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Developmental Expression and Cellular Origin of the Laminin α2, α4, and α5 Chains in the Intestine

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Laminins are extracellular matrix glycoproteins that are involved in various cellular functions, including adhesion, proliferation, and differentiation. In this study, we examine the expression patterns and the cellular origins of the laminin α2, α4, and α5 chains in the developing mouse intestine and in *in vitro* mouse/chick or chick/mouse interspecies hybrid intestines. *In situ* hybridization and Northern blot analysis revealed that mRNA levels for all three laminin α chains are highest in the fetal intestine undergoing intense morphogenetic movements. Laminin α4 mRNA and polypeptide are associated with mesenchyme-derived cell populations such as endothelium and smooth muscle. In contrast, laminin α2 and α5 chains participate in the structural organization of the subepithelial basement membrane and, in the mature intestine, show a complementary pattern of expression. All three laminin α chains occur in the smooth muscle basement membrane, with a differential expression of laminin α5 chain in the circular and longitudinal smooth muscle layers. The cellular origin of laminin α2 and α5 chains found in the subepithelial cell basement membrane was studied by immunocytochemical analysis of mouse/chick or chick/mouse interspecies hybrid intestines at various stages of development using mouse-specific antibodies. Laminin α2 was found to be deposited into the basement membrane exclusively by mesenchymal cells, while the laminin α5 chain was deposited by both epithelial and mesenchymal cells in an apparently developmentally regulated pattern. We conclude that (1) multiple laminin α chains are expressed in the intestine, implying specific roles for individual laminin isoforms during intestinal development, and (2) reciprocal epithelial/mesenchymal interactions are essential for the formation of a structured subepithelial basement membrane.

Key Words: **intestinal development; laminin α chains; epithelial/mesenchymal interactions; basement membrane; muscle differentiation.**

INTRODUCTION

Ontogenic regulation by epithelial/mesenchymal interactions has been clearly demonstrated for a number of organ systems. Using several experimental models, it has been demonstrated that the mesenchyme plays a permissive role in the morphogenesis and cytodifferentiation of the digestive tract endoderm. This conclusion has been drawn from data from interspecies recombinants composed of chick and rodent intestinal anlagen, in combination with species-specific morphological and biochemical analyses (Kedinger *et al.*, 1981; and for a review, see Kedinger *et al.*, 1999). Epithelial differentiation, assessed by the expression of cytoskeletal proteins (such as villin) and digestive enzymes (sucrase–isomaltase, maltase) requires the participation of a mesenchymal support. In turn, the endoderm induces the associated mesenchymal cells to organize into smooth muscle-like layers and into the connective tissue axis of the villi and to express specific markers such as α smooth muscle actin (Kedinger *et al.*, 1990). These close and inductive interactions persist in the mature organ in the crypt regions where continuous epithelial cell renewal from stem cells occurs. Thus, the intestine represents an excellent model for studying the precise molecular mechanisms mediating heterotypic cell interactions.

Another crucial component of this epithelial–stromal unit is the basement membrane located at the interface between the two cell populations. Comprehensive immunohistological studies have revealed changes in the spatial distribution of some extracellular matrix molecules which correlate with morphogenetic processes or with cell renewal in the adult (for reviews see Simon-Assmann *et al.*, 1995, 1998; Beaulieu, 1997; Kedinger *et al.*, 1999). Among these molecules are the laminins, large cross- or T-shaped

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heterotrimeric glycoproteins. Members of this ever-growing family (11 laminin variants have been discovered to date) are often expressed in tissue-specific or developmentally regulated manners, suggesting that they are functionally distinct (Delwel and Sonnenberg, 1996; Engvall and Wewer, 1996; Timpl and Brown, 1996; Miner *et al.*, 1997; Sorokin *et al.*, 1997a,b; Frieser *et al.*, 1997; Aumailley and Smyth, 1998). To date three laminin isoforms have been identified in the mouse intestine. The first laminin isoform identified in the intestinal basement membrane was laminin-1, composed of α1, β1, and γ1 chains. Using specific monoclonal antibodies, we have shown that in the rodent adult intestine laminin β1/γ1 chains are homogeneously distributed in the crypt and villus subepithelial basement membrane, while the laminin α1 chain is restricted to the crypt zone. Laminin-1 is detected at the boundary between epithelial cells and the stromal compartment throughout the developmental period (Simo *et al.*, 1991). In contrast, laminin-2, composed of the α2 chain associated with β1 and γ1 chains, is present exclusively in the mature stages in the basement membrane that underlies the crypt epithelial cells (Simon-Assmann *et al.*, 1994). Laminin-5 has a unique chain composition consisting of α3, β3, and γ2 chains. The distribution of the individual chains of laminin-5 is intriguing. At early stages of development only γ2 and β3 chains are found, decorating the endodermal/mesenchymal interface, while in the adult intestine laminin γ2 chain shows a continuous expression along the villus and laminin α3 and β3 chains are restricted to the very top of the villus where terminally differentiated cells are shed into the lumen. Yet, RT-PCR has shown that the three genes are transcribed at all stages of development (Orian-Rousseau *et al.*, 1996).

Preliminary studies on the expression of the laminin α4 chain have been performed on various mouse tissues (Frieser *et al.*, 1997; Iivanainen *et al.*, 1997; Miner *et al.*, 1997; Sorokin *et al.*, 1997b; Salmivirta *et al.*, 1997). To date laminin-8, composed of α4, β1, and γ1 chains, has been convincingly shown to exist only in basement membranes of endothelium (Sorokin *et al.*, 1994, 1997b; Frieser *et al.*, 1997). However, *in situ* hybridization and immunofluorescence studies suggest the expression of this isoform also in basement membranes of embryonic cardiac, skeletal, and smooth muscle; axons of peripheral nerves; and fat tissue (Frieser *et al.*, 1997; Iivanainen *et al.*, 1997; Sorokin *et al.*, 1997b). Laminin-9, composed of α4, β2, and γ1 chains, may exist in basement membranes of arterioles and at the neuromuscular junction (Sorokin *et al.*, 1997a; Ringelmann *et al.*, 1999). In contrast to the somewhat restricted expression pattern of most laminin α chains, the laminin α5 chain has a more widespread distribution in adult tissues (Miner *et al.*, 1995, 1997; Durkin *et al.*, 1997; Patton *et al.*, 1997; Sorokin *et al.*, 1997a) and combines with β1 and γ1 chains to form laminin-10. In addition, the α5 chain has been shown recently to be secreted by normal and carcinoma lung epithelial cells (Pierce *et al.*, 1998; Kikkawa *et al.*, 1998) and by choriocarcinoma cells (Church and Alpin, 1998). Sequence analysis has revealed a close relationship between laminin α5 and the *Drosophila* α chain. Apart from its main expression by epithelial and endothelial cells, the laminin α5 chain has also been shown to be expressed by smooth muscle cells (Sorokin *et al.*, 1997a). Interestingly the α5 chain is found mainly in epithelial sheets that produce very little α1 chain (Durbeej *et al.*, 1996; Sorokin *et al.*,1997a,b).

Both laminin α4 and α5 have been described to be expressed in the intestine (Frieser *et al.*, 1997; Iivanainen *et al.*, 1997; Sorokin *et al.*, 1997a); however, there has been no precise description of their cellular expression patterns or whether developmental changes in expression occur. Similarly, the laminin α2 chain is known to be expressed in the intestinal crypts (Beaulieu and Vachon, 1994; Simon-Assmann *et al.*, 1994; Leivo *et al.*, 1996), but there has been no investigation of the cellular origin of this laminin chain. The cellular expression patterns and developmental changes in expression patterns of laminin chains may provide us with hints to the functions of the different laminin isoforms and indications on the nature of cells that have to be targeted in order to study the function of laminins by specific inhibiting experiments. For this reason we use here complementary approaches to define the expression and distribution of laminin α2, α4, α5 chains during mouse intestinal morphogenesis and differentiation. The current study provides evidence for the first time that laminin α2 and α5 chains actively participate in the structural organization of the subepithelial basement membrane through reciprocal and complementary epithelial/mesenchymal interactions.

MATERIAL AND METHODS

Animals

Fetuses from pregnant IOPS/OF1 mice bred in our laboratory were removed by cesarean section at various stages between the 12th day of gestation and birth. Gestational age of fetuses was calculated taking the day on which a vaginal plug was found as day 0. Small intestinal segments were taken from fetal and suckling mice of different ages and from adult mice.

White Leghorn chick embryos were used for grafting experiments. The chicken eggs were incubated at 38 ± 1°C, and the developmental stages were referred to as days of incubation.

Northern Blot Analysis

Total RNA was prepared using the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987) with two slight modifications. First, an extra centrifugation step was performed (at 10,000*g*, 4°C, 10 min) before addition of chloroform–isoamyl in order to remove cellular debris and chromosomal DNA. The supernatant was then treated with chloroform–isoamyl as described in the protocol. Second, at the end of the procedure an additional precipitation step was included using sodium acetate (0.3 M final concentration, pH 5.5) plus 2.5 vol of absolute ethanol. After being washed with 70% ethanol, the pellet was resuspended in dimethylpyrocarbonate water. Ten micrograms of total RNA or 3 μg of 0.24- to 9.5-kb RNA ladder (Gibco BRL, Life Technologies

SARL, Cergy Pontoise, France) was then fractionated by electrophoresis on 1% agarose gels in the presence of 2 M formaldehyde, transferred to nylon membranes (Boehringer Mannheim, Meylan, France), and bound to the membrane with a UV crosslinker (Bioblock Scientific, Illkirch-Graffenstaden, France). The RNA integrity was checked by methylene blue staining of blots.

Plasmids (pBluescript SK(+); Stratagene, La Jolla, CA) containing specific probes for mouse laminin α2 covering nucleotides 6420–6895 (Schuler and Sorokin, 1995), laminin α4 covering nucleotides 4720–5311 (Frieser *et al.*, 1997), and laminin α5 covering nucleotides 3962–4623 (Sorokin *et al.*, 1997a) were employed. Anti-sense DIG RNA-labeled probes were synthesized using 1 μg of linearized plasmid DNA with the DIG RNA labeling kit according to the manufacturer's instructions (Boehringer Mannheim). Probe concentrations were estimated by serial dilutions of the labeling mix compared to an RNA labeling DIG standard (Boehringer Mannheim).

Prehybridization was performed at 68°C in 10 ml of DIG Easy Hyb solution (Boehringer Mannheim) for at least 1 h. For hybridization, probes (50 ng/ml) were denatured for 5 min at 100°C, chilled immediately on ice, and added to 10 ml of prewarmed (68°C) DIG Easy Hyb. Following overnight hybridization at 68°C, the membranes were washed 2 × 15 min in 2× SSC, 0.1% SDS at 68°C and 2 × 30 min in 0.1× SCC, 0.1% SDS at 68°C. Chemiluminescence detection was performed using the DIG wash and block buffer set with CDP-Star as substrate according the manufacturer's instructions (Boehringer Mannheim). The blot was then exposed to X-ray film (Lumi-Film; Boehringer Mannheim) for 15 to 30 min.

In Situ Hybridization

In situ hybridizations were performed as described in Schnürch and Risau (1991) with the mouse laminin α-chain-specific probes described above. Frozen sections (5–10 μm) of whole mouse embryos of day 14, 16, and 18 of gestation (E14, E16, and E18) were fixed immediately after cutting in 4% paraformaldehyde and stored at −70°C until used. Sections were incubated overnight at 48°C with ^{35}S-labeled single stranded anti-sense DNA (5×10^4 cpm/μl) in hybridization buffer (50% formamide, 5 mM EDTA, 2× SSC, 0.1 mM UTP, 10% dextran sulfate, 150 μg/ml *Escherichia coli* (RNase free) tRNA, 10 mM Tris, pH 7.5, 10 mM $NaHPO_4$, pH 6.8). On each slide, a control section was hybridized with ^{35}S-labeled sense DNA under identical conditions. Sections were washed at 37°C with washing buffer (50% formamide, 2× SSC, 10 mM β-mercaptoethanol) and RNase buffer (500 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5). Autoradiography was performed using Kodak NTB-2 emulsion. Exposure times varied from 7 to 30 days. Sections were stained with Giemsa and mounted. Control sections were hybridized with ^{35}S-labeled corresponding sense probes and did not show any specific binding.

Interspecies Intestinal Recombinants

Associations between mouse and chick intestinal tissue components were performed using a previously described experimental procedure (Kedinger *et al.*, 1981). Briefly, 5-day chick embryonic and 12-day fetal mouse intestinal anlagen were isolated. Mesenchyme was separated from the endoderm after incubation of the intestinal segments in a 0.03% solution of collagenase (1 h at 37°C). Two types of interspecies recombinations of the isolated endodermal and mesenchymal components were prepared: chick mesenchyme/mouse endoderm (Cm/Me) and mouse mesenchyme/chick endoderm (Mm/Ce). After overnight culture on agar-solidified medium to ensure their cohesion, the hybrid intestines were grafted into the coelomic cavity of 3-day chick embryos. The hybrid intestines were harvested at various time points, up to 17 days, after grafting.

Antibodies

Rat monoclonal antibodies specific for mouse laminin α2 (mAb 4H8-2; Schuler and Sorokin, 1995), α4 (mAb 341; Ringelmann *et al.*, 1999), and α5 (mAb 4G6; Sorokin *et al.*, 1997a) chains were used for immunodetection on cryosections. To follow differentiation of epithelial cells in the hybrid intestines composed of mouse endoderm, affinity-purified polyclonal anti-villin antibodies (generous gift from Dr. S. Robine, Institut Curie, Paris; Robine *et al.*, 1985) were used. Polyclonal smooth muscle myosin antibodies were kindly provided by Professor Gabbiani (Université de Genève, Switzerland; Benzonana *et al.*, 1988).

Immunofluorescence and Morphological Analysis

Mouse small intestinal segments at different developmental stages and hybrid intestines were processed similarly. They were embedded in Tissue-Tek compound (Miles Laboratories, Inc.), frozen in isopentane cooled in liquid nitrogen, and stored at −70°C until use. Transverse sections (5–6 μm thick) cut at −25°C were placed onto gelatin-coated slides. Incubations with rat monoclonal antibodies to laminin α2, α4, or α5 chains were carried out for 1 h at room temperature in a moist chamber. Bound antibodies were visualized using anti-rat secondary antibody conjugated with fluorescein isothiocyanate (1:50; The Jackson Laboratory). Some sections were double stained by consecutive incubations of sections with anti-laminin α5, mAb 4G6, followed by polyclonal anti-myosin antibody. After washing, sections were incubated with a mixture of the appropriate FITC- and TRITC- (rhodamine) labeled secondary antibodies.

The differentiation of Cm/Me hybrid intestines was analyzed by using polyclonal anti-villin antibodies, which specifically react with this cytoskeletal protein in the mouse epithelial cells. Sections were subsequently counterstained with periodic–acid–Schiff for histological observation. After being mounted in a glycerol/PBS/phenylenediamine solution, the slides were examined using a Zeiss Axiophot Microscope (Zeiss, Oberkochen, Germany).

Control sections were processed as above, with omission of the primary antibodies, and revealed some nonspecific fluorescence within the lamina propria, mainly in the adult organ. Controls also included the staining of chick intestinal sections with anti-laminin α2, α4, and α5 antibodies, which reveal no cross-reaction.

RESULTS

Expression of Laminin α2, α4, and α5 mRNA during Mouse Intestinal Development

Northern Blot Analysis

Expression of the laminin α2, α4, and α5 chain transcripts during mouse intestinal development was studied using Northern blot analysis (Fig. 1). The approximately 9.5-, 7-, and 11-kb mRNAs for laminin α2, α4, and α5 chains,

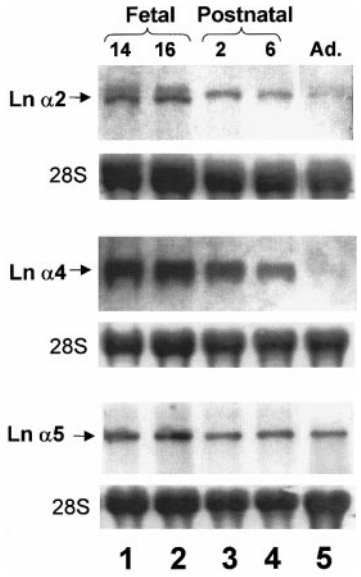


FIG. 1. Northern blot analysis of laminin α2, α4, and α5 mRNA expression during mouse intestinal development. Total RNA from intestines was prepared at 12–14 and 16 days of gestation (lanes 1 and 2), at 2 and 6 days after birth (lanes 3 and 4), and at the adult stage (lane 5). The filters were hybridized with anti-sense DIG-labeled riboprobes. The lower blots are methylene blue staining of the same filter showing the 28S rRNA. The results revealed a strong expression of the three laminin genes throughout embryonic and perinatal development and a weaker expression in the adult organ.

respectively, were strongly expressed in embryonic and perinatal intestines (Fig. 1, lanes 1–3) and were progressively down regulated from the postnatal period to the adult stage (lanes 4 and 5), at which only faint signals were detectable for α2 and α4 chains.

In Situ Hybridization

14-day mouse embryos. The intestine differentiates according to a cranial (proximal) to caudal (distal) gradient. By 12 days of gestation, the embryonic mouse intestinal anlage is a simple undifferentiated tube of stratified endodermal cells surrounded by mesenchymal cells. At 14 days of gestation, onset of differentiation of the mesenchymal tissue occurs, giving rise successively to the presumptive circular (inner) and longitudinal (outer) muscle cell layers. This stage precedes villus formation.

In situ hybridization of E14 mouse embryos revealed a strong laminin α2 mRNA signal in the mesenchymal tissue, while epithelium was negative. Figures 2A and 2B illustrate a cross section through the distal portion of an E14 intestine showing expression of laminin α2 mRNA in the mesenchymal cells underlying the endoderm and a weaker signal covering the presumptive circular muscle. Laminin α4 mRNA has an expression pattern similar to that of laminin α2 in the E14 intestine; it is also expressed in mesenchyme of the E14 intestine and is not detectable in epithelium (Figs. 3A and 3B). Laminin α5 mRNA is expressed in the intestinal endodermal cells at this stage of development (Figs. 4A and 4B), but also exhibits strong expression in the mesenchymal cell layer surrounding the endoderm and in the presumptive circular (inner) layer of the muscular coat. In addition, laminin α5 mRNA is expressed in the serosa.

16-day mouse embryos. Between E15 and E17 villus primordia begin to form. In addition a second morphologically distinct ring of smooth muscle myoblasts appears between the developing circular muscle layer and the serosa. This corresponds to the presumptive longitudinal (outer) smooth muscle layer of the muscular coat (Kedinger *et al.*, 1990; McHugh, 1995). In the E16 embryos examined, laminin α2 mRNA expression was concentrated in the mesenchyme underneath the epithelium and in the smooth muscle layer region (Figs. 2C and 2D). Laminin α4 mRNA was expressed in the mucosal connective tissue (the lamina propria) including the protruding villus core (Figs. 3C and 3D). Laminin α5 expression at this stage was restricted to mesenchymal cell populations, such as the lamina propria of the villi, especially at their tips (Figs. 4C and 4D). Laminin α5 mRNA was still detected in the smooth muscle coat.

18-day mouse embryos. From E18 to 2 days postnatally, villus elongation proceeds and crypts form by epithelial down growth into the surrounding mesenchyme. At E18 laminin α2 mRNA expression was restricted to the mucosal connective tissue at the base of the nascent crypts (Figs. 2E and 2F). Laminin α4 mRNA expression occurred over the entire lamina propria of the villi and some expression was evident in mesenchymal cells at the base of villi. Laminin α4 mRNA expression also occurred over the smooth muscle layers (Figs. 3E and 3F). Laminin α5 mRNA was strongly expressed in the lamina propria mainly at the villi tips and in the inner circular smooth muscle layer (Figs. 4E and 4F).

Immunolocalization of Laminin α2, α4, and α5 Polypeptides in the Developing Mouse Intestine

Immunolocalization of laminin α2, α4, and α5 chains was investigated using the rat monoclonal antibodies 4H8-2, 341, and 4G6, respectively.

The laminin α2 chain was not detected until gestational day 16 in the mouse (day 12 is illustrated in Fig. 5A) and was restricted to the nascent crypt basement membrane at this stage (not illustrated). Clear and strong laminin α2 expression, as revealed by mAb 4H8-2, became evident at the end of the gestational period (Fig. 5B). At this stage, immunoreactivity was concentrated in the subepithelial basement membrane at the bottom of the crypt primordia and also occurred weakly in the mucosal connective tissue, in the

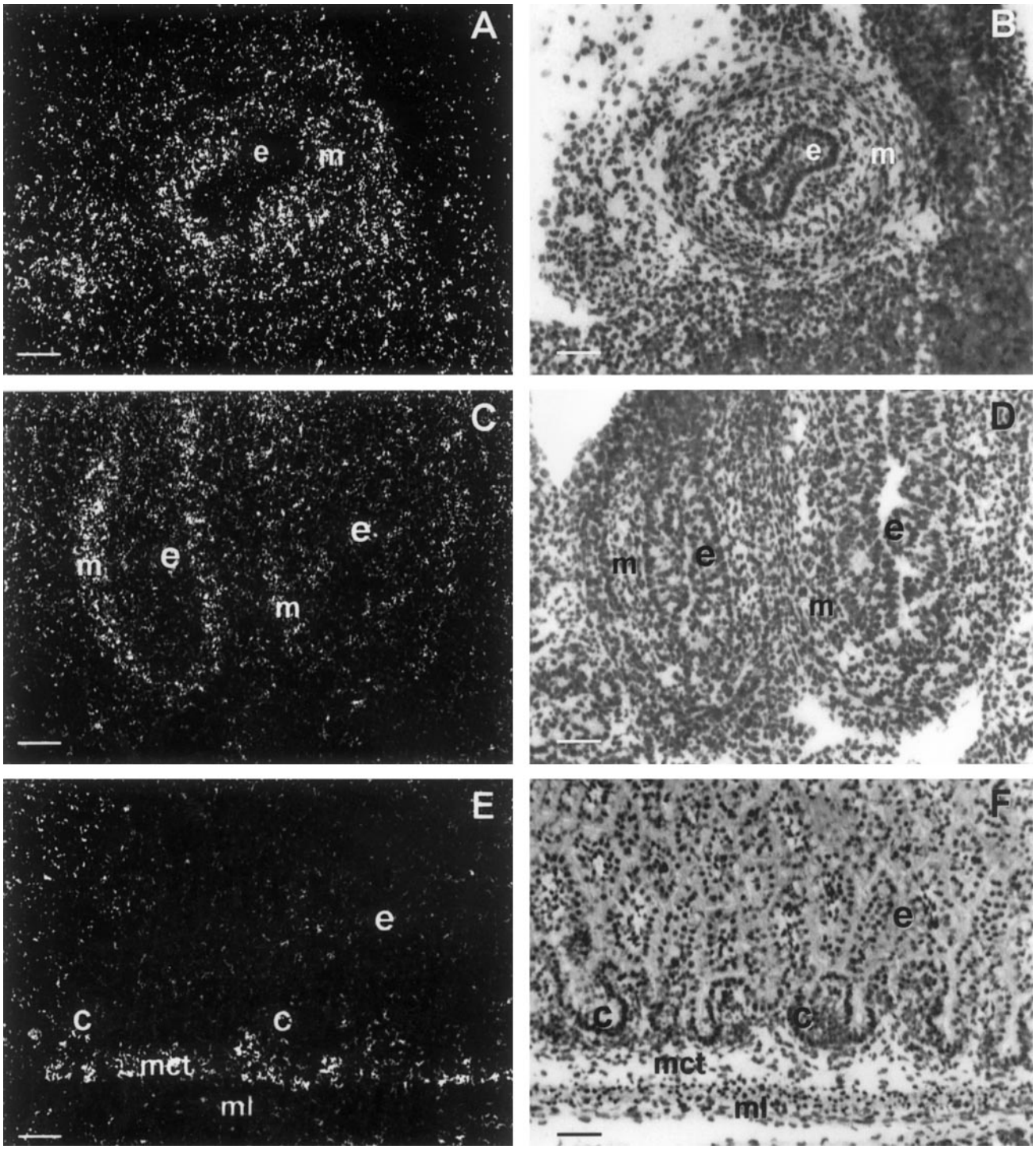


FIG. 2. Localization of laminin $\alpha 2$ mRNAs by *in situ* hybridization in embryonic intestines using anti-sense RNA riboprobes. Dark-field illuminations are on the left, and corresponding light-field illuminations are on the right. At E14 (A and B) and E16 (C and D), expression of $\alpha 2$ laminin mRNAs is detected in the mesenchymal cells surrounding the endoderm. At E18 (E and F) expression is confined to the mesenchyme underneath the presumptive crypt region, corresponding to the mucosal connective tissue. No specific signal above background could be detected in the epithelial layer. m, mesenchyme; e, endoderm or epithelium; mct, mucosal connective tissue; ml, muscle layers; c, nascent crypts. Bars, 50 μm.

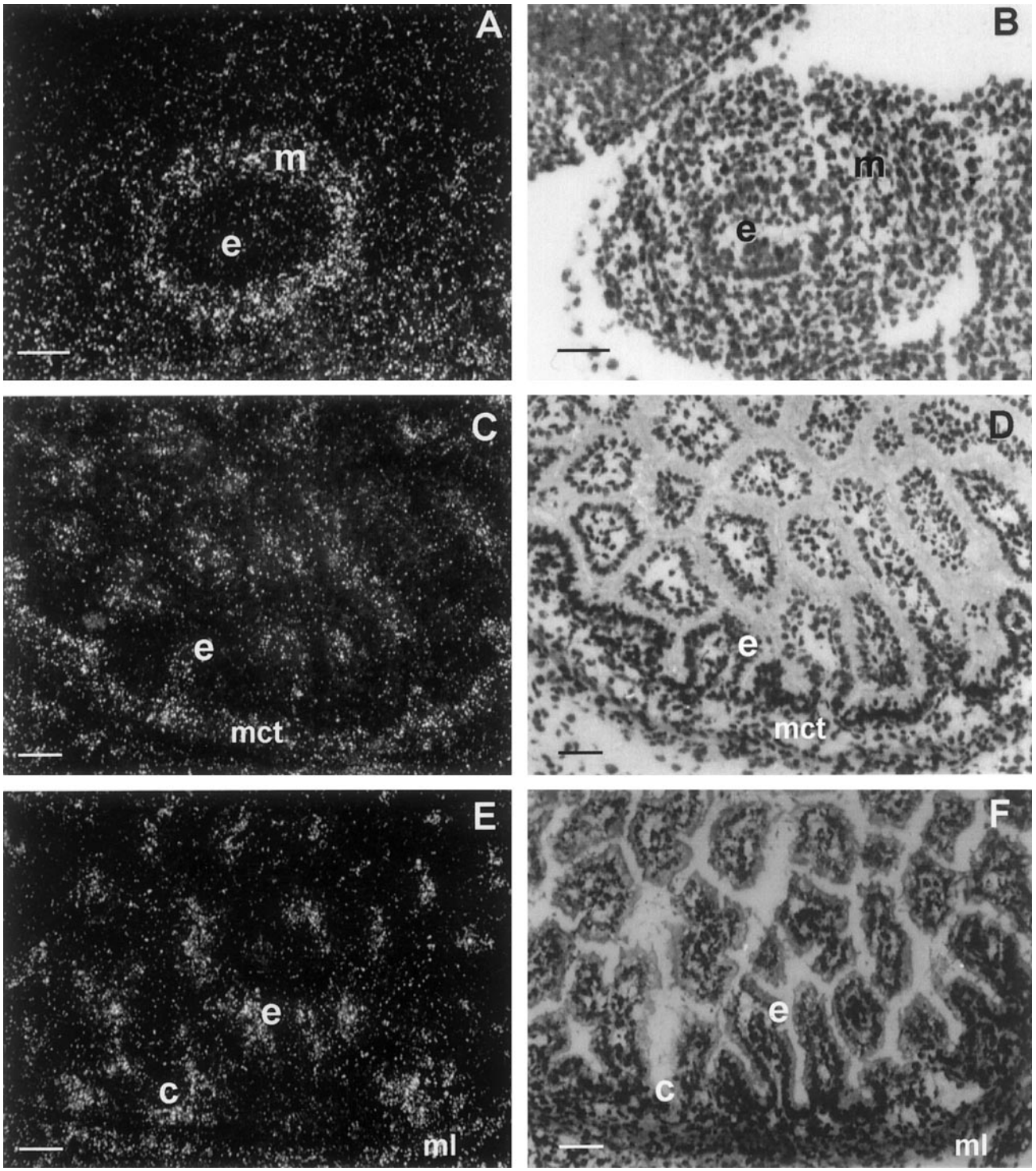


FIG. 3. Localization of laminin α4 mRNAs by *in situ* hybridization in embryonic intestines using anti-sense RNA riboprobes. At early stages of development, expression is seen in the mesenchyme surrounding the intestinal endoderm (E14, A and B) and subsequently in the mucosal connective tissue at the base of intestinal villi and in the villus core as well as in the smooth muscle layers (E16, C and D; E18, E and F). For further details see Fig. 2. Bars, 50 μm.

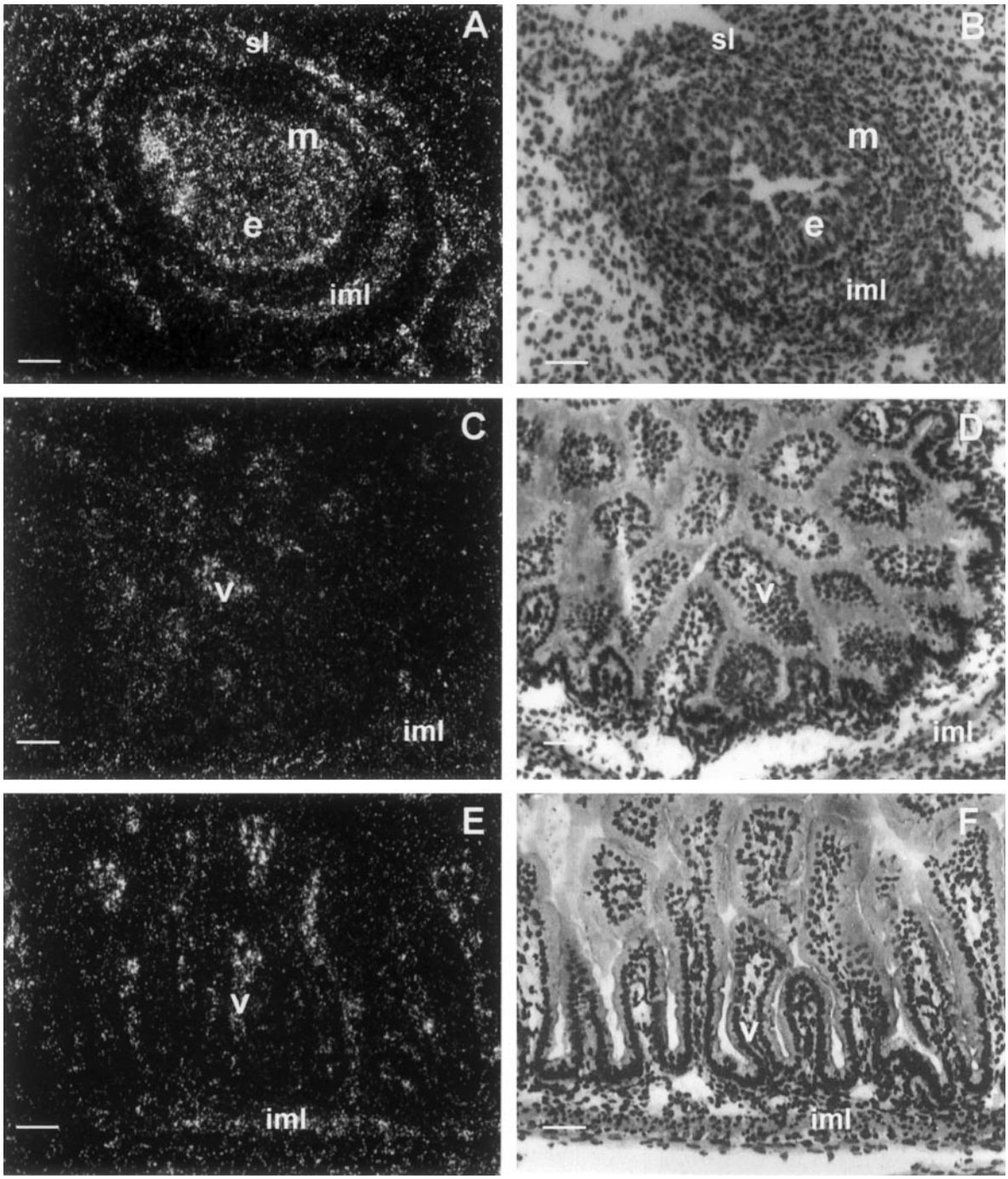


FIG. 4. Localization of laminin α5 mRNAs by *in situ* hybridization in embryonic intestines using anti-sense RNA riboprobes. At E14 (A and B), expression of laminin α5 mRNA is found in the endodermal and in the mesenchymal layers; accumulation of laminin α5 transcripts is also found in the presumptive inner muscular layer and in the serosal layer (A and B illustrate a section throughout the proximal intestine). At E16 (C and D) and E18 (E and F), the hybridization signal is concentrated in the lamina propria at the top of the protruding villi and in the inner muscle layer. iml, inner muscular cell layer; sl, serosal cell layer; v, villus. For further details see Fig. 2. Bars, 50 μm.

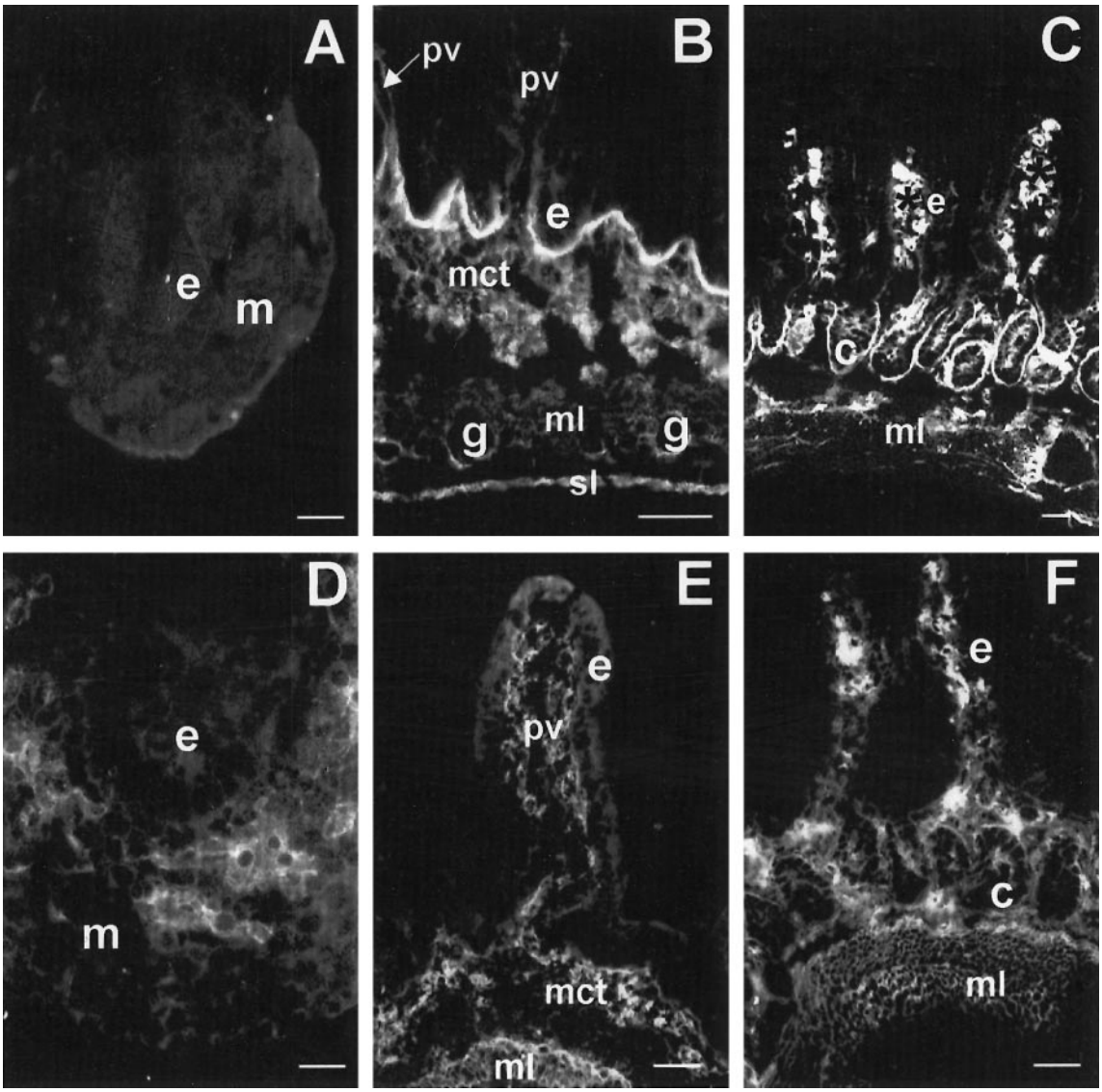


FIG. 5. Immunostaining of laminin α2 (A–C) and α4 (D–F) chains during mouse intestinal development. Intestinal segments were analyzed at 12 (A and D) and 19 (B and E) days of gestation and in the adult (C and F). No laminin α2 chain is detected at early stages of development (A). Once expressed, the laminin α2 chain is found at the subepithelial basement membrane level exclusively at the base of the protruding villi (B), corresponding to the crypt region in the adult (C). Laminin α2 staining is also found in the mucosal connective tissue, around the enteric ganglia, and faintly decorating the muscle cells. Staining at the core of the adult villi is nonspecific (* in C). At all stages studied (D–F), laminin α4 chain staining was restricted to the mesenchyme or mesenchyme-derived cells and increased progressively in the muscle coat (E and F). e, endoderm or epithelium; m, mesenchyme or mesenchyme-derived tissues; c, crypt; pv, protruding villi; g, enteric ganglia; mct, mucosal connective tissue; ml, muscle layers; sl, serosal cell layer. Bars, 30 μm.

serosal layer, and around the enteric ganglia located between the muscle layers. It is worth noting that during the intense phase of villus formation, laminin α2 staining was also visible along the protruding villi, albeit faintly (see Fig. 5B). In the mature adult organ, laminin α2 staining was restricted to the crypt area with no distinct staining along the villus (Fig. 5C). A weak immunoreactivity was noted in association with the differentiating/differentiated smooth muscle cells (Figs. 5B and 5C).

Throughout development the laminin α4 chain was found only in the mesenchymal compartment. At 12 days of gestation the laminin α4 chain-specific antibody, 341, weakly stained structures which probably correspond to blood vessels in the innermost part of the mesenchyme (Fig.

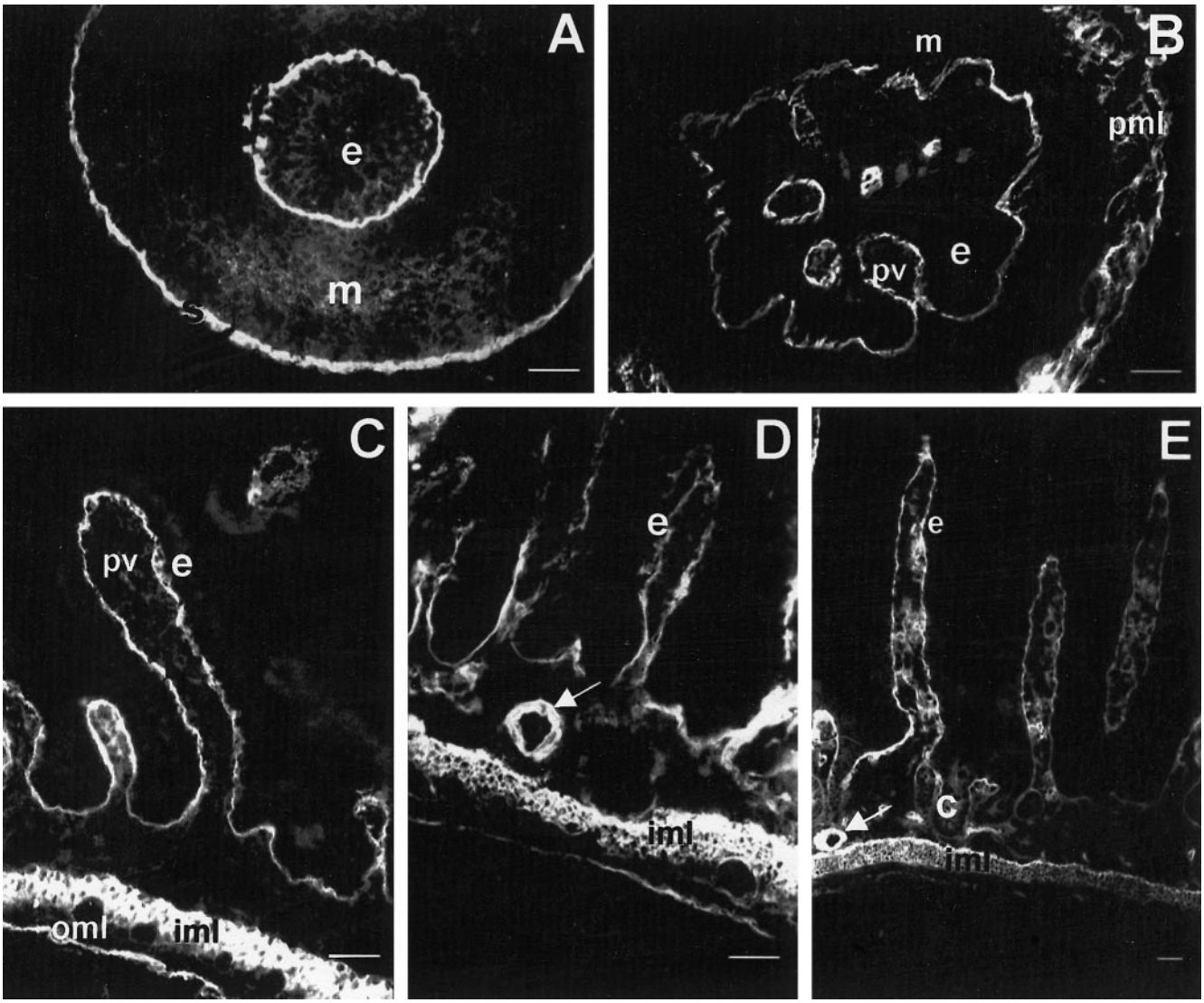


FIG. 6. Immunostaining of laminin α5 chain during mouse intestinal development. Intestinal segments were analyzed at 12 (A), 16 (B), and 19 (C) days of gestation; at postnatal day 4 (D); and in the adult (E). At the earliest stage studied (A) the laminin α5 chain staining underlined the endodermal/mesenchymal interface and the serosal layer around the mesenchyme. At 16 days of gestation (B) immunoreactivity appeared in the newly formed muscular layers. From 19 days of gestation onward (C–E) the laminin α5 chain present in the muscle coat is clearly concentrated in the inner circular layer. Staining of large blood vessels became evident after birth (D and E). In the adult, basement membrane staining occurred from the upper part of the crypts to the villus tip. e, endoderm or epithelium; m, mesenchyme or mesenchyme-derived tissues; c, crypt; pv, protruding villi; pml, presumptive muscular layer; iml, inner muscle layer; oml, outer muscle layer; s, serosal cell layer; arrows in (D) and (E) mark blood vessels. Bars, 30 μm.

5D). Thereafter, laminin α4 chain staining increased progressively in basement membranes of the differentiating muscle cells of the muscle coat and was found expressed within the lamina propria (Fig. 5E). In the mature organ, laminin α4 expression clearly delineated the cells of the muscle coat and was most intense at the mesenchymal area at the crypt region surrounding the crypt epithelial cells (Fig. 5F).

At 12 days of gestation laminin α5 chain, as revealed by 4G6, appeared as a continuous linear band at the intestinal epithelial/mesenchymal junction and was strongly expressed in the peripheral-most serosal layer (Fig. 6A). From 16 days of gestation to birth, continuous staining for laminin α5 along the protruding villi (Figs. 6B and 6C) and in the future muscle coat (Fig. 6B) was apparent and, later, in the well-defined circular or inner muscle layer, while only a

weak fluorescent signal occurred in the outer longitudinal muscle layer (Figs. 6C–6E). At postnatal day 4, laminin α5 was also found in basement membranes of large blood vessels (Fig. 6D). Thereafter, once crypt formation was completed, a differential staining for laminin α5 was evident in the subepithelial basement membrane with an increasing gradient of intensity from crypt to villus tip, especially evident in the mature intestine (Fig. 6E).

Cellular Origin of Laminin α2, α4, and α5 Polypeptides Using Mouse/Chicken Hybrid Intestines and Mouse-Specific Antibodies

General Features of the Hybrid Intestines

The cellular origin of the three laminin α chains was studied using hybrid intestines consisting of mouse and chick tissues. The secreted laminin polypeptides deposited at the epithelial/mesenchymal interface were identified with mouse-specific antibodies. The antibodies employed, 4H8-2, 341, and 4G6, were raised against the mouse laminin α2, α4, and α5 chains, respectively, and show no reaction with the chick proteins.

In general, both types of hybrid intestines (chick mesenchyme/mouse endoderm and mouse mesenchyme/chick endoderm) gave rise to well-vascularized small intestinal segments. This system mimics well the *in vivo* cell–cell interactions that occur during intestinal development as progressive epithelial and muscle cell differentiation occurs, starting from early embryonic tissue rudiments. Histological investigation revealed well-differentiated epithelial and muscular layers by 9 days of grafting in all hybrid intestines (Figs. 7A and 7B). Differentiation of the hybrid intestines was further assessed by examination of the expression patterns of villin, a cytoskeletal marker present in the epithelial apical brush border membrane (Fig. 7C), and smooth muscle myosin, a marker for smooth muscle cells.(Fig. 7D).

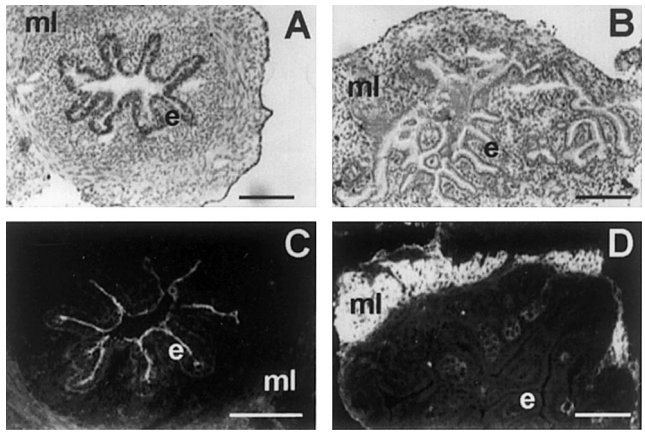


FIG. 7. Histology (A and B) of intestinal segments and immunodetection of villin (C) and smooth muscle myosin (D). Segments analyzed are from a chick mesenchyme/mouse endoderm association (Cm/Me; A and C) and from a mouse mesenchyme/chick endoderm association (B and D), 9 (A and C) or 13 (B and D) days after grafting. The use of an anti-villin antibody recognizing exclusively the mouse antigen localizes this cytoskeletal protein at the apical pole of the epithelial cells that have differentiated from mouse endoderm in the Cm/Me association (C). In parallel, anti-mouse smooth muscle myosin detects only the differentiated muscle cells in the inverse association (D). e, epithelial cells; ml, muscular layers. Bars, 100 μm.

Both Epithelial and Mesenchymal Cells Synthesize and Secrete the Laminin α5 Chain

In both types of hybrid intestines (Cm/Me and Mm/Ce) grafted for at least 9 days, mAb 4G6 against the laminin α5 chain gave a continuous staining of the subepithelial basement membrane, which is indicative of the presence of mesenchymal- and epithelial-derived molecules (Figs. 8A and 8C). From the 13th day after grafting, when villi are well formed, interesting differences in the pattern of laminin α5 chain expression between the two types of hybrid intestines were apparent: in the Cm/Me associations, laminin α5 staining became restricted to the villus base, whereas in the inverse association, Mm/Ce, a clear-cut gradient of intensity of laminin α5 staining occurred from the base to the tip of the villi (illustrated at 17 days after grafting in Figs. 8B and 8D), which was similar to that seen *in vivo* in adult intestines (compare Fig. 8D to 6E). It should be pointed out that the mouse laminin α5 chain antibody also decorated various mesenchyme-derived cells, including the muscle layers and some structures within the lamina propria of the Mm/Ce hybrid intestines (Figs. 8C and 8D). These data demonstrate that the laminin α5 chain is deposited into the basement membrane by both the endoderm and the mesenchyme and, at advanced stages of differentiation, in a complementary fashion along the crypt–villus axis.

Laminin α2 Chain Located in the Subepithelial Basement Membrane Is a Mesenchymal Product Whose Expression Is Regulated by Endoderm

Immunocytochemical experiments similar to those described above were performed using the 4H8-2 antibody against the mouse laminin α2 chain. None of the Cm/Me hybrid intestines were stained by the laminin α2 chain-specific antibody, regardless of their state of differentiation (Fig. 8E). In the inverse hybrid associations (Mm/Ce), 4H8-2 showed discontinuous staining of basement membranes as early as 3 days after grafting (Fig. 8G). At later stages of development, a clear-cut predominance of laminin α2 staining was noted in the crypt basement membrane, as well as a fainter staining along some villus axes (Fig. 8H). This expression pattern contrasts with the delayed and more restricted expression of laminin α2 in the crypt region *in vivo* in the mouse intestine (see Figs. 5A–5C). These data

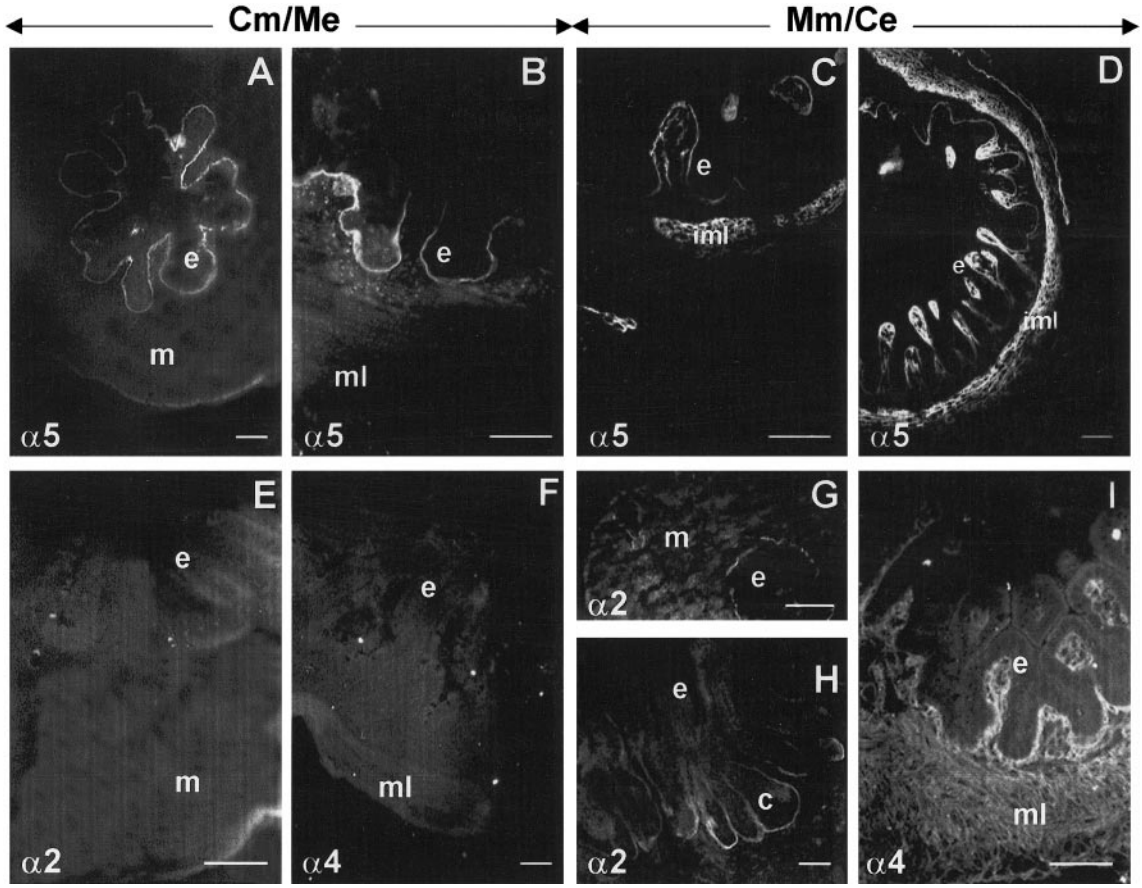


FIG. 8. Immunodetection of laminin chains with monoclonal antibodies recognizing mouse antigens in chimeric hybrid intestines: chick mesenchyme/mouse endoderm (A, B, E, and F) and mouse mesenchyme/chick endoderm (C, D, and G–I) associations developed in the coelomic cavity of chick embryos for 3 (G), 9 (A, C, and E), 13 (F), and 17 (B, D, H, and I) days. Rat monoclonal antibodies specific for mouse laminin α5 (A–D), α2 (E, G, and H), and α4 (F and I) chains were employed. e, endoderm or epithelium; m, mesenchyme; c, crypt region; ml, muscular layers; iml; inner muscular layer. Bars, 50 μm.

show the exclusive deposition of the laminin α2 chain into the basement membrane by the mesenchymal cell population and suggest an inhibitory regulating effect exerted by the homologous endoderm.

Laminin α4 Chain Is Strictly a Mesenchymal Product

Regardless of the differentiation state of the Mm/Ce hybrid intestines examined, antibody 341, specific for mouse laminin α4, uniformly stained the mesenchymal cell compartment (Fig. 8I). In well-differentiated Mm/Ce hybrid intestines, where smooth muscle layers were clearly defined, distinct laminin α4 staining occurred around the individual muscle cells. No deposition of laminin α4 was evident at the level of the subepithelial basement membrane (Fig. 8I). The inverse associations, Cm/Me, were not stained by antibody 341 at all developmental stages examined (Fig. 8F).

Comparative Analysis of Laminin α5 Chain Expression and Smooth Muscle Myosin

The progressive preferential expression of laminin α5 in association with the smooth muscle cells of the inner circular layer may be indicative of differences in the differentiation state of the inner circular and the outer longitudinal smooth muscle layers. To test this possibility double immunofluorescence was performed with antibodies against smooth muscle myosin, a smooth muscle marker, and the laminin α5 chain. In the adult mouse intestine, in contrast to the nonhomogeneous staining obtained with the laminin α5 chain antibody, myosin was detected at similar intensities in both inner and external muscle layers (Figs. 9A and 9B). This difference between myosin and laminin α5 staining was also apparent in the Mm/Ce hybrid intestines, in particular in 17-day-old grafts (Figs. 9C and 9D). In these grafts, bright myosin staining occurred throughout the muscle layers, whereas laminin α5 staining was apparent

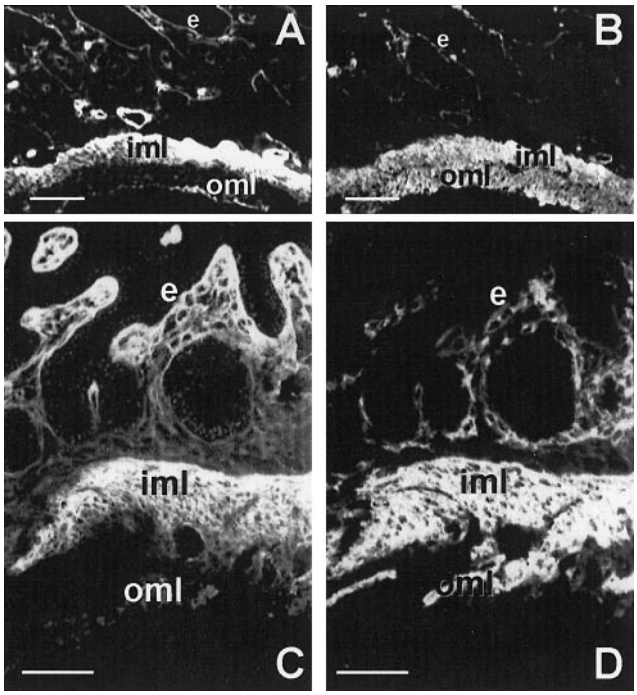


FIG. 9. Double staining of adult mouse intestine (A and B) and a mouse mesenchyme/chick endoderm association developed as an intracoelomic graft for 17 days, using antibodies recognizing the laminin α5 chain (A and C) and smooth muscle myosin (B and D). Note that in both samples, the outer longitudinal cell layer is only weakly stained with anti-laminin α5 chain antibodies, compared to myosin antibodies. e, endoderm or epithelium; iml, inner muscular cell layer; oml, outer muscular cell layer. Bars, 50 μm.

only within the inner muscle coat. Similarly, in the lamina propria, the myosin-positive subepithelial myofibroblasts found at the base of the protruding villi were not stained by the anti-laminin α5 antibody.

DISCUSSION

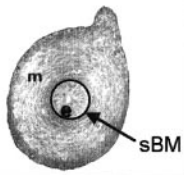
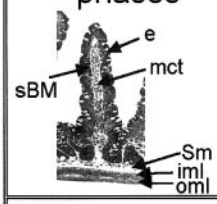
Using *in situ* analyses and an *in vitro* chick–mouse intestinal model, we show here that laminin α2, α4, and α5 chains display distinct expression patterns in the intestine which vary with development. The data also strengthen the view that the subepithelial basement membrane is composed of molecules that are produced by different cell types. The major findings are compiled in Fig. 10. The reciprocal induction of mesenchymal cells by epithelium, and vice versa, via specific signals seems to control the sequential synthesis of individual basement membrane components and, hence, the progressive onset of the intestinal steady state.

Among the three laminin α chains studied, the laminin α5 chain showed the broadest distribution in the intestine. This is consistent with the view that the laminin α5 chain is one of the major laminin α chains in various mouse organs (Miner *et al.*, 1995; Sorokin *et al.*, 1997a,b). In support of this, multiple defects were observed in the *Lam a5* $-/-$ homozygotes, including failure of anterior neural tube closure, failure of digit septation, and dysmorphogenesis of the placental labyrinth (Miner *et al.*, 1998). A major finding in the present study was that laminin α5 chain is expressed throughout embryonic and perinatal life in the intestine, being deposited into the basement membrane by both epithelial and mesenchymal cells. The previously reported absence of immunoreactivity for the laminin β2 chain in intestinal villus basement membranes (Lohi *et al.*, 1996) suggests that only laminin-10 ($\alpha5\beta1\gamma1$) is present in this region. The concept that laminin α1 chain is essential for epithelial cells at early stages of development, whereas laminin α5 predominates in more mature epithelial cells, emerges from data from various organs (Durbeej *et al.*, 1996; Sorokin *et al.*, 1997a,b). In the intestine, the situation is somewhat different as both laminin α1 and α5 chains are expressed at early stages of development. Immunocytochemical studies have localized the laminin α1 chain to the intestinal basement membrane underlying epithelial cells during the developmental period and crypt epithelial cells in the mature organ. The fact that laminin α1 is replaced gradually by laminin α5 along the adult villi may reflect a role for laminin-10 in the maintenance of the fully differentiated phenotype, while the laminin α1 chain is more likely to be important for the polarization of epithelial cells, as has been shown for other epithelial cell types (Klein *et al.*, 1988; Matter and Laurie, 1994; Kadoya *et al.*, 1995; De Arcangelis *et al.*, 1996).

Northern blot and *in situ* hybridization revealed that laminin α5 mRNA expression is highest during the late gestational period, correlating with a phase of intense intestinal morphogenetic events, i.e., villus emergence and smooth muscle cell differentiation. Although the immunocytochemical signals were strong in the adult organ (especially for the laminin α5 chain), only faint hybridization signals were observed in Northern blot analysis for the three laminin α chains in the adult intestine compared to embryonic intestines. This indicates that the subepithelial and the smooth muscle basement membranes are rather stable structures once organogenesis is complete. A higher expression of basement membrane molecules during the developmental period, compared to the postnatal and adult stages, is a general phenomenon that has also been noted for collagen IV (Simon-Assmann *et al.*, 1990) and laminin-1 (Simo *et al.*, 1991). Yet, while the laminin α5 protein is synthesized and deposited into the subepithelial basement membrane already during early phases of development, the onset of laminin α2 chain expression is apparent only when crypt formation occurs around birth (Simon-Assmann *et al.*, 1994), suggesting a role for laminin α2 in this morphogenetic event.

The use of interspecies recombinants allows conclusions to be made about the contributions of different cellular compartments to basement membrane formation. Previous

A

		α2 chain	α4 chain	α5 chain
Undifferentiated stages 	e	-	-	+
	sBM	-	-	+
	m	+	+	+
Morphogenetic phases 	e	-	-	-
	sBM	+ (crypts)	-	+
	mct	+ (crypt primordia)	+	+ (++: villus tip)
	Sm	-	-	-
	iml	±	+	++
	oml	±	+	±

B

sBM deposition		α2 chain	α5 chain
early stages	e	-	+
	m	+	+
later stages	e	-	-
	m	+	+

C

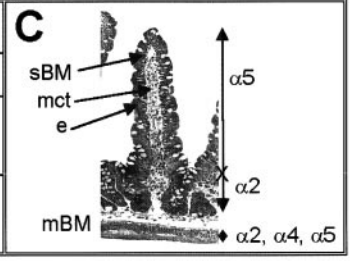


FIG. 10. Summary of the main results obtained by *in situ* hybridization and immunodetection of laminin α2, α4, and α5 chains in the undifferentiated intestine and in the intestine undergoing morphogenetic movements (A). (B) A recapitulation of the cell compartments responsible for the deposition of laminin α2 and α5 into the subepithelial basement membrane, as determined by immunocytochemical analysis of interspecies hybrid intestines at early and late stages of development. (C) The laminin α chains present at the subepithelial and muscle basement membranes in the adult mouse intestine. e, endoderm or epithelium; m, mesenchyme; mct, mucosal connective tissue; Sm, submucosa; iml, inner (circular) muscle layer; oml, outer (longitudinal) muscle layer; sBM or mBM, subepithelial or muscle cell basement membrane.

data from our laboratory (Simo *et al.*, 1992) allowed us to conclude that the β1 and γ1 chains are deposited in the subepithelial basement membrane by both epithelial and mesenchymal cells whatever the developmental stage. Here, we show that, at early stages, the α5 chain, characteristic of laminin-10, is secreted by both epithelial and mesenchymal cells while laminin-1, identified by its α1 chain, is deposited as shown in Simo *et al.* (1992) by epithelial cells only. Later in development, once villi have formed, the deposition of laminin α5 into the subepithelial basement membrane is attributable mainly to the mesenchymal cells. Concurrently, other laminin chains, such as laminin α1 and γ2 chains, are triggered to be deposited into the subepithelial basement membrane by the mesenchymal compartment (Simo *et al.*, 1992; Orian-Rousseau *et al.*, 1996). The situation for laminin-2, characterized by the α2 chain, is somewhat different. The present observations emphasize the exclusive production of the laminin α2 chain by mesenchymal cells. This contrasts with the data obtained by Perrault *et al.* (1998) who showed the presence of laminin α2 mRNA in human intestinal epithelial cells after dissociation from the underlying mesenchymal cells. Yet, various studies based on *in situ* hybridization have shown that the expression of the laminin α2 chain mRNA is confined to cells of mesenchymal origin in several organs (Vuolteenaho *et al.*, 1994; Sorokin *et al.*, 1997b). In addition, the restricted deposition of the laminin α2 chain into the basement membrane in the crypt region is probably due to unique synthetic properties of the myofibroblasts present in this region which also express α and γ smooth muscle actins (Kedinger *et al.*, 1990) and myosin (Joyce *et al.*, 1987; Plateroti *et al.*, 1998). Similarly, in lung, myofibroblasts have been implicated as major laminin α2-producing cells (Flores-Delgado *et al.*, 1998).

The analysis of the chimeric intestines composed of mouse mesenchyme and chick intestinal endoderm re-

vealed surprising differences from the *in vivo* laminin α2 expression pattern. First, the laminin α2 chain was precociously expressed before crypt formation and, second, α2 immunoreactivity was no longer restricted to the crypt region but extended along the villus epithelium. One can hypothesize that in the intestine *in vivo* the fetal endodermal cells, as well as the differentiated villus epithelial cells, exert a repressive effect on the mesenchymal expression of laminin α2 chain, which is lifted when the mouse intestinal endoderm is replaced by the chick endoderm. Such species differences in the inductive properties of human versus animal intestinal mesenchymal cells on endodermal cells have been reported previously (Lacroix *et al.*, 1984).

Like laminin α2, laminin α4 is a predominantly mesenchymal cell product. Comparison of interspecies intestine chimera and the *in vivo* data show that epithelial cells are clearly negative for laminin α4 expression in the intestine. This laminin chain is expressed by mesenchymal cells, probably endothelial cells (Frieser *et al.*, 1997) and enteric neuroblasts within villi. Laminin α4 is also strongly expressed by the smooth muscle cells of the intestine at all stages of development examined and in the mature organ, where it colocalizes with laminin α2 and laminin α5 mostly in the inner muscle layer. Laminin α4 has been shown to be expressed in the peripheral nervous system (Frieser *et al.*, 1997; Iivanainen *et al.*, 1997). It is therefore probable that the widespread expression of laminin α4 mRNA in the mesenchyme is associated with different functions such as innervation of the intestine, development of smooth muscle cells, and blood vessel maturation.

Few studies have been conducted to define the laminin isoforms present in the developing and mature intestinal smooth muscle cells whose role in the overall physiology of the gastrointestinal tract is determinant. So far the presence of α1, β1, β2, and γ1 laminin chains has been found in smooth muscle of mouse and human intestines (Simo *et al.*, 1991; Glukhova *et al.*, 1993; Lohi *et al.*, 1996). Interestingly, an increased expression of laminin-1 in the smooth muscle layers observed in the aganglionic intestine of *ls*/*ls* mice may account for the failure of neural crest cells to complete their colonization of the colon (Rothman *et al.*, 1996). In the present paper we provide evidence that intestinal smooth muscle cells contain multiple laminin α chains including α2, α4, and α5. Immunocytochemistry revealed a progressive increase in staining intensity for all three laminin α chains that parallels the differentiation of certain mesenchymal cells into smooth muscle cells. The most interesting finding is that while laminin α2 and α4 chains are equally expressed in both smooth muscle layers, expression of laminin α5 predominates in the inner (circular) smooth muscle layer. Particularly noteworthy is the fact that no smooth muscle cell marker (smooth muscle α actin and myosin) or basement membrane molecule studied to date shows such an imbalanced deposition between the two cell layers (Simon-Assmann *et al.*, 1986; Kedinger *et al.*, 1990). This argues for heterogeneity in the smooth muscle cell phenotype similar to that described for fibroblasts in the intestinal lamina propria (Fritsch *et al.*, 1997, 1999). A down regulation of laminin α5 expression with development has been reported for skeletal and cardiac muscle, implicating this molecule in myogenesis (Sorokin *et al.*, 1997b). It may be, therefore, that the selective absence of laminin α5 from the outer (longitudinal) smooth muscle layer plays a role in the differentiation and/or maturation of this muscle layer.

In the intestine, the presence of multiple laminin variants whose expression is developmentally regulated suggests that each variant has a specific role in differentiation/proliferation and migration events during intestinal morphogenesis. The differential expression of laminin α5 chain is of particular interest and could provide a basis for the study of the regulators implicated in the myogenic program. Furthermore, on the basis of the knowledge of the precise cell expression of the various laminin α chains gained by the present analysis, our goal is now to create models that will allow analysis of the role of specific laminin isoforms *in vivo* by intestine-specific gene targeting.

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