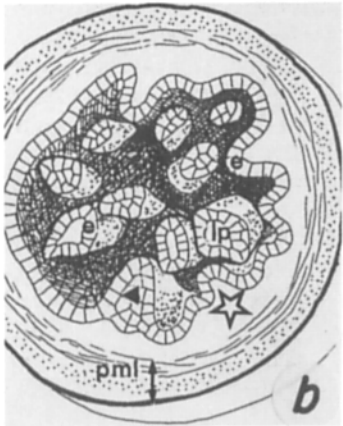
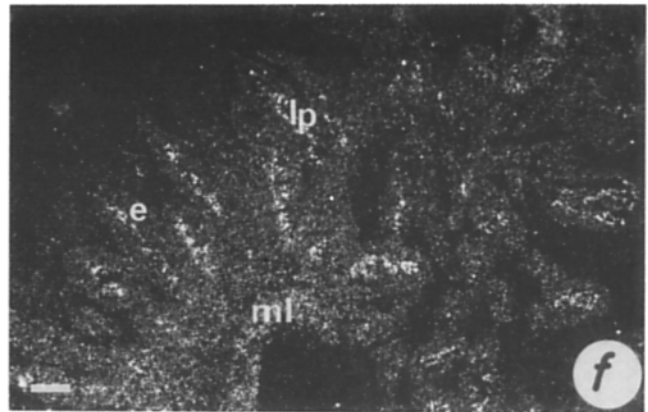
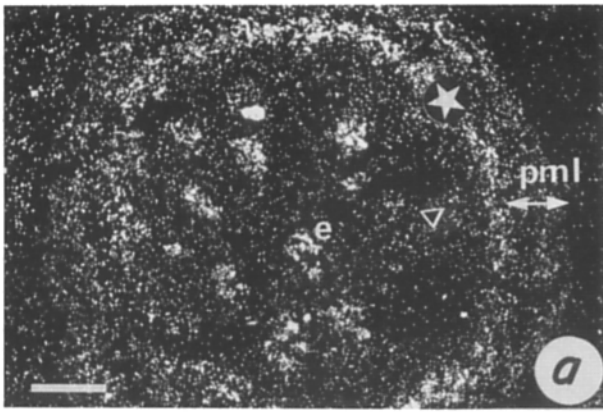
The DNase-treated ³H-labeled DNA was resuspended in the hybridization solution containing 50% formamide, 0.6 M NaCl, 1 × Denhardt's solution, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 500 μg/ml of yeast tRNA, 100 μg/ml poly (A), 10% dextran sulfate. The double-stranded probe was heat-denatured immediately before use and pipetted directly onto the sections, which were topped with a siliconized coverslip. Slides were incubated overnight at 35°C in humid chambers equilibrated with 50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. After hybridization, coverslips were removed by flotation in the same solution. The slides were washed thoroughly during 18 h at 35°C and dehydrated by two 5-min incubations in 70% ethanol-0.3 M ammonium acetate, and then in 95% ethanol-0.3 M ammonium acetate. Sections were finally air dried, and exposed for autoradiography from 3 to 7 wk.

Controls

Nonspecific and emulsion background signals were routinely estimated by hybridizing adjacent tissue sections with a probe consisting of a fragment of plasmid pSP65 or pEMBL (Dente et al., 1983) of the same length as the specific probe; it has to be noted that background was always higher over tissue than in the surrounding emulsion.

Figure 2. Localization of type IV collagen mRNA by in situ hybridization with the pFAC probe. Dark field views of (a) 12- and (d and e) 14-d fetal mouse intestinal oblique (a) or cross sections; 12-d fetal mouse intestinal section hybridized with the control pEMBL probe (h). Photographic emulsion was exposed between 4 and 7 wk. 5-μm cryosections of (b) 12- and (g) 14-d fetal mouse intestines stained with hematoxylin-eosin. Immunolabeling of type IV collagen in (c) 12-, and (f) 14-d fetal mouse intestines. e, endoderm; m, mesenchyme; pml, presumptive muscular layers. Bars, (a, b, d, e, g, and h) 80 μm; (c and f) 50 μm.





Immunolocalization of Type IV Collagen

Tissues were frozen without prior fixation and 5- μ m cryosections were stained with purified antibodies against mouse type IV collagen (Institut Pasteur, Lyon, France) as described previously (Simon-Assmann et al., 1986, 1988).

Results

In the present study, we used a cDNA fragment contained in plasmid pFAC that was cloned from a cDNA library constructed using mRNA extracted from mouse F9 cells (Oberbäumer et al., 1985). As shown by Northern blot analysis (Fig. 1, *a* and *b*), this probe hybridized in 2-d-old murine intestinal extracts to a single \sim 6.5 kb mRNA species consistent in size with the major messenger found in the collagen IV-producing PYS-2 cells (Oberbäumer et al., 1985). Yet, in adult intestinal extracts, this messenger was much less abundant, although still detectable. For a fixed content of actin mRNA, and according to calculation of areas on the densitometric profiles of autoradiograms exposed for 3 wk, we estimated that collagen type IV messenger accumulated at least 50 times more in the neonatal intestine than in the adult organ (Fig. 1 *b*).

The localization of type IV collagen mRNA was analyzed by in situ hybridization with the corresponding cDNA probe. Autoradiographs of cross-sections through the intestinal wall at various developmental stages are illustrated in Figs. 2–5.

At 12–13 d of gestation (i.e., the first stages studied), the intestinal anlage consists of a simple tube composed of a stratified layer of radially arranged endodermal cells surrounded by mesenchymal cells (Fig. 2 *b*). There was an intense accumulation of silver grains over the whole thickness of the mesenchyme (Fig. 2 *a*). When sections were stained with type IV collagen antibodies, the antigen was shown to be present throughout the mesenchyme and at the epithelial-mesenchymal interface (Fig. 2 *c*) as in all subsequent developmental stages. Virtually no hybridization signal above nonspecific background (Fig. 2 *h*) was detectable over epithelial cells at this stage as well as at all developmental stages studied. Later on, at 14–15 d of gestation, just before villus formation and when the intestinal tube was still undifferentiated (Fig. 2 *g*), the highest grain concentration was seen over 2–3 layers of mesenchyme cells located just beneath the epithelial cells (Fig. 2, *d* and *e*); immunocytochemistry demonstrated that this region was stained heavily with type IV collagen antibodies (Fig. 2 *f*).

When villus primordia start to develop \sim 16 d of gestation in the mouse (Fig. 3 *c*), the hybridization signal was concentrated over three distinct areas: the presumptive muscular cell layers, the mesenchymal cells underlying the epithelium, and the lamina propria of the protruding villi, espe-

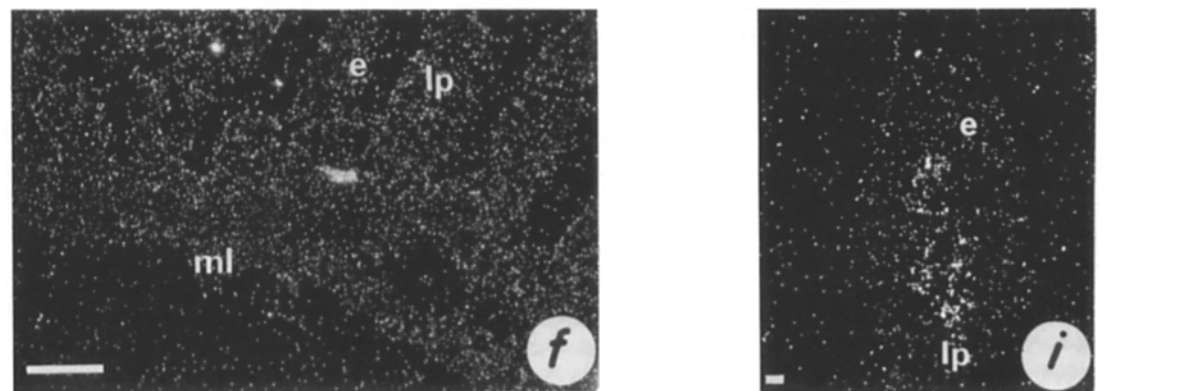
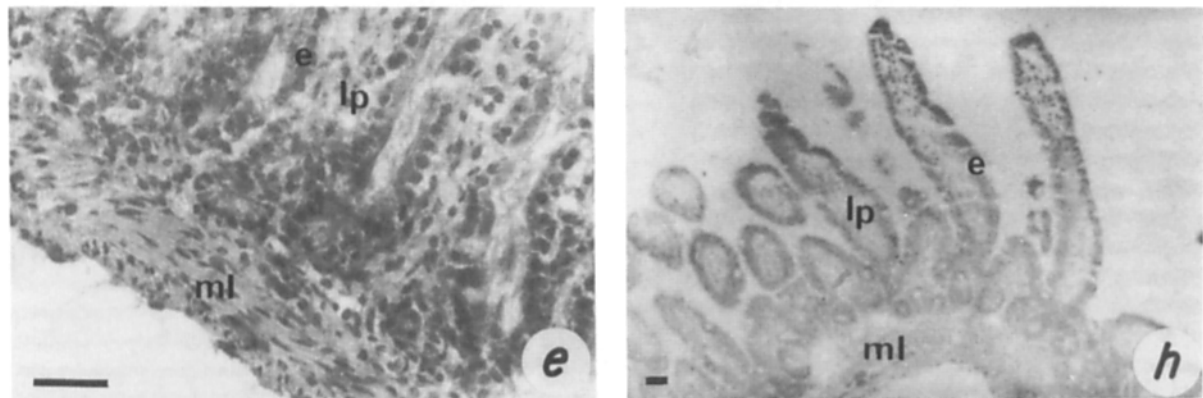
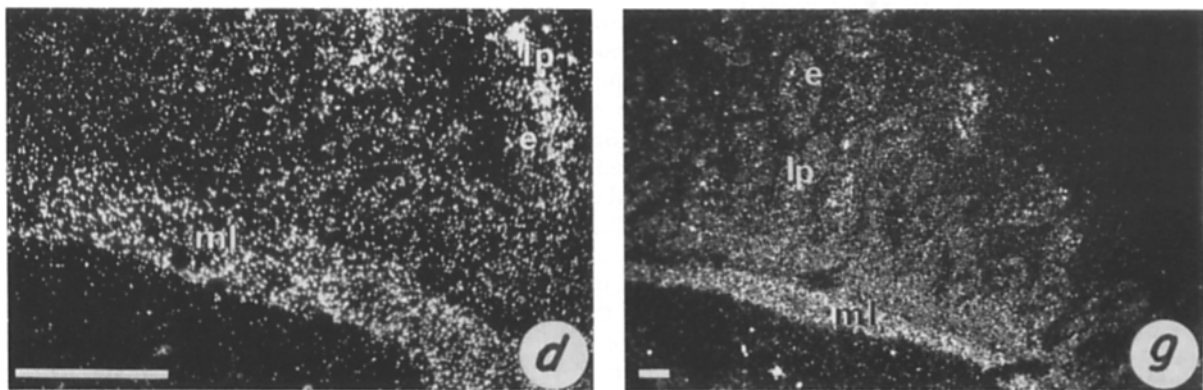
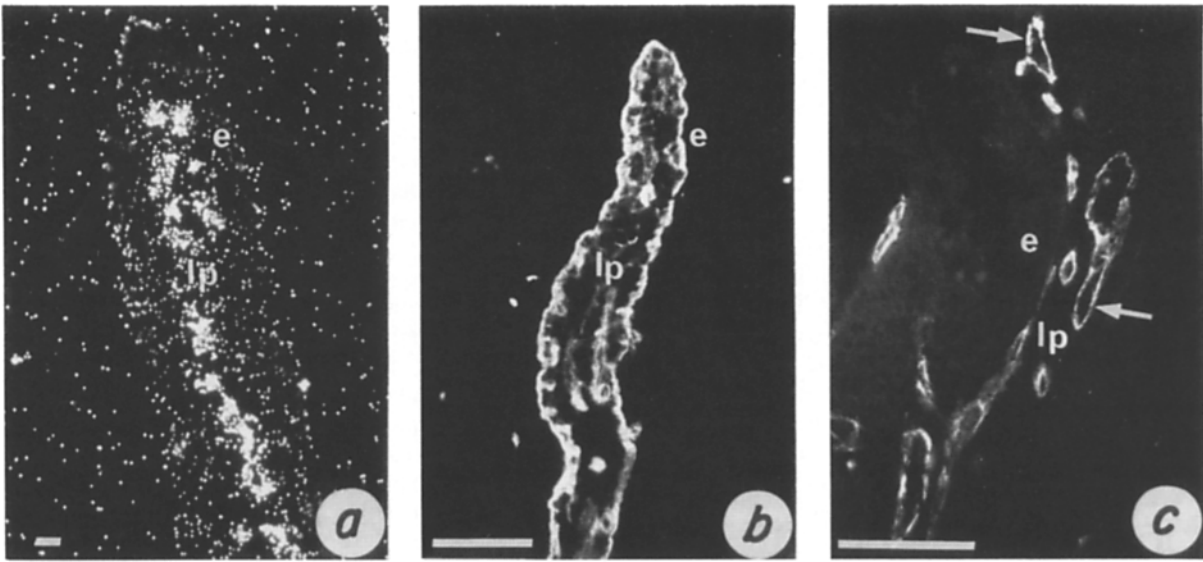
cially at the top of them (Fig. 3, *a* and *b*). The staining pattern of type IV collagen in the mesenchyme was superimposable to the distribution of the corresponding transcripts (Fig. 3, *d* and *e*). From 18 d of gestation until 2 d after birth, a phase during which villus elongation proceeds and crypts develop by epithelial downgrowth into the surrounding mesenchyme (Fig. 3 *g*), type IV collagen mRNA accumulated intensively and evenly over the entire lamina propria of the villi (Fig. 3, *f*, *i*, and *j*, and Fig. 4 *a*). In parallel, the muscular layers showed a clear increase in signal, although far lower than that found in the lamina propria (Fig. 4, *d* and *e*). At the next stage studied, postnatal day 10, the specific signal was restricted to the upper part of the lamina propria within the still growing villi and to the muscular layers (Fig. 4, *g*–*i*). Finally, in the adult organ, no hybridization signal above the background was virtually observed in any cell compartment of the small intestine (Fig. 5, *a* and *b*). This correlates with the low rate of accumulation of the type IV collagen mRNA determined by Northern blot hybridization. Longer exposure up to 3 mo of the sections of adult intestines still led to a uniform signal, but with a higher nonspecific background (not illustrated).

From the 18th d of gestation onwards, type IV collagen was found (in addition to its localization at the epithelium/lamina propria interface) associated with cellular and fibrillar structures within the lamina propria (Figs. 3 *h*, 4 *b*, and 5 *d*), among which were found blood vessels (Fig. 4 *c*), and with the cells of the muscle coat (Figs. 3 *h* and 5 *c*).

Discussion

In this work, we showed by Northern hybridization that the collagen IV cDNA probe, pFAC, detects in the murine intestine a single mRNA species of size corresponding to α 1 type IV collagen mRNA as described in PYS-2 cells (Oberbäumer et al., 1985). In situ hybridization was used to locate this mRNA at tissular levels in the developing intestine. This methodology allowed us to demonstrate the widespread presence of type IV collagen messenger in the intestinal embryonic mesenchyme and in the differentiated mesenchymally derived compartments. In contrast, no convincing expression of type IV collagen message by epithelial cells in the intestine was obvious at any time during pre- and postnatal life. Our present data demonstrate that mesenchymal cells direct the synthesis of basement membrane type IV collagen. Although the basement membrane is generally considered to be an epithelial product, the absence of role of the epithelial cells in type IV collagen deposition at the basement membrane region is substantiated by the use of species-specific antibodies on chimeric chick/rat intestines; in these hybrid intestines, basement membrane type IV collagen molecules

Figure 3. Localization of type IV collagen mRNA by in situ hybridization in (a) 16-d, (f, i, and j) 18-d fetal mouse intestines using the pFAC probe. Bright light field (j) and the corresponding dark field (i) autoradiographs of a longitudinal section through the villus of a 18-d fetal mouse intestine. Photographic emulsion was exposed for 6–7 wk. In b, schematic representation of the three-dimensional architecture of the small intestinal section illustrated in a. In c and g, 5- μ m cross cryo-sections stained with hematoxylin-eosin of respectively the 16- and 18-d fetal intestinal wall. In a–c (\blacktriangle), area corresponding to a section tangential to a villus where only epithelial cells are obvious; (\star) subepithelial mucosal connective tissue. Immunolabeling of type IV collagen in (d and e) 16-d and (h) 18-d fetal mouse intestines; in e, cross section through villi. e, epithelium; lp, lamina propria; pml, presumptive muscular layers; ml, muscular layers. Bars, (a, c, f, g, i, and j) 80 μ m; (d, e, and h) 50 μ m.



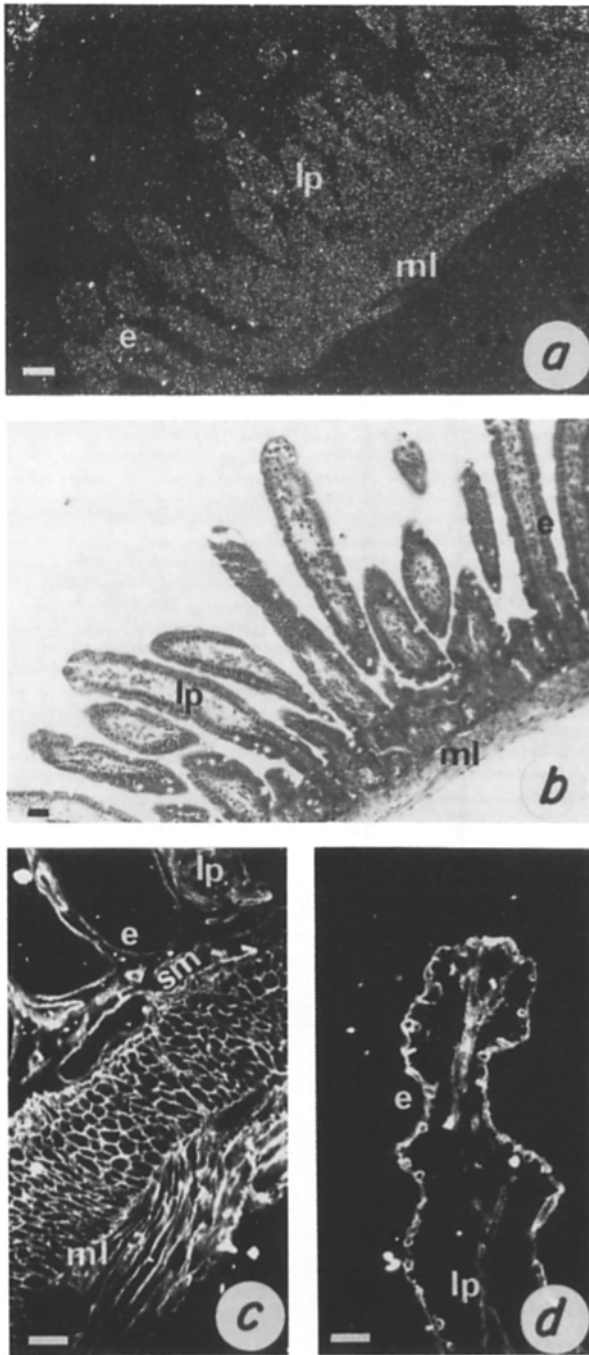


Figure 5. In situ hybridization with pFAC probe for $\alpha 1$ type IV collagen mRNA on a 20-d postnatal mouse intestine (a); the mature mouse intestine shows uniform labeling similar to nonspecific background. Photographic emulsion was exposed for 40 d. Sections are

were labeled only with the antibodies directed against the species from which the mesenchyme was taken (Simon-Assmann et al., 1988).

The accumulation of mRNA corresponding to type IV collagen into the mesenchymal compartment is not an exception. Indeed in *Drosophila*, haemocytes as "fibroblastic" cells have been shown to accumulate transcripts of the DCG1 gene, a type IV related collagen (Knibiehler et al., 1987; Mirre et al., 1988). Interestingly, Senior et al. (1988) showed by in situ hybridization in murine intestine that another basement membrane component, laminin, is also a product of the mesenchymal tissue. However, the idea that the mesenchyme could be responsible for the production of basement membrane cannot be extended to all components, nor to all tissues. Indeed, in the intestine, heparan sulfate proteoglycan molecules are deposited at the basement membrane region by epithelial cells (Simon-Assmann et al., 1989). In the kidney, organ where epithelialization of the mesenchymal cells takes place, it has been shown that expression of detectable levels of laminin mRNA is restricted to the epithelial cells (Senior et al., 1988). In addition, in a few instances, cellular cooperation in the deposition of a single basement membrane molecule species has been demonstrated. For example, in the developing kidney, both the endothelial and epithelial cells contribute to the production of glomerular basement membrane type IV collagen (Sariola et al., 1984). In the intestine, although endothelial cells synthesize type IV collagen molecules, as assessed by the deposition of these molecules at the blood vessel basement membrane (Fig. 4 c), the observed accumulation of type IV collagen mRNA and of the corresponding antigen within the whole lamina propria can be attributed only partly to the endothelial cells. Furthermore, examination of an hybrid chick/rat intestine invaded by the host vasculature (Fig. 4 c) clearly shows that cell types other than endothelial ones present in the mesenchyme are responsible for the deposition of type IV collagen at the epithelial/lamina propria interface.

The accumulation of type IV collagen mRNA in the muscular cells is in accordance with other reports. Indeed muscle fibroblasts have been shown to synthesize type IV collagen as well as laminin (Kühl et al., 1982, 1984). The production of basement membrane molecules studied im-

illuminated under dark field. In b, 5- μ m transversal cryosection of the 20-d postnatal intestinal wall stained with hematoxylin-eosin. Immunolabeling of type IV collagen in the muscle layers (c), and in the upper part of a villus (d). e, epithelium; lp, lamina propria; ml, muscular layers; sm, submucosae. Bars, (a and b) 80 μ m; (c and d) 50 μ m.

Figure 4. In situ hybridization with pFAC probe for $\alpha 1$ type IV collagen mRNA on (a and d) 2-d and (g and i) 10-d postnatal mouse intestines. 2-d postnatal intestine hybridized with a control probe (f). Specific signal in g is restricted to the upper part of the villus lamina propria (detail in i) and to the muscular layers. Photographic emulsion was exposed for 3 (a and d) and 6 (f, g, and i) wk. Sections are illuminated under dark field. 5- μ m transversal cryosections of the (e) 2- and (h) 10-d postnatal mouse intestinal wall stained with hematoxylin-eosin. Immunolabeling of type IV collagen in a 2-d postnatal mouse intestinal villus (b). Immunodetection of type IV collagen (c) on a chick mesenchyme/rat endoderm hybrid intestine developed under the kidney capsule of a nude mouse (experimental procedure described in Simon-Assmann et al., 1989). Owing to the species-specificity of the antibody (Simon-Assmann et al., 1988), the following conclusions arise from this figure: only the invading mouse host capillaries were decorated (arrows); and no type IV collagen deposition by the rat epithelial cells at the epithelium-lamina propria interface could be observed. e, epithelium; lp, lamina propria; ml, muscular layers. Bars, (a and d-i) 80 μ m; (b and c) 50 μ m.

munohistochemically or biochemically in muscle cell and organ cultures has been reviewed by Timpl and Dziadek (1986).

In studying the production and distribution of type IV collagen during development, it is important to correlate the localization of the mRNA by *in situ* hybridization versus the localization of the corresponding antigens studied by immunocytochemistry. First, reorganization of type IV collagen antigens (which became in the mesenchyme confined just beneath the epithelial/mesenchymal interface and in its most peripheral zone) parallels a similar segregation of type IV mRNA. Later on, when intestine undergoes villus outgrowth, a period at which an irregular deposition of basement membrane molecules at the upper part of the villi occurs (Simon-Assmann et al., 1986) high levels of mRNA were seen in the protruding villi suggesting that new basement membrane material has to be laid down. Finally, in the mature adult organ, although collagen IV was still detectable by immunocytochemistry (at the basement membrane region and around some structures scattered within the lamina propria) no collagen type IV mRNA accumulation could be reliably demonstrated by *in situ* hybridization. Yet, Northern blot hybridization revealed the presence of collagen IV mRNA in the adult intestine although at a much lesser amount than at developmental stages where intense morphogenetic movements take place.

The fact that embryonic and perinatal tissues exhibit a strong hybridization signal (while the mature adult intestine is devoid of specific signal) indicates that the biosynthesis of type IV collagen is more active during the strategic phases of development when remodeling of connective tissue and tissue growth occur. Related to this, one can assume that the turnover of the basement membrane collagen IV is very low in the adult intestine. Indeed, it has been shown that in adult organisms basement membranes are stable structures with a turnover rate of 40 d in the colon (Timpl and Dziadek, 1986). This observation indicates that epithelial cell migration that occurs along the crypt-villus axis is not accompanied by a renewal of the underlying basement membrane, but rather that the epithelial cell population slides along a stable basement membrane.

In conclusion, the data of *in situ* hybridization in combination with our previous immunocytochemical observations (Simon-Assmann et al., 1988) clearly demonstrate that the type IV collagen molecules present at the epithelial/mesenchymal interface derive from the mesenchyme. They further bring arguments about the regulation of pattern formation and differentiation in the intestine via basement membrane molecules.

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