

Inhibition of Laminin α 1-Chain Expression Leads to Alteration of Basement Membrane Assembly and Cell Differentiation

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Abstract. The expression of the constituent α 1 chain of laminin-1, a major component of basement membranes, is markedly regulated during development and differentiation. We have designed an antisense RNA strategy to analyze the direct involvement of the α 1 chain in laminin assembly, basement membrane formation, and cell differentiation. We report that the absence of α 1-chain expression, resulting from the stable transfection of the human colonic cancer Caco2 cells with an eukaryotic expression vector comprising a cDNA fragment of the α 1 chain inserted in an antisense orientation, led to (a) an incorrect secretion of the two

other constituent chains of laminin-1, the β 1/ γ 1 chains, (b) the lack of basement membrane assembly when Caco2-deficient cells were cultured on top of fibroblasts, assessed by the absence of collagen IV and nidogen deposition, and (c) changes in the structural polarity of cells accompanied by the inhibition of an apical digestive enzyme, sucrase-isomaltase. The results demonstrate that the α 1 chain is required for secretion of laminin-1 and for the assembly of basement membrane network. Furthermore, expression of the laminin α 1-chain gene may be a regulatory element in determining cell differentiation.

BASEMENT membranes (BM)¹ are specialized, sheet-like extracellular matrices dividing tissues into compartments; they form the supporting structure on which epithelial cells lie. BM consists of ubiquitous components: type IV collagen, laminin, nidogen (also known as entactin), and heparan sulfate proteoglycan (perlecan) that are secreted locally by epithelial or parenchymal as well as fibroblastic cells. The BM functions as a dynamic structure in tissular morphogenesis, differentiation, and maintenance of the mature structural and functional steady states; its constituent molecules are able to regulate different types of cell behavior such as adhesion, proliferation, and maintenance of cell polarity either directly or via the delivery of growth/migration signals.

Laminin-1, a major constituent of the basement membranes, is the earliest molecule produced in embryogenesis; its potential importance has been largely stressed in the last decade (Timpl and Brown, 1994). Indeed, laminin-1, as well as type IV collagen, has been shown to self-assemble,

leading to independent networks favoring the hypothesis that at least one of these molecules will define the basic scaffold structure of BMs (Yurchenco and Schittny, 1990; Yurchenco et al., 1992). Laminin-1 is a heterotrimeric glycoprotein isolated in 1979 by Timpl et al. from the murine Engelbreth-Holm-Swarm (EHS) tumor. The three chains (α 1, β 1, and γ 1) are encoded by three different genes (*LAMAI*, *LAMBI*, *LAMCI*) located on different chromosomes. Several major functions have been assigned to the α 1 chain (for reviews see Paulsson, 1992; Yurchenco et al., 1993; Timpl and Brown, 1994), among which are polarization of developing tubules (Klein et al., 1988), lung alveolar formation (Matter and Laurie, 1994), and differentiation in the mammary gland system (Streuli et al., 1995). These signal effects could be driven by binding proteins such as integrins known to interact with the COOH-terminal end of the α 1 chain (Deutzmann et al., 1990; for review see Timpl and Brown, 1994). A potential role of this latter domain in the assembly of BM molecules is supported by the ability of fragment E3 (distal end of the globular COOH-terminal domain) to bind BM heparan sulfate proteoglycan (Battaglia et al., 1992; Sorokin et al., 1992).

The intestine represents a fascinating dual model of proliferation, commitment, and differentiation during development and in the continuously renewing adult organ (for reviews see Henning, 1994; Kedinger, 1994; Madara and Trier, 1994). During the last few years, several major characteristics of BM molecular localization and composition

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1. *Abbreviations used in this paper:* AS, antisense; BM, basement membrane; DPPIV, dipeptidylpeptidase IV; EHS, Engelbreth-Holm-Swarm; LN, laminin; RT, reverse transcription.

in the gut have been defined (for review see Simon-Assmann et al., 1995). The following observations stress the potential importance of laminin-1 in gut morphogenesis and in the maintenance of the adult steady state between proliferation and differentiation compartments. A peak of laminin-1 production occurs during the period of villous formation as rat intestinal development proceeds with a concomitant rise of the relative proportion of the $\alpha 1$ chain versus $\beta 1/\gamma 1$ chains (Simo et al., 1991). The $\alpha 1$ chain is secreted solely by epithelial cells during the initial establishment of BM that precedes epithelial cell differentiation, as opposed to $\beta 1/\gamma 1$ chains that are secreted and deposited by both epithelial and mesenchymal cells (Simo et al., 1992a).

To determine the biological role of laminin more directly, we have designed an antisense RNA strategy to stably block the production of laminin $\alpha 1$ chain in epithelial cells; this will allow us to analyze consequences on the long term behavior of the cells. As established intestinal cell lines depicting the normal characteristics of *in situ* cells do not exist at present, we used in this study human colonic cancer cells. The key observation underlying this work is that colonic cancer cell lines differing in their differentiation potential exhibit various adhesion properties to fibroblasts concomitantly with differences in BM formation (Bouziges et al., 1991). In coculture, the well-differentiated Caco2 cells form a monolayer on the fibroblastic cells; in parallel, laminin-1 and collagen IV are deposited at the heterotypic cell interface. In contrast, the undifferentiated HT29 cells do not spread, but grow as clusters on the fibroblasts and do not form a BM. While the three constituent chains of laminin-1 ($\alpha 1$, $\beta 1$, and $\gamma 1$) are expressed in Caco2 cells, $\alpha 1$ chain is not detected in HT29 cells (De Arcangelis et al., 1994). Therefore, we transfected Caco2 cells with an eukaryotic expression vector comprising a cDNA fragment of the $\alpha 1$ chain inserted in an antisense position under the control of the cytomegalovirus promoter. In the present study, we have addressed the role of $\alpha 1$ chain in laminin assembly, BM formation, and cell differentiation. The results demonstrate (a) the importance of $\alpha 1$ chain in the secretion of laminin-1 molecule, (b) the need of laminin-1 for the subsequent correct assembly of the BM molecules at the epithelial-fibroblastic interface, and (c) the necessity of laminin-1 to complete cell differentiation.

Materials and Methods

Construction of the Antisense Laminin $\alpha 1$ -chain Expression Vector

A 1.4-kb BamHI-HindIII cDNA (Deutzmann et al., 1988) that consisted of the COOH-terminal part of fragment T2 and almost the entire E3 fragment of mouse laminin $\alpha 1$ chain was inserted in the antisense orientation into the BglIII-HindIII sites of the expression vector pCB6 (kindly provided by Dr. Russell, University of Texas, Southwestern Medical Center, Dallas, TX). Restriction enzyme analysis and sequencing by the dideoxy chain-termination method (Pharmacia, Uppsala, Sweden) were used to confirm that the insert was in the correct orientation. The pCB6 vector carries a cytomegalovirus promoter, polyadenylation signal sequences (hGH terminator), and the neomycin phosphotransferase gene (Neo[®]) that confers resistance to the antibiotic G418 (geneticin; Gibco-BRL, Gaithersburg, MD). The recombinant vector was designated pCB6/AS LN (antisense laminin).

Formation of a heteroduplex between human laminin $\alpha 1$ sense RNA and mouse laminin $\alpha 1$ antisense RNA has been tested *in vitro*. Equimolar amount of sense and antisense RNA has been hybridized under stringent conditions (50% formamide, 42°C) in an hybridization buffer used for RNase protection assay (Sambrook et al., 1989). The formation of a heteroduplex was seen after blotting on Hybond membrane (Amersham Intl., Little Chalfont, UK) followed by hybridization with an $\alpha 1$ human cDNA probe ³²P-labeled by random priming (Feinberg and Vogelstein, 1983) (data not shown). This probe corresponds to a 1.6-kb PCR fragment from the 3' part of the $\alpha 1$ laminin cDNA (nucleotides 7715 to 9304) (Haaranta et al., 1991).

Cell Culture and Transfection of Caco2 Cells

The human colon Caco2 cells (Fogh et al., 1977) were cultured in DME (Gibco-BRL) supplemented with 10% heat-inactivated FCS (Gibco-BRL) and 1% nonessential aminoacids (Gibco-BRL). Caco2 cells were transfected by the calcium phosphate precipitation method. Briefly, Caco2 cells were plated on 100-mm culture dishes in the standard culture medium at a density of 2×10^6 cells per dish. The next day at preconfluency, cells were transfected with either pCB6 (controls) or pCB6/AS LN at 10 μ g of DNA per dish prepared as calcium phosphate precipitates. After a 48-h period, selection was started by adding G418 (1.2 mg/ml) to the culture medium. After 2 wk, resistant clones were isolated by capillary duct aspiration, transferred one by one into 96-well plates, and then individually amplified. From passages seven to eight, stably transfected lines were routinely maintained in 600 μ g G418 per ml, a concentration that is toxic for the majority of the cells. For some clones that exhibited a reversion in phenotype, the geneticin concentration was reestablished to 1.2 mg/ml.

Coculture Experiments

This experimental model (Kedinger et al., 1987) permits the study of the interactions between transfected Caco2 cells and fibroblastic cells, and of the establishment of the basement membrane at the interface of both cell populations. Skin fibroblastic cells were obtained by enzymatic dissociation of 20-d-old fetal rat aponeurosis after incubation for 1 h at 37°C in Ca²⁺/Mg²⁺-free Ham's F10 (Gibco-BRL) in the presence of 0.01% trypsin (Calbiochem-Novabiochem Corp., La Jolla, CA) and 0.01% collagenase (CLS 4, 222 U/mg; Worthington Biochem. Corp., Freehold, NJ). Isolated cells were cultured in medium composed of a mixture (1:1) of DME and Ham's F12 (Gibco-BRL) supplemented with 15% heat-inactivated FCS. Subcultures were performed after trypsinization of confluent monolayers in Ca²⁺/Mg²⁺-free PBS with 0.25% trypsin (Gibco-BRL) and 0.02% EDTA for 10–15 min at 37°C. Cells were seeded at a plating density of 2×10^4 cells per cm² and used between the first and third passage. Stable Caco2 clones obtained after transfection with pCB6/AS LN or with the vector pCB6 alone were trypsinized and seeded at 2×10^4 cells on the preformed confluent monolayers of skin fibroblastic cells. Cocultures were maintained in the basic Caco2 culture medium up to 17 d.

Indirect Immunofluorescent Staining

Cultured Cells. Detection of surface or intracellular laminin was performed on transfected cells grown for 7 d on glass coverslips. Cells were fixed with 1% paraformaldehyde in PBS for 10 min. For intracellular detection of laminin, cells were permeabilized with 1% Triton X-100 for 10 min before incubation with the antibody. The two following antibodies have been used: a rabbit polyclonal antibody raised against mouse EHS laminin recognizing the three constituent chains, $\alpha 1$, $\beta 1$ and $\gamma 1$ (Simo et al., 1991) and a mouse anti-human mAb recognizing specifically the $\alpha 1$ -chain laminin (mAb 1924; Chemicon Int., Inc., Temecula, CA). Secondary antibodies were respectively goat anti-rabbit IgG (Nordic Immunological Laboratories, Capistrano Beach, CA) and sheep anti-mouse IgG (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), both FITC labeled. Possible modifications of cell differentiation resulting from the transfection were analyzed after 10 d using differentiation markers such as digestive enzymes, i.e., sucrase and dipeptidylpeptidase IV (DPPIV) detected with mouse mAbs (HBB2/614/88 and HBB3/775/42, respectively, kindly provided by Dr. Hauri [Biozentrum, Basel, Switzerland]) or specific components of brush border cytoskeleton like villin detected with a mouse monoclonal anti-villin antibody (M α villin BDID₂C₃, kindly provided by Dr. Robine [Institut Pasteur, Paris, France]). Secondary antibodies were FITC-labeled sheep anti-mouse IgG (Sanofi Diagnostics Pasteur). Cells were examined with a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY).

Cocultures. Sheets of the cocultures were detached mechanically from the culture dishes, embedded in Tissue-Tek (Labonord, Villeneuve d'Ascq, France), and frozen with isopentane cooled by liquid nitrogen. Transverse 5- μ m cryosections were prepared and incubated with the above-mentioned specific polyclonal anti-laminin, monoclonal anti-laminin α 1 chain, anti-sucrase, anti-DPPIV, anti-villin antibodies, and with polyclonal anti- α 1(I) type IV collagen antibodies (Timpl et al., 1983) or polyclonal anti-type IV collagen antibodies (prepared in our laboratory and proven to be specific of α 1(I) type IV collagen chains by immunoblotting and displaying no cross-reactivity for laminin). Appropriate FITC-labeled secondary antibodies were used for the detection of antigen-antibody complexes.

Immunoprecipitation of Laminin

Cell cultures were radiolabeled for 24 h with 100 μ Ci of the Trans 35 S-labelTM metabolic labeling reagent containing [35 S]L-methionine and [35 S]L-cysteine (ICN Biomedicals, Orsay, France) (sp act 1092 Ci/mmol) in methionine-cysteine-free DME containing 2% FCS after a 1-h starvation in the methionine-free medium. The culture medium was then collected, and protease inhibitors were added (2 mM *N*-ethylmaleimide, 1 mM PMSF). The cells were washed twice in PBS plus protease inhibitors, solubilized in RIPA buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM cysteine, 2 mM methionine, 250 μ M PMSF, 1 mM *N*-ethylmaleimide, 0.5% NP-40, 0.05% Triton X-100, 0.3% sodium deoxycholate, 0.1% BSA, 150 mM NaCl) containing 0.1% SDS, and sonicated. After centrifugation at 330 g (Biofuge; Heraeus-Amerisil, Inc., Sayreville, NJ) for 10 min, the culture medium and the cell extracts were either stored at -80°C or used immediately.

Samples were first preincubated for 1 h at 4°C with protein A-Sepharose (CL-4B; Sigma Chemical Co., St. Louis, MO). In parallel, the affinity-purified polyclonal anti-laminin-1 antibodies were incubated for 1 h at 37°C with the protein A-Sepharose beads. The pellets containing the antibodies bound to the beads were washed twice with RIPA buffer and incubated with the precleared samples at 4°C for 18 h. Immune complexes were washed five times with RIPA buffer and resuspended in Laemmli buffer containing β -mercaptoethanol. Pellets were boiled and subjected to 5% SDS-PAGE gels. The gels were dried down and exposed to medical x-ray films (Fuji Photo Film Co., Ltd., Tokyo, Japan) for at least 3 d at -80°C . Gels were calibrated with ^{14}C -labeled protein standards (Amersham Intl.), and apparent molecular masses were determined by linear scanning with a densitometer (Shimadzu Corp., Kyoto, Japan).

Immunoblot Analysis of Villin

Cells grown up to confluency (7 d) were washed in PBS containing 1 mM PMSF. Proteins were extracted with the following buffer: 0.01 M Tris, pH 7.4, 0.1% Triton X-100, 1 mM PMSF, 0.1% β -mercaptoethanol. After sonication, proteins were fractionated by SDS-PAGE analysis (7% gels) and then subjected to Western blotting using polyclonal antibodies to villin (Robine et al., 1985). After incubation with affinity-purified alkaline phosphatase-conjugated secondary antibody, the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate toluidine salt/*p*-Nitroblue tetrazolium chloride-color reagents.

Morphological Analysis

For conventional histology and transmission EM, specimens were fixed 1 h at 4°C in 0.2 M cacodylate-buffered 2% glutaraldehyde, pH 7.4, postfixed in cacodylate-buffered 1% osmium tetroxide, pH 7.4, for 30 min at 4°C , dehydrated, and embedded in araldite. Semithin 0.5- μ m sections were stained with toluidine blue for histological observation. Ultrathin sections were stained with uranyl acetate and lead citrate before ultrastructural observation using an electron microscope (CM-10; Philips Electronic Instruments, Mahwah, NJ).

For scanning electron microscope analysis, transfected cells were grown for 10 d on Thermanox coverslips. Specimens were fixed in 0.2 M cacodylate-buffered 2% glutaraldehyde, pH 7.4, for 1 h at 4°C . They were then dehydrated, dried in a critical-point drier, and coated with gold using a sputter coater (Balzers; Hudson, NH). The specimens were examined with a scanning electron microscope (501B; Philips Electronic Instruments).

Genomic DNA Preparation and PCR Amplification

Genomic DNA was prepared from confluent cultures of various transfected clones (including AS and controls); cells were washed twice with

PBS and then treated with lysis buffer (50 mM Tris HCl, pH 7.5, 5 mM EDTA, 0.2 M NaCl, 1% SDS, 250 μ g/ml proteinase K) overnight at 60°C . The lysate was then extracted twice with phenol, twice with phenol/chloroform, and once with chloroform. The DNA was precipitated by ethanol and resuspended in sterile water.

3 μ g of each purified genomic DNA extract was used in a standard 100- μ l PCR assay (Gibco-BRL). The endogenous laminin α 1 gene was detected as control using a pair of oligonucleotides specific of the human α 1-chain sequence (Haaparanta et al., 1991), designated LN3 (5'-AGGACTCG-GTCCCAGGACAG-3') for the 3' primer and LN4 (5'-GTTGGCGCT-GAAAGTCCACACA-3') for the 5' primer, respectively. These sequences were designed to span at least one intron by comparison with the mouse genomic sequence.

The antisense cDNA fragments potentially inserted into the cellular genome after transfection were searched using two specific primers of the mouse α 1-chain sequence (Deutzmann et al., 1988). The 3' and 5' primers were designated LN5 (5'-TACAAAGTTCGATTGGATTTA-3') and LN2 (5'-TTGCCATCACAGAGAGCTCTG-3'), respectively. The predicted length of the PCR product is 206 bp. The reaction mixture was heated at 94°C for 2 min. The amplification in the presence of Taq DNA polymerase (Gibco-BRL) for both endogenous laminin α 1 gene and antisense α 1 cDNA consisted of 32 cycles of denaturation for 30 s at 94°C , annealing for 30 s at 60°C for the endogenous laminin α 1 gene and at 51°C for the antisense cDNA, and extension for 1 min at 72°C in a DNA thermal cycler apparatus (Eppendorf North America, Inc., Madison, WI). At the completion of the PCR run, 16 μ l of each reaction mixture was analyzed on a 3% agarose gel.

Southern Blot Hybridization Analysis

DNA isolation, restriction endonuclease digestion, 1% agarose gel electrophoresis, and Southern blotting onto Hybond N membrane (Amersham Intl.) were all performed as described by Sambrook et al. (1989). Southern blots were hybridized under stringent conditions (50% formamide, 42°C) with the mouse laminin α 1 cDNA (used for antisense experiment) or with the human laminin α 1 cDNA probe (as described previously) ^{32}P -labeled by random priming (Feinberg and Vogelstein, 1983). Washings were performed in $2\times$ SSC, 0.1% SDS at 22°C , followed by $0.1\times$ SSC, 0.1% SDS at 55°C .

RNA Preparation and Reverse Transcription (RT) PCR Analysis

Cells were grown up to 7 d on 60-mm tissue-culture dishes, and total cellular RNA was extracted using the method of Peppel and Baglioni (1990). Briefly, after washing, cells were lysed in a solution composed of 2% SDS, 200 mM Tris-HCl, pH 7.5, and 1 mM EDTA. DNA and proteins were precipitated for 2 min on ice by adding an ice-cold potassium acetate solution (42.9 g potassium acetate, 11.2 ml acetic acid, and water to 100 ml). Samples were centrifuged (5 min, 12,000 g), and supernatant was extracted twice with chloroform/isoamyl-alcohol (24:1). RNA was precipitated with ice-cold isopropanol for 10 min on ice and collected by centrifugation for 5 min. The pellet was washed with 70% ethanol, dried, and resuspended in sterile water. To exclude the possibility of genomic DNA contamination, RNA was treated for 30 min at 37°C with RNase-free DNase I (1U DNase/ μ g RNA; Boehringer Mannheim, Mannheim, Germany).

cDNA synthesis by reverse transcription was performed according to the manufacturer's recommendations (Promega, Biotec, Madison, WI). For the antisense RNA detection, the specific oligonucleotide LN5 (sequence as defined above) from the murine α 1-chain sequence was used. Reverse transcription of α 1-chain sense mRNA was realized with oligo(dT)₁₇ primers. 3 μ g of purified total RNA from the different clones was incubated 5 min at 70°C in a final volume of 25 μ l reaction mixture containing 5 μ l of avian myeloblastosis virus-RT reaction $5\times$ buffer, 8 μ l of a 10 mM mix of all four deoxynucleotide triphosphates and 50 pmol of LN5 or oligo(dT)₁₇ primer. 10 U per reaction of avian myeloblastosis virus-RT was added to the reaction mixture and incubated at 42°C for 60 min. Samples were next heated at 94°C for 5 min and chilled quickly on ice.

The PCR amplification step was as detailed previously for the genomic DNA. 2 μ l of the resulting cDNA of the reverse-transcribed RNAs was used with 50 pmol of each of the appropriate 3' and 5' primers in the standard 100- μ l PCR assay. The two specific primers LN5 and LN2 of the mouse α 1 chain sequence were used for the antisense cDNA amplification. The predicted length of the PCR product is, as before, 206 bp. Sense cDNA were detected using the specific oligonucleotides LN3 and LN4 of

the human $\alpha 1$ -chain sequence. The calculated size of the PCR product is 235 bp.

To quantify the antisense and $\alpha 1$ mRNA levels, a competitive PCR method was realized according to Förster (1994). In this technique, an internal standard DNA fragment was generated from the cDNA to be quantified and subcloned into the pGEM-T vector (Promega Biotec). In the following step, this fragment was used as a competitor of the cDNA, its amplification requiring the same primers but yielding a shorter PCR product. The sizes of the antisense and sense competitors were 135 and 160 bp, respectively. Constant amounts of the cDNA products were coamplified with serial dilutions of the competitor. The competition was realized using the ThermojeT[®] apparatus (Eurogentec, Seraing, Belgium). Resultant PCR products were analyzed by electrophoresis on a 3% agarose gel. When both the internal standard and the PCR product to be quantified show equivalent band strength on the gel, they contain approximately the same copy number.

Results

Human colonic adenocarcinoma Caco2 cells were transfected with the expression vector pCB6/AS LN designed to produce the antisense RNA from a cDNA corresponding to the COOH-terminal part of the mouse laminin-1 $\alpha 1$ chain. In parallel, transfection of Caco2 cells with the pCB6 vector alone was performed as control. 2 wk after selection with G418, a total of 192 AS clones and 96 control clones were isolated and transferred into 96-well plates. Only 37 AS clones from the 192 could be further amplified. All of these AS clones were screened, as well as eight control clones.

General Characteristics of the Antisense-transfected Caco2 Cells

In the course of cell amplification, the clones exhibited variable growth kinetics as first exemplified by the time needed to attain the fifth passage. 10 AS clones and all controls reached the fifth passage between 40 and 54 d. The remaining 27 antisense-laminin $\alpha 1$ -chain transfected clones were characterized by a slower rate of division, since they reached the fifth passage only between 68 and 80 d. Fig. 1 shows representative growth curves of three stably transfected Caco2 clones being part of the 27 slowly growing AS clones (AS 12, 40, and 51) from passage 5 onward, as compared to pCB6 controls. Illustration of these three AS clones has been provided, given their interesting

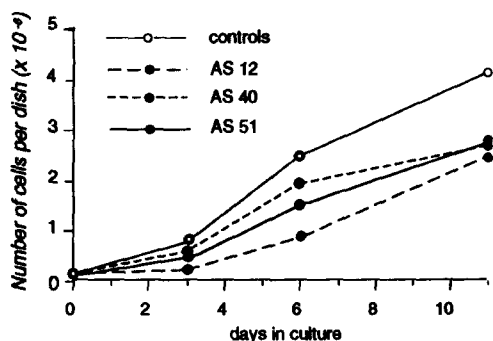


Figure 1. Cell growth of transfected cells. Proliferation of three antisense laminin $\alpha 1$ -chain transfected clones (AS12, AS40, and AS51) as compared to clones transfected with the pCB6 vector alone (mean value of five different clones). Cells were seeded at 0.15×10^6 per dish. Data represent the mean of duplicate cultures that were counted on days 3, 6, and 11 after plating.

characteristics detailed in the following sections. The slower growth rate of the $\alpha 1$ -deficient clones can be related to data obtained by Schuger et al. (1995) who demonstrated that an mAb directed against the cross region of the laminin $\alpha 1$ chain inhibited proliferation of epithelial cells selectively.

The morphology of the various AS clones was quite similar to that depicted by control cells. At confluency, three distinct aspects could be distinguished (Fig. 2): (a) monolayer of regular polygonal cells (Fig. 2, A and D), (b) presence of intra- or intercellular vacuoles that conferred a very irregular arrangement to the cell cultures (Fig. 2, B and E), and (c) areas of cells displaying loose cell-cell and cell-substrate contacts (Fig. 2, C and F).

Inhibition of Laminin $\alpha 1$ Chain Expression Leads to an Altered Secretion of $\beta 1/\gamma 1$ Chains

The expression of $\alpha 1$ chain was first analyzed in the various clones by indirect immunofluorescence. Intracellular and extracellular $\alpha 1$ chain was visualized using a specific monoclonal anti-human laminin $\alpha 1$ antibody on cells cultured on glass coverslips for 7 d. Among the 37 antisense

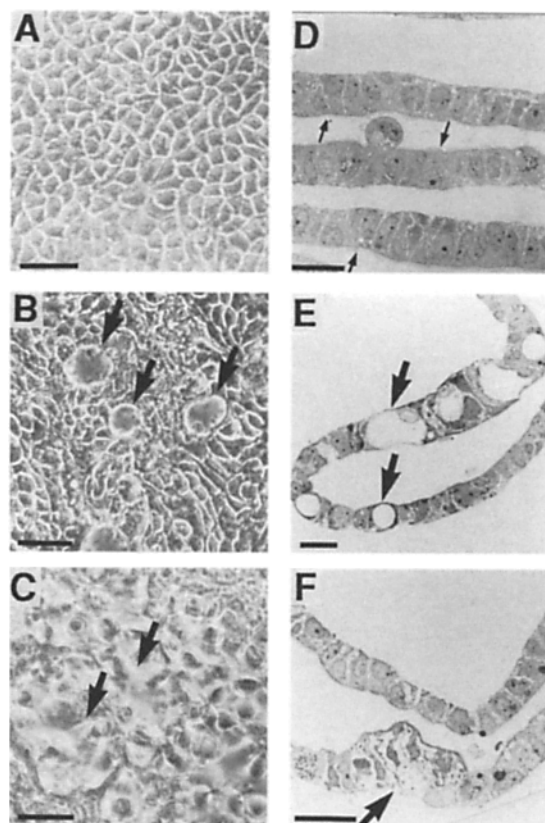


Figure 2. Morphological aspect of transfected cells at confluency. (A–C) Phase-contrast microscopy and (D–F) semithin sections of Caco2 clones depicting various aspects of the culture. (A and D) Monolayer of regular polygonal cells; polarization is assessed by the presence of apical microvilli (arrows in D). (B and E) Monolayer of cells in which intercellular or intracellular vacuoles (arrows) are found. (C and F) Some clones are characterized by focal loosening of cells from the culture dish (arrows in C); note the elongated aspect of some cells associated with important intercellular dilatations within the monolayer (arrows in F). Bars: (A–C) 50 μm ; (D–F) 30 μm .

clones tested, three exhibited the expected phenotype that is repression of α 1-chain expression. Indeed, in contrast with all controls and the majority of antisense cells where laminin α 1-chain staining appeared located in the peripheral cytoplasm of the cells (Fig. 3 E) and in the intercellular spaces (Fig. 3 F), no labeling was detected for the three antisense clones designated AS12, AS40, and AS51 (Fig. 3, A and B). For these three clones, it was noted that the maintenance of the cells in a culture medium containing 0.6 mg/ml of G418 (instead of 1.2 mg/ml) from passage 7 onward led to a reexpression of the α 1 chain. By increasing the amount of the drug used for selection back to 1.2 mg/ml, we were able to restore the original phenotype.

The various transfected clones were examined for the

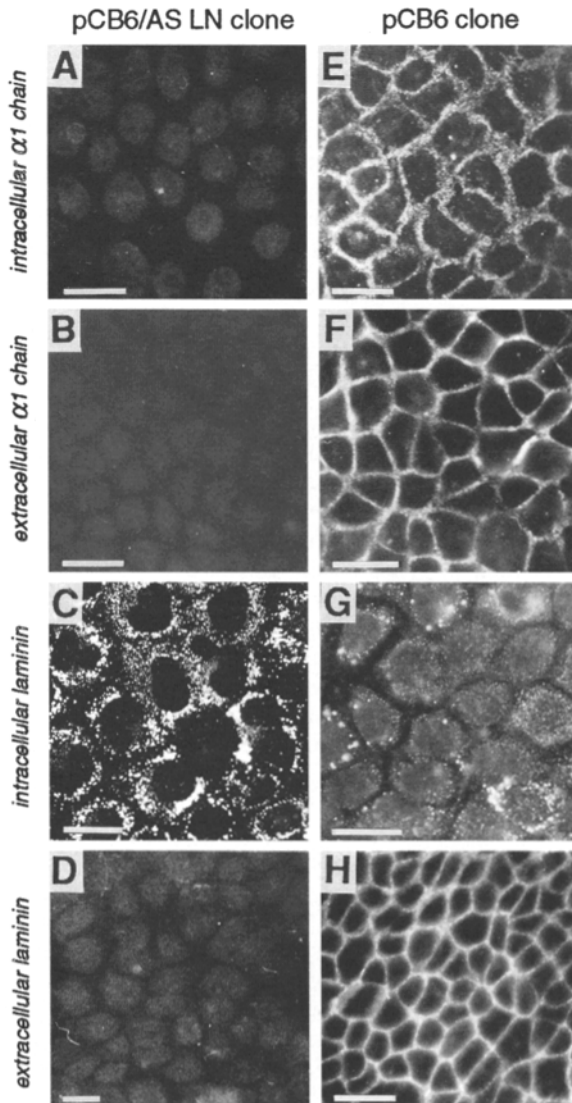


Figure 3. Expression of the constituent chains of laminin-1. Immunofluorescent staining on Triton X-100 permeabilized cells (A, C, E, and G) or on the cell surface (B, D, F, and H) with a monoclonal anti-human laminin α 1 antibody (A, B, E, and F) or with a polyclonal anti-laminin-1 antibody (C, D, G, and H). Representative pictures showing the absence of α 1 chain (A and B), the intracellular accumulation (C), and the lack of extracellular labeling of β 1/ γ 1 chains in a pCB6/AS LN clone as compared to cells transfected with the pCB6 vector alone (E–H). Bars, 30 μ m.

presence of the other laminin-1 constituent chains, the β 1/ γ 1 chains, by indirect immunofluorescence using a polyclonal anti-laminin-1 antibody. AS12, AS40, and AS51 which had no detectable α 1 chains, contained extensive intracellular deposits of β 1/ γ 1 chains (Fig. 3 C); the staining was obvious within the whole cytoplasm of the cells as a granular fluorescent pattern that was significantly higher than in control cells (Fig. 3 G). Immunolabeling on non-permeabilized α 1-negative cells did not allow detection of extracellular laminin material (Fig. 3 D). On the contrary, in the control cells, laminin deposits formed a honeycomb pattern that appeared to outline the intercellular spaces (Fig. 3 H). These results show that the absence of α 1 chain was accompanied by the lack of extracellular staining of the two other constituent chains of laminin-1.

The expression of the various chains was further assessed by immunoprecipitation using the affinity-purified antiserum against laminin-1 (Fig. 4). In a control pCB6 clone (Fig. 4, lanes 1 and 5), the α 1 chain (\sim 350 kD) and the two β 1/ γ 1 chains migrating as a doublet (215–200 kD) could be detected in the cell fraction and in the culture medium; in addition, a band migrating at \sim 280 kD was co-precipitated corresponding to a nonspecific product (see Fig. 4, lanes 4 and 8) also found in other cell lines (Sorokin et al., 1994). The smaller size of the α 1 subunit as compared to that present in the murine EHS tumor (\sim 400 kD) was also obtained by immunoblot experiments (not shown); this observation has also been noticed in Caco2 cells by Vachon and Beaulieu (1995).

For the AS12 clone, in which α 1-chain immunostaining was absent, only the β 1/ γ 1 chains were synthesized and expressed in the cell fraction (Fig. 4, lane 3). In the AS12 culture medium, no distinct bands were found that might cor-

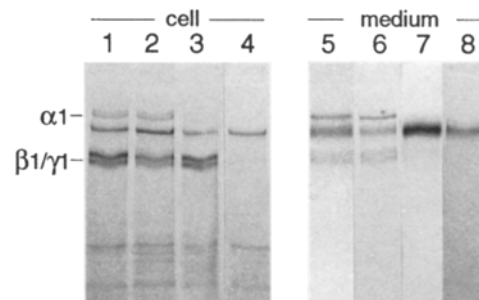


Figure 4. Detection of laminin-1 constituent chains by immunoprecipitation. After a 24-h metabolic labeling period, cell extracts (lanes 1–4) and conditioned culture media (lanes 5–8) from a control clone transfected with the pCB6 vector alone (lanes 1 and 5) and from two clones transfected with the pCB6/AS LN vector (AS22, lanes 2 and 6; AS12, lanes 3 and 7) were processed for immunoprecipitation with the polyclonal anti-laminin-1 antibody (lanes 1–3 and 5–7) or with a nonimmune serum (lanes 4 and 8). Samples were reduced and subjected to 5% SDS-PAGE. Protein bands were visualized by fluorography. These immunoprecipitation experiments demonstrated that the AS12 clone synthesized only the β 1/ γ 1 chains, but no laminin α 1 chain, in contrast with AS22 cells and with the control pCB6 clone. Note that the β 1/ γ 1 chains that migrate as a doublet at 215–200 kD were present within the AS12 cells (lane 3), but not found secreted into the culture medium (lane 7). The nonspecific precipitated band migrating at \sim 280 kD (lanes 4 and 8) was not detected by immunoblot analysis (not shown).

respond to $\alpha 1$ or $\beta 1/\gamma 1$ chains (Fig. 4, lane 7). The AS22 clone that depicted $\alpha 1$ immunostaining presented the same immunoprecipitation profile as the control pCB6 clone (Fig. 4, lanes 2 and 6).

Together these data reveal that the absence of $\alpha 1$ chain does not modify the synthesis of the $\beta 1/\gamma 1$ chains but leads to the lack of secretion of the dimeric $\beta 1/\gamma 1$ complexes.

Differential Expression of $\alpha 1$ -Chain Sense and Antisense RNAs in Transfected Cells

The insertion of the $\alpha 1$ antisense cDNA into the cellular genome of AS transfected clones was checked by analysis of the genomic DNA from some AS and control cell lines by PCR using specific primers, LN2 and LN5, of the $\alpha 1$ antisense sequence. Results depicted in Fig. 5 demonstrate the presence of the antisense transgene in AS12 and AS51 clones as assessed by the expected 206-bp signal (Fig. 5, lanes 6 and 7), whereas no band was detected in pCB6 clones (Fig. 5, lanes 8 and 9). The endogenous laminin $\alpha 1$ gene selected as a control using the specific oligonucleotides LN3 and LN4 was revealed in both AS and pCB6 clones, yielding the same signal whatever the clone examined (Fig. 5, lanes 1–4). In addition, Southern blot hybridization of EcoRI- or BamHI-digested genomic DNA from antisense transfected clones, using a 1.4-kb mouse or a 1.6-kb human laminin $\alpha 1$ probe, demonstrated the random integration of the antisense construction (data not shown).

RT-PCR analysis of total RNA extracted from various transfected Caco2 clones was carried out to define the relative expression of the laminin $\alpha 1$ chain mRNA and to search for the $\alpha 1$ antisense RNA. Results demonstrated that $\alpha 1$ -chain mRNA, detected with the specific 5' and 3' primers LN3 and LN4, was present in both AS and control cell lines. However, in contrast with pCB6 control cells that exhibited a rather constant signal of the expected 235-bp PCR product (Fig. 6 A, lanes 10–12), AS clones showed variable $\alpha 1$ mRNA expression (Fig. 6 A, lanes 1–8). For the three clones AS12, AS40, and AS51, in which $\alpha 1$ -chain

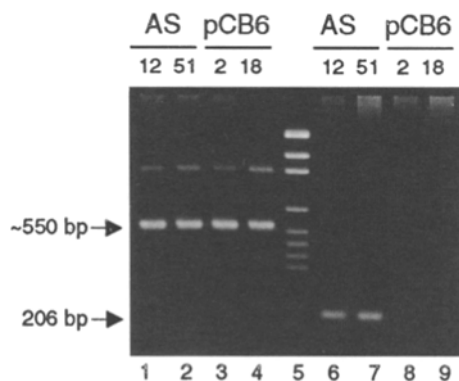


Figure 5. Integration of the $\alpha 1$ antisense transgene into the AS clones genome. PCR amplification of endogenous laminin $\alpha 1$ gene (lanes 1–4) and of $\alpha 1$ antisense transgene (lanes 6–9) was performed using the specific primers, described in Materials and Methods, on the genomic DNA of AS12 (lanes 1 and 6), AS51 (lanes 2 and 7), and control (lanes 3, 4, 8, and 9) clones. PCR products were electrophoresed through a 3% agarose gel. Arrows indicate PCR products amplified respectively from the endogenous laminin $\alpha 1$ gene (~550 bp) and from the $\alpha 1$ antisense transgene (206 bp). (Lane 5) pGEM marker (Promega Biotec).

peptide was not detected, the $\alpha 1$ -corresponding RT-PCR product was weak (Fig. 6 A, lanes 1, 7, and 8). To perform relative quantification of the $\alpha 1$ -chain transcripts, a competitive PCR approach was used. For the control pCB6/18 clone and for the AS22 clone that expressed $\alpha 1$ chain, equal amounts of both $\alpha 1$ mRNA (235 bp) and the internal competitor (160 bp) were obtained in presence of about 10^{-3} pg/ml of competitor (Fig. 6 B). As exemplified for the AS12 clone, equivalent signal was obtained using a lower amount of competitor (10^{-5} pg/ml), confirming the decrease of $\alpha 1$ mRNA. These data show that the absence of the laminin $\alpha 1$ protein correlates with a decrease of the laminin $\alpha 1$ mRNA.

Expression of the mouse $\alpha 1$ antisense RNA was determined by RT-PCR using the two specific 5' and 3' primers LN2 and LN5. By the competitive PCR method, high variability in the expression of the $\alpha 1$ antisense RNA was noted depending on clones (exemplified for the AS12 and the AS22 clones in Fig. 6 C). Indeed, in the AS12 clone equivalent amounts of antisense RNA (206 bp) and of competitor (135 bp) were obtained when the competitor concentration was 0.9×10^{-12} pg/ml (Fig. 6 C, lane 6) while in the AS22 clone, the amount needed reached 0.9×10^{-4} pg/ml (Fig. 6 C, lane 2). No signal was seen in the control pCB6 clones (not shown).

Absence of the $\alpha 1$ Chain Results in Alteration of Basement Membrane Formation

Cocultures of transfected cells and fibroblasts have been used to follow the formation of the basement membrane as well as the deposition of constituent molecules at the interface between both cell populations. After seeding on top of preformed confluent fetal skin fibroblastic cultures, all controls and the majority of AS clones (36 out of 37) were able to attach and to spread. However, histological examination of sections through the coculture sheets revealed different aspects depending on clones. Cocultures comprising control clones exhibited well-organized epithelial monolayers on the top of the fibroblastic cells (Figs. 7 C and 8 J), whereas those including AS clones were characterized by a disturbed arrangement of Caco2 cells (illustrated for AS12 in Figs. 7 A and 8 E). Concomitantly, the underlying fibroblasts did not exhibit the typical elongated shape and characteristic orientation found in control cocultures (Fig. 7 C vs 7 A).

The analysis of transverse sections of the cocultures comprising AS12, AS40, or AS51 clones with mAb 1924 recognizing specifically human $\alpha 1$ chain confirmed the lack of $\alpha 1$ chain at the Caco2 cells/fibroblasts interface (Fig. 8 A). Simultaneously, there was no deposition of $\beta 1/\gamma 1$ chains between both cell populations, despite the production of these chains by the fibroblastic layer (Fig. 8 B). The most striking feature was the absence of deposition of collagen IV (Fig. 8 C) and of nidogen (Fig. 8 D) in the cocultures comprising the $\alpha 1$ chain-deprived cells. In all other clones, a continuous deposition of laminin-1, collagen IV, and nidogen at the epithelial/fibroblastic interface was seen (Fig. 8, G–I) as in parental cells (Bouziges et al., 1991). Ultrastructural analysis of the coculture comprising AS12 clone confirmed the absence of an organized structure corresponding to a basement membrane (Fig. 7

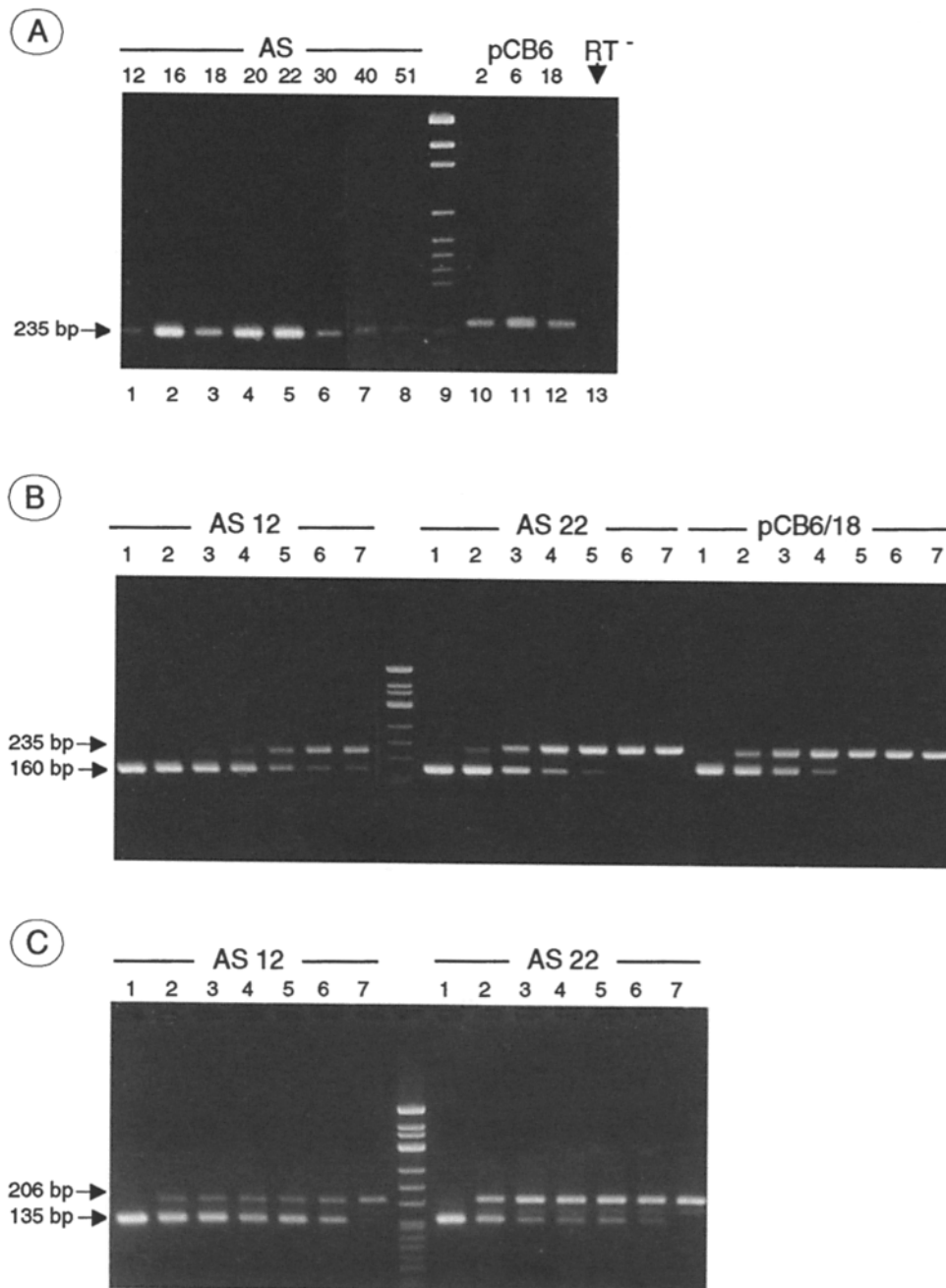


Figure 6. Expression of $\alpha 1$ -chain mRNA and antisense $\alpha 1$ -chain transcripts. (A) Analysis of $\alpha 1$ -chain mRNA expression by RT-PCR was performed using the primers described in Materials and Methods on total RNA extracted from various clones transfected with the pCB6/AS LN vector (lanes 1–8), or from control clones transfected with the pCB6 vector alone (lanes 10–12). (Lane 13) Representative RT reaction performed without addition of reverse transcriptase as negative control. The PCR products were electrophoresed on a 3% agarose gel. The arrow indicates the amplified 235-bp $\alpha 1$ -chain specific fragment. (Lane 9) pGEM-3 DNA digested with *Hinf*I, *Rsa*I, and *Sin*I used as marker. (B) Quantitative analysis of $\alpha 1$ mRNA by competitive PCR. The PCR amplification was realized in presence of constant concentration of the $\alpha 1$ cDNA and serial dilutions of the internal standard. The competitor concentration decreased 10-fold in each sample from lanes 1 (10^{-1} pg/ml) to 6 (10^{-6} pg/ml); lanes 7 contained 10^{-8} pg/ml. The upper and lower bands represent respectively the $\alpha 1$ PCR product (235 bp) and the competitor amplified fragment (160 bp). Data obtained with AS12, AS22, and pCB6/18 clones were illustrated. Equivalent signals of both PCR products were obtained at a competitor concentration of $\sim 10^{-3}$ pg/ml in AS22 and pCB6/18 clones (lanes 3) and of 10^{-5} pg/ml in AS12 clone (lane 5). $\phi \times 174$ digested with

*Hinf*I was used as marker. (C) Quantification of the $\alpha 1$ antisense transcripts. Competitive PCR method was applied generating an antisense PCR fragment of 206 bp (upper band) and a competing product of 135 bp (lower band). The coamplification was realized with constant amounts of the antisense cDNA and decreasing concentrations of the competitor (from $0.9 \cdot 10^{-2}$ pg/ml in lanes 1 to $0.9 \cdot 10^{-14}$ pg/ml in lanes 7, with a serial dilution of 100-fold per lane). Equivalent band strength was obtained with a high concentration of competitor ($0.9 \cdot 10^{-4}$ pg/ml) in the AS22 clone (lane 2), while the AS12 clone required very low amounts ($0.9 \cdot 10^{-12}$ pg/ml; lane 6). $\phi \times 174$ digested with *Hinf*I was used as marker.

B). In addition, AS transfected epithelial cells exhibited numerous processes that penetrated the underlying fibroblastic culture, conferring a very irregular and perturbed arrangement at the interface of both cell types (not shown). In contrast, control cocultures displayed a regular and continuous basement membrane assessed by the deposition of an electron dense material between the well-arranged control Caco2 cells and the fibroblasts (Fig. 7 D). These data demonstrate that the lack of a structured deposition of col-

lagen IV and nidogen is associated with an inability of AS cells to secrete mature laminin.

Modifications of the Structural and Functional Polarity in $\alpha 1$ -deficient Clones

Cells were examined by scanning and transmission EM 10 d after seeding to get a general overview of the state of morphological differentiation. The most obvious difference

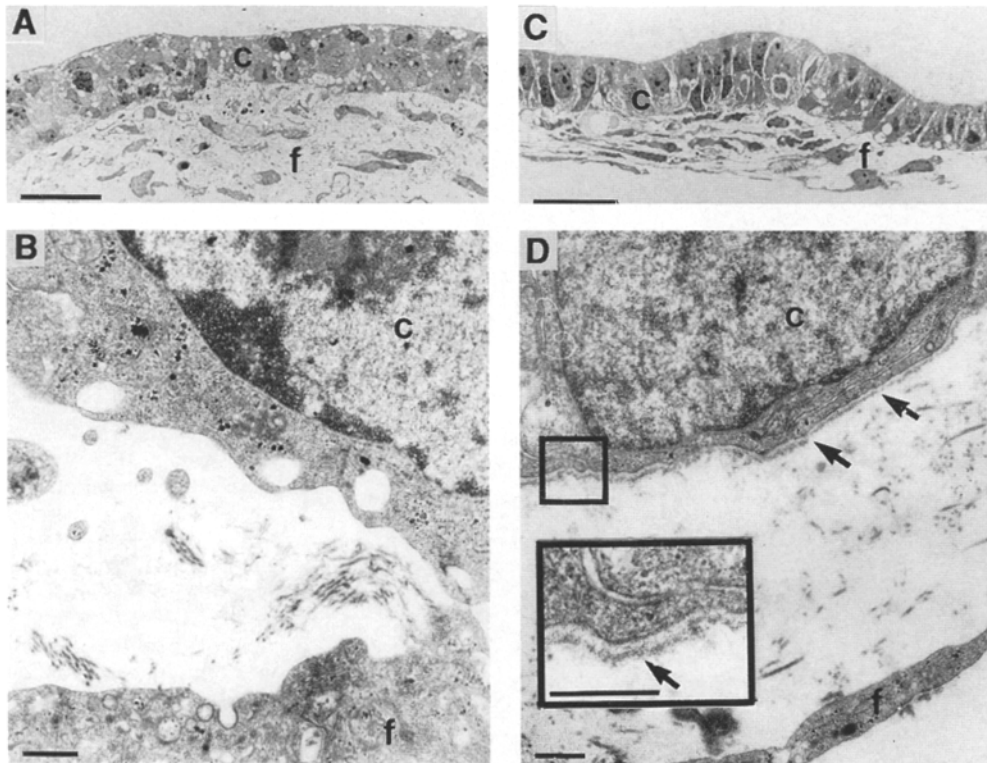


Figure 7. Behavior of transfected cells in coculture. Transfected Caco2 cells lacking the $\alpha 1$ chain (A and B) and control Caco2 cells transfected with the pCB6 vector alone (C and D) were cultured on top of confluent skin fibroblastic cells for 17 d. (A and C) Semithin sections through the cocultures show the anarchic arrangement of the AS12 cells (A) contrasting with the well-arranged organization of control Caco2 cells (C) on top of fibroblasts. (B and D) Transmission electron micrographs of the corresponding cocultures depicted in A and C; lack of electron-dense material at the AS12 cells/fibroblasts interface (B) contrary to coculture with control Caco2 cells depicting a well-formed continuous basement membrane at the contact side with fibroblasts (arrows). Caco2 (c) and fibroblastic (f) cells; basement membrane deposition (arrows). Bars: (A and C) 30 μm ; (B and D) 0.5 μm .

between AS and control clones concerned the apical surface domain. In general, cells transfected with the pCB6 vector alone were covered by brush border microvilli found regularly and densely packed within the monolayer (Fig. 9, B, D, and F) as in the case of parental Caco2 cells (Pinto et al., 1983). The apical surface domain of the cells from AS12, AS40, or AS51 clones (Fig. 9, A, C, and E) was far more heterogeneous. Indeed, the microvilli appeared often short, rare, and perturbed. To eliminate the possibility that AS cells did not express denser microvilli due to a growth-related delay, they have been cultured for 17 d. In these conditions, we did not observe any major changes in the organization and density of the apical brush border (not shown).

Differences in the structural polarity between AS clones and controls were paralleled by changes in the expression of sucrase-isomaltase, an apical digestive enzyme, as assessed by indirect immunofluorescence staining (Fig. 10). While control cells, like parental cells, displayed an apical sucrase-isomaltase distribution visualized as a punctiform immunoreactivity (Fig. 10 B), cells from the AS clones were devoid of apical (Fig. 10 A) and intracellular (not shown) labeling. In contrast, no significant differences could be observed in the staining pattern of two other apical differentiation markers, an enzyme, DDPIV (Fig. 10 C), and a cytoskeletal protein, villin (Fig. 10 D). Similarly, there was no difference in villin expression as determined by Western blotting in between the various AS clones, including AS12, AS40, and AS51 clones (Fig. 10 E).

Besides the microvilli, the overall differentiation pattern

of $\alpha 1$ chain-deprived cells is quite similar to that depicted by control Caco2 cells. In particular, well-developed junctional complexes, such as desmosomes and apical tight junctions, have been found located on lateral plasma membranes (Fig. 9 G). Specific organelles called annulate lamellae, bearing a close resemblance to the structure of the nuclear envelope, were detected in the transfected Caco2 cells (Fig. 9 H). Although the significance and function of this organelle remains unknown at present, it has been postulated that it might be involved with the mobilization of stored gene products (for review see Ghadially, 1988).

Discussion

In this study, we used the antisense RNA strategy to examine the effect of a forced reduction of one constituent chain of laminin, the $\alpha 1$ chain, on basement membrane assembly as well as on epithelial cell morphological and functional polarization, using the human colon carcinoma Caco2 cells. We have demonstrated that the absence of $\alpha 1$ -chain expression in Caco2 cells leads to (a) an incorrect secretion of the other constituent chains of laminin, the $\beta 1/\gamma 1$ chains, (b) the lack of basement membrane formation in a coculture system, and (c) alterations in the structural and functional polarity of cells. These results provide direct proof that laminin, through its $\alpha 1$ constituent chain, plays an important role in the constitution of the basement membrane at the epithelial/mesenchymal interface and in cell differentiation.

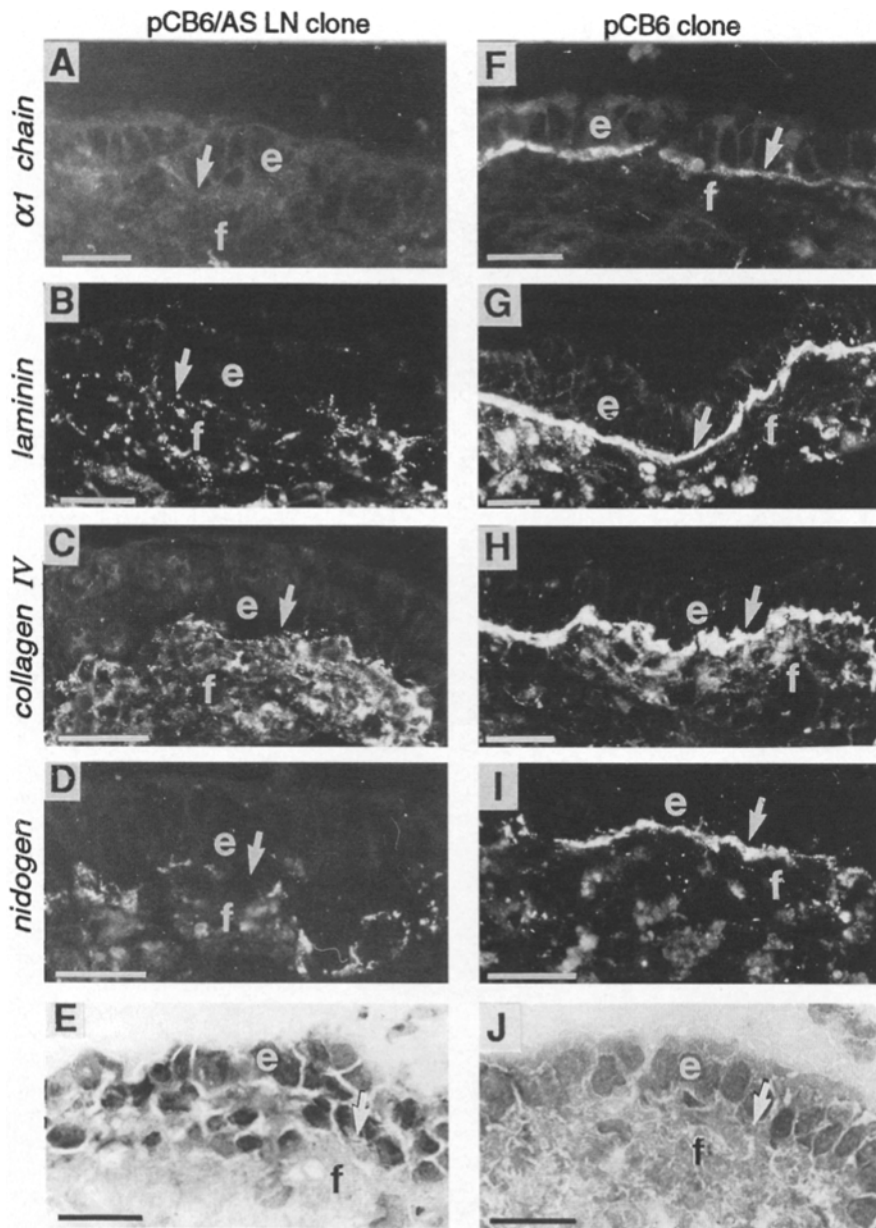


Figure 8. Lack of deposition of basement membrane molecules in coculture. Patterns of deposition of laminin α 1 chain (A and F), laminin-1 (B and G), collagen IV (C and H), and nidogen (D and I) in cocultures of α 1-deprived Caco2 cells (A–D) or of control Caco2 cells (F–I) and skin fibroblasts. Cryosections from the cocultures were stained with hematoxylin/eosin (E and J). Deposition of basement membrane molecules between the two cell populations occurs only when α 1 chain was present. Laminin-1, collagen IV, and nidogen are also detected within the fibroblastic layer. Epithelial (e) and fibroblastic (f) cell layers. (Arrows) Caco2 cells/fibroblasts interface. Bars, 30 μ m.

α 1 Chain Inhibition by Antisense RNA

Antisense DNA or RNA techniques are increasingly used mainly due to their potency in control of specific gene expression. The major advantage of this strategy is that it permits long-term studies in culture and in vivo that cannot be achieved with either antisense oligonucleotides or antibodies. In the present study, we transfected Caco2 cells with an expression vector carrying a 1.4-kb antisense cDNA that includes the E3 fragment of α 1 laminin chain. The latter fragment is comprised within the G domain of laminin and represents the most COOH-terminal part of the α 1 chain. This part of the sequence has been chosen according to the fact that the cDNA sequence encoding the NH₂-terminal part of the α 1 chain is highly homologous to the corresponding NH₂-terminal part of the β 1 chain (Hartl et al., 1988). In addition, antisense RNAs complementary to the 5' or the 3' end of the mRNA have been shown to be equally effective (Kioussis and Grosfeld, 1986).

The immunocytochemical analysis of the transformants resistant to G418 showed that the α 1-chain expression could be completely abolished in three clones (AS12, AS40, and AS51). In parallel, the amount of detectable α 1-chain mRNA determined by quantitative RT-PCR amplification decreased as compared to control clones transfected with the pCB6 vector alone. It should be pointed out that the phenotypic changes in antisense α 1-chain transfected clones was rather unstable. This phenomenon has also been reported in other antisense studies (Knecht, 1989; Fernández et al., 1993). In our conditions, a reversion of the blocked phenotype could be observed by lowering the antibiotic concentration. Yet, this phenotype could be restored by elevating back the antibiotic dose. This may be linked to the selection of a random duplication event of the resistance gene including the antisense construct and leading to a higher expression of the antisense RNA. It is worth noting that the exact mechanism by

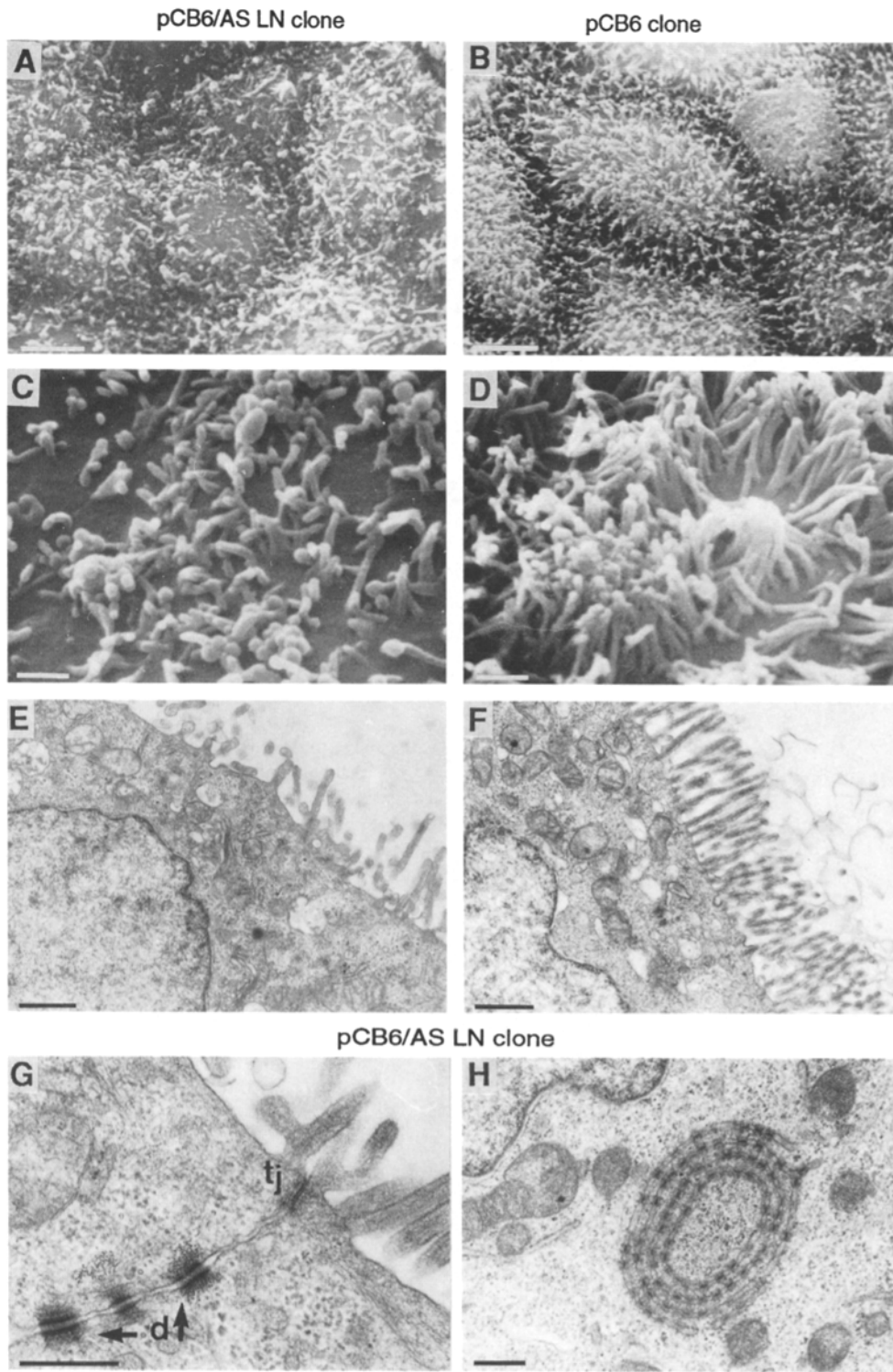


Figure 9. Perturbation of the apical microvilli in $\alpha 1$ chain-deprived clones. (A–D) Apical view at the scanning electron microscope of $\alpha 1$ -deprived Caco2 cells (A and C) and of control cells (B and D) after 10 d in culture showing different patterns of brush border organization. In A and C, microvilli are less densely packed and shorter as compared with controls. (E–H) Transmission electron micrographs of $\alpha 1$ -deprived Caco2 cells (E, G, and H) and of control cells (F) after 10 d in culture, showing that the general polarity of cells has been retained. The antisense clones are characterized by cells possessing few microvilli at their apical surface (E); well-developed desmosomal structures (d) and tight junctions (tj) located on lateral plasma membrane were observed (G) as in control cells. Note the presence of large intracytoplasmic annulate lamellae organized as circular profiles in the AS40 clone (H). Bars: (A and B) 5 μm ; (C–F) 1 μm ; (G and H) 0.5 μm .

which antisense inhibition works is still unclear and may vary from one clone to the other depending on the plasmid integration site (for reviews see Knecht, 1989; Erickson, 1993).

$\alpha 1$ Chain Inhibition Leads to an Impaired Secretion of $\beta 1/\gamma 1$ Chains

The inhibition of $\alpha 1$ chain in Caco2 cells was accompanied by an intracellular accumulation of the other constituent

chains of laminin, $\beta 1$, and $\gamma 1$ subunits, as visualized by immunocytochemistry and immunoprecipitation. While $\beta 1$ and $\gamma 1$ chains are known to interact and assemble within the cell to form a double coiled-coil (Hunter et al., 1992), structural analysis indicates that the COOH-terminal portion of the laminin $\alpha 1$ chain is essential for the formation of a triple-stranded structure and for the chain-specific assembly (Nomizu et al., 1994; Utani et al., 1994). In addition, the fact that the G domain, which distinguishes $\alpha 1$

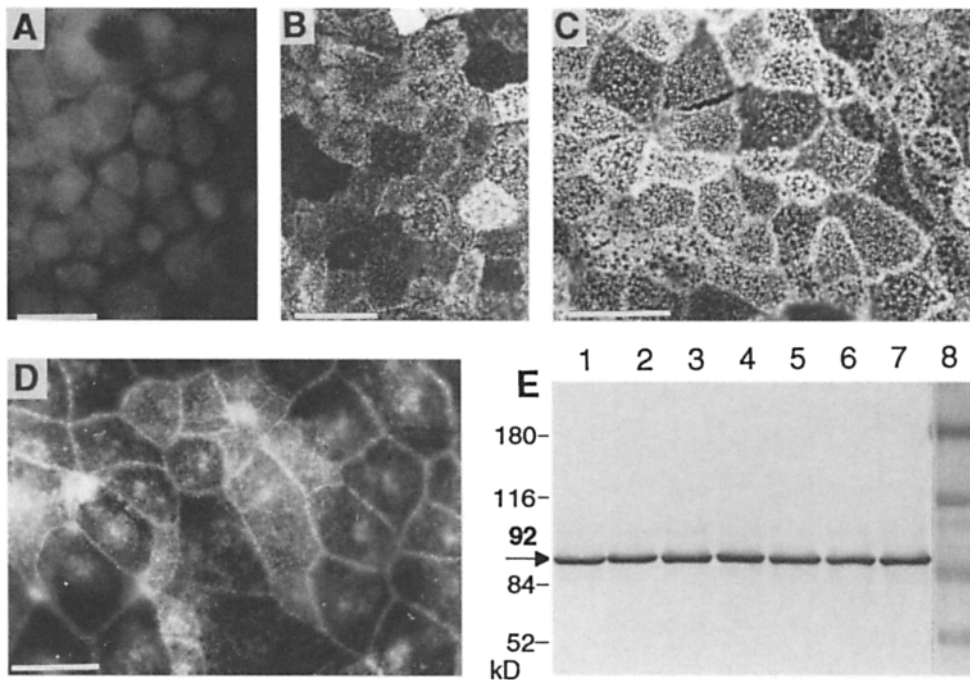


Figure 10. Sucrase-isomaltase staining is abolished in $\alpha 1$ chain-deprived clones. Immunofluorescent staining with anti-human sucrase-isomaltase (A and B), DPPIV (C), and with anti-villin (D) antibodies at the apical cell surface of confluent cultures at day 10. Note the inhibition of sucrase expression in $\alpha 1$ -deprived clones vs control cells (A vs B), while DPPIV (C) and villin (D) are less affected as exemplified for one AS clone. Western blot analysis of cell extracts (E) from control clones transfected with the pCB6 vector alone (lanes 1 and 2) or from various clones transfected with the pCB6/AS LN vector (lanes 3–7) including AS40, AS51 and AS12 clones (lanes 5–7), confirming that villin expression (arrow) was not affected in the antisense-expressing cells. (Lane 8) Molecular weight markers. Bars, 30 μ m.

chain from $\beta 1/\gamma 1$ chains, is made up of G loops that are sequence motifs found in a variety of secreted and cell surface proteins supports its involvement in the secretion of the molecule (for references see in Kusche-Gullberg et al., 1992). Interestingly, correlations between the expression of $\alpha 1$ -chain and laminin secretion have been reported in nonmanipulated colonic cancer cells, lung carcinoma cells, and MDCK cells (Ecay and Valentich 1992; De Arcangelis et al., 1994; Narumi et al., 1994). Similarly, during development, the appearance of immunodetectable extracellular laminin coincides with the appearance of newly synthesized laminin $\alpha 1$ chain in the cytoplasm of the 16-cell morula (Leivo et al., 1980).

In the clones in which $\alpha 1$ chain was inhibited, some of the $\beta 1\gamma 1$ dimers are nevertheless able to be exported out of the cells; these heterodimers were detected, albeit at low levels, as soluble forms in the culture medium (as determined by Western blots, not shown) but not associated with the cell surface, in contrast to the controls (see Fig. 4). By studying the biosynthesis, processing, and secretion of laminin by a choriocarcinoma cell line (JAR) expressing noticeable amounts of $\alpha 1$ chain, Peters et al. (1985) demonstrated that the mature form of laminin is rapidly externalized upon completion, being either secreted into the culture medium (25%) or associated with the cell surface (75%). Therefore, our data emphasize on the one hand, the role of $\alpha 1$ chain in laminin secretion, and on the other hand, the necessity of $\alpha 1$ chain to link and/or stabilize the $\beta 1\gamma 1$ chains to the cell surface.

Laminin Forms the Basic Network of the Basement Membrane

Assembly and organization of basement membranes result

from homophilic and heterophilic interactions. The necessity to know how these molecules fit together is dictated by existence of pathologies in which basement membrane structure is disorganized. Since type IV collagen and laminin have been shown to self-assemble, leading to independent networks (Yurchenco and Schittny, 1990), it is not clearly established which one of these molecules forms the primary scaffold. From the coculture model used herein, it can be concluded that when laminin deposition is blocked, neither type IV collagen nor nidogen are found at the Caco2 cells/fibroblasts interface despite the fact that both of these molecules are produced by the fibroblasts. In addition, no morphologically distinct basement membrane could be visualized. Yurchenco and co-workers (1992, 1993) showed that self-assembly of laminin *in vitro* is mediated through the short lateral-arm globular domains. The necessity of the three chains is confirmed by our present experiments; indeed, the sole secretion of soluble $\beta 1\gamma 1$ dimers does not allow the formation of a stable structural frame to support binding of other basement membrane molecules. It is worth noting that type IV collagen molecules, shown to be deposited by mesenchymal cells in the intestine (Simon-Assmann et al., 1988, 1990), are not able to compensate for the laminin deficiency. Data available from the literature, showing that basement membrane-like structures may be present without type IV collagen, strengthen this assumption (for references see Mosher et al., 1992; Yurchenco et al., 1992). Therefore, one can clearly conclude that formation of laminin networks are a prerequisite for basement membrane assembly in the intestinal system. It should be pointed out that this process results from a continuous and progressive interplay between epithelial and mesenchymal cell populations. The cells themselves, subsequent to inductive and

reciprocal interactions, control the assembly process by modifying the amount and nature of the secreted molecules. Based on the analysis of hybrid intestines developed *in vivo*, one can assume that intestinal epithelial cells may first control the deposition of $\alpha 1$ chains (Simo et al., 1992a); nidogen, produced by the mesenchymal cells (Simon-Assmann et al., 1995), could then ensure the link between laminin and type IV collagen. The essential role of nidogen in the assembly of basement membranes forming a link between laminin and type IV collagen networks has been demonstrated recently (Aumailley et al., 1993). Related to this, Ekblom et al. (1994) showed that antibodies, recognizing the nidogen binding site on laminin, inhibited kidney tubule development and lung branching by perturbing the extracellular nidogen–laminin complex.

Lack of Basement Membrane Leads to Altered Cell Polarity

There is now good evidence that the extracellular matrix, besides its prime effect on tissue maintenance, plays an important role in regulation of gene expression in various tissues. A good example is provided by the mammary gland, an organ in which β -casein transcription can be up-regulated by laminin substratum (Streuli et al., 1991, 1995; for review see Boudreau et al., 1995). The situation encountered in the present study using colonic cancer Caco2 cells is somewhat different since these cells, grown in the absence of inducers, exhibit spontaneously a high degree of structural and functional polarization/differentiation (Pinto et al., 1983; for review see Zweibaum et al., 1991). One may assume that laminin-1 secretion by the cells plays a role in autocrine regulation leading to cell differentiation. This is supported by an increase in biosynthetic levels of laminin up to 10 d of culture and by a gradual deposition of the $\alpha 1$ chain paralleling the cell differentiation event (De Arcangelis et al., 1994; Levy et al., 1994; Vachon and Beaulieu, 1995).

The phenotypic changes obtained in antisense laminin transfected clones were confined to certain critical aspects of cell morphology. It can be postulated that transformants with more dramatic phenotypic changes may well have been created but did not survive during the time of amplification. Indeed, out of 192 clones selected, only 37 could be amplified. Analysis of cell properties from the three $\alpha 1$ -deprived clones revealed mainly a general disorganization of the apical brush border membrane, a more “anarchic” behavior on top of fibroblasts, and a shutdown of one apical digestive enzyme, sucrase. The other apical markers studied, villin (a cytoskeleton protein sustaining the microvilli) and dipeptidylpeptidase IV, were less altered than sucrase. This indicates that although the overall polarity of the cells was perturbed, various features of polarized cells were maintained, such as the presence of apical junctional complexes and targeting of some synthesized apical markers. Complementary experiments in which Caco2 cells were cultured on exogenous human laminin used as a substratum showed a stimulation of the expression of sucrase and lactase but no variation in DPPIV (Vachon and Beaulieu, 1995). Two hypotheses could account for the absence of sucrase in $\alpha 1$ -deficient clones contrasting with the continued DPPIV apical targeting.

First, sucrase is known to be highly affected by stress conditions. As an example, Quaroni et al. (1993) demonstrated that culture of Caco2 cells at 42.5°C inhibited the maturation of sucrase that was degraded intracellularly while DPPIV was unaffected. Related to this, it is worth noting that the two enzymes are transported asynchronously to the cell surface and by different routes (Stieger et al., 1988; Le Bivic et al., 1990). Second, sucrase expression may be more directly regulated by the laminin via “extracellular matrix response elements” as described for the β -casein gene in mammary epithelial cells and for the albumin gene in hepatocytes (see Boudreau et al., 1995). This latter hypothesis is supported by several arguments. The fact that none of the pCB6 control clones analyzed presented a lack of sucrase immunoreactivity argues against a possible transfection stress-induced shutdown of this enzyme; in our experimental conditions, no intracellular accumulation of sucrase was revealed. In contrast, suppression of villin expression by antisense RNA in Caco2 cells prevents the insertion or stabilization at the apical membrane of sucrase that remains intracellular (Costa De Beauregard et al., 1995). Finally, when cultures comprising normal intestinal embryonic epithelial cells were incubated with anti-laminin antibodies, expression of lactase, another apical marker, was abolished (Simo et al., 1992b).

From the present model, one can assume that laminin $\alpha 1$ chain may stabilize rather than induce the Caco2 cell polarity. However, the use of spontaneously differentiated cancer colonic cells might be a limitation. Indeed, during development, it has been shown that $\alpha 1$ chain of laminin-1 is important for initiation of cell polarity that occurs during kidney (Klein et al., 1988) as well as lung alveolar formation (Matter and Laurie, 1994). To get more insight into the mechanisms involved in normal tissue, it will be necessary to analyze the consequences of a knockout of the $\alpha 1$ laminin gene directed to a specific tissue. Indeed, in *Drosophila*, complete loss of function mutations of the *LAMA* gene lead to late embryonic lethality (Henchcliffe et al., 1993). Another important question that remains to be addressed is whether *LAMA1* gene could be considered as a tumor suppressor gene, like APC or DCC genes that both encode cell adhesion molecules. For these latter genes, mutation or inhibition leads to anarchic cell proliferation and acquisition of malignant properties (Fearon and Vogelstein, 1990; Lawlor et al., 1992; Hülsken et al., 1994).

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References

- Aumailley, M., C. Battaglia, U. Mayer, D. Reinhardt, R. Nischt, R. Timpl, and J.W. Fox. 1993. Nidogen mediates the formation of ternary complexes of basement membrane components. *Kidney Int.* 43:7-12.
- Battaglia, C., U. Mayer, M. Aumailley, and R. Timpl. 1992. Basement-membrane heparan sulfate proteoglycan binds to laminin by its heparan sulfate chains and to nidogen by sites in the protein core. *Eur. J. Biochem.* 1:359-366.
- Boudreau, N., C. Myers, and M.J. Bissell. 1995. From laminin to lamin: regulation of tissue-specific gene expression by the ECM. *Trends Cell Biol.* 5:1-4.
- Bouziges, F., P. Simo, P. Simon-Assmann, K. Haffen, and M. Kedinger. 1991. Altered deposition of basement-membrane molecules in co-cultures of colonic cancer cells and fibroblasts. *Int. J. Cancer.* 22:101-108.
- Costa De Beauregard, M.-A., E. Pringault, S. Robine, and D. Louvard. 1995. Suppression of villin expression by antisense RNA impairs brush border assembly in polarized epithelial intestinal cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:409-421.
- De Arcangelis, A., P. Simo, T. Lesuffleur, M. Kedinger, and P. Simon-Assmann. 1994. Laminin expression is correlated to differentiation of colonic cancer cell lines. *Gastroenterol. Clin. Biol.* 18:630-637.
- Deutzmann, R., J. Huber, K.A. Schmetz, I. Oberbäumer, and L. Hartl. 1988. Structural study of long arm fragments of laminin. Evidence for repetitive C-terminal sequences in the A-chain, not present in the B-chains. *Eur. J. Biochem.* 177:35-45.
- Deutzmann, R., M. Aumailley, H. Wiedemann, W. Pysny, R. Timpl, and D. Edgar. 1990. Cell adhesion, spreading and neurite stimulation by laminin fragment-E8 depends on maintenance of secondary and tertiary structure in its rod and globular domain. *Eur. J. Biochem.* 31:513-522.
- Ecay, T.W., and J.D. Valentich. 1992. Basal lamina formation by epithelial cell lines correlates with laminin A-chain synthesis and secretion. *Exp. Cell Res.* 203:32-38.
- Eklom, P., M. Eklom, L. Fecker, G. Klein, H.Y. Zhang, Y. Kadoya, M.L. Chu, U. Mayer, and R. Timpl. 1994. Role of mesenchymal nidogen for epithelial morphogenesis in vitro. *Development (Camb.)* 120:2003-2014.
- Erickson, R.P. 1993. The use of antisense approaches to study development. *Dev. Genet.* 14:251-257.
- Fearon, E.R., and B. Vogelstein. 1990. A genetic model for colorectal tumorigenesis. *Cell.* 61:759-767.
- Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Fernández, J.L.R., B. Geiger, D. Salomon, and A. Ben-Ze'ev. 1993. Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility, and anchorage-dependent growth of 3T3 cells. *J. Cell Biol.* 122:1285-1294.
- Fogh, J., J.M. Fogh, and T. Orfeo. 1977. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.* 59:221-225.
- Förster, E. 1994. An improved general method to generate internal standards for competitive PCR. *Biotechniques.* 16:18-20.
- Ghadially, F.N. 1988. Annulate lamellae. In *Ultrastructural Pathology of the Cell and Matrix*. 3rd ed. Butterworths, London. 573-587.
- Haaparanta, T., J. Uitto, E. Ruoslahti, and E. Engvall. 1991. Molecular cloning of the cDNA encoding human laminin A-chain. *Matrix.* 11:151-160.
- Hartl, L., I. Oberbäumer, and R. Deutzmann. 1988. The N terminus of laminin A chain is homologous to the B chains. *Eur. J. Biochem.* 173:629-635.
- Henchcliffe, C., L. Garcia-Alonso, J. Tang, and C.S. Goodman. 1993. Genetic analysis of laminin-A reveals diverse functions during morphogenesis in *Drosophila*. *Development (Camb.)* 118:325-337.
- Henning, S.J., D.C. Rubin, and R.J. Shulman. 1994. Ontogeny of the intestinal mucosa. In *Physiology of the Gastrointestinal Tract*. Vols. 1 and 2. 3rd ed. 571-610.
- Hülken, J., J. Berhens, and W. Birchmeier. 1994. Tumor-suppressor gene products in cell contacts: the cadherin-APC-armadillo connection. *Curr. Opin. Cell Biol.* 6:711-716.
- Hunter, I., T. Schulthess, and J. Engel. 1992. Laminin chain assembly by triple and double stranded coiled-coil structures. *J. Biol. Chem.* 267:6006-6011.
- Kedinger, M. 1994. Growth and development of intestinal mucosa. In *Small Bowel Enterocyte Culture and Transplantation*. R.G. Landers, editor. RG Landers Company, Austin, TX. 1-31.
- Kedinger, M., P. Simon-Assmann, E. Alexandre, and K. Haffen. 1987. Importance of a fibroblastic support for in vitro differentiation of intestinal endodermal cells and for their response to glucocorticoids. *Cell Differ.* 20:171-182.
- Kioussis, D., and F. Grosveld. 1986. Naturally occurring complementary transcripts: do they function as anti-sense RNA? *Trends Genet.* 304.
- Klein, G., M. Langegger, R. Timpl, and P. Eklom. 1988. Role of laminin A chain in the development of epithelial cell polarity. *Cell.* 55:331-341.
- Knecht, D. 1989. Application of antisense RNA to the study of the cytoskeleton: background, principles, and a summary of results obtained with myosin heavy chain. *Cell Motil. Cytoskeleton.* 14:92-102.
- Kusche-Gullberg, M., K. Garrison, A.J. Mackrell, L.I. Fessler, and J.H. Fessler. 1992. Laminin-A chain: expression during *Drosophila* development and genomic sequence. *EMBO J.* 11:4519-4527.
- Lawlor, K.G., N.T. Telang, M.P. Osborne, R.Q. Schaapveld, K.R. Cho, B. Vogelstein, and R. Narayanan. 1992. Antisense RNA to the putative tumor suppressor gene "deleted in colorectal cancer" transforms fibroblasts. *Antisense Strategies.* 660:283-285.
- Le Bivic, A., A. Quaroni, B. Nichols, and E. Rodriguez-Boulan. 1990. Biogenetic pathways of plasma membrane proteins in Caco2, a human intestinal epithelial cell line. *J. Cell Biol.* 111:1351-1361.
- Leivo, I., A. Vaheri, R. Timpl, and J. Wartiovaara. 1980. Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol.* 76:100-114.
- Levy, P., O. Loreal, A. Munier, Y. Yamada, J. Picard, G. Cherqui, B. Clement, and J. Capeau. 1994. Enterocytic differentiation of the human Caco2 cell line is correlated with down-regulation of fibronectin and laminin. *FEBS Lett.* 7:272-276.
- Madara, J.L., and J.S. Trier. 1994. The functional morphology of the mucosa of the small intestine. In *Physiology of the Gastrointestinal Tract*. Vols. 1 and 2. 3rd ed. 1577-1622.
- Matter, M.L., and G.W. Laurie. 1994. Novel laminin E8 cell adhesion site required for lung alveolar formation in vitro. *J. Cell Biol.* 124:1083-1090.
- Mosher, D.F., J. Sottile, C. Wu, and J.A. McDonald. 1992. Assembly of extracellular matrix. *Curr. Biol.* 4:810-818.
- Narumi, K., K. Satoh, M. Isemura, T. Sakai, T. Abe, S. Shindo, T. Kikuchi, K. Matsushima, M. Motomiya, and T. Nukiwa. 1994. Variety of laminin expressions in murine neoplastic cell lines. Neuroblastoma NA cells produce only laminin-B2 chain. *Int. J. Oncol.* 4:133-136.
- Nomizu, M., A. Otaka, A. Utani, P.P. Roller, and Y. Yamada. 1994. Assembly of synthetic laminin peptides into a triple-stranded coiled-coil structure. *J. Biol. Chem.* 48:30386-30392.
- Paulsson, M. 1992. Basement membrane proteins: structure, assembly, and cellular interactions. *Crit. Rev. Biochem. Mol. Biol.* 27:93-127.
- Peppel, K., and C. Baglioni. 1990. A simple and fast method to extract RNA from tissue culture cells. *Biotechniques.* 9:711-713.
- Peters, B.P., R.J. Hartle, R.F. Krzesicki, T.G. Kroll, F. Perini, J.E. Balun, I.J. Golstein, and R.W. Ruddon. 1985. The biosynthesis, processing, and secretion of laminin by human choriocarcinoma cells. *J. Biol. Chem.* 260:14732-14742.
- Pinto, M., S. Robine-Leon, M.-D. Appay, M. Kedinger, N. Triadou, E. Dusaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh et al. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco2 in culture. *Biol. Cell.* 47:323-330.
- Quaroni, A., E.C.A. Paul, and B.L. Nichols. 1993. Intracellular degradation and reduced cell-surface expression of sucrose isomaltase in heat-shocked Caco2 cells. *Biochem. J.* 15:725-734.
- Robine, S., C. Huet, R. Moll, C. Sahuquillo-Merino, E. Coudrier, A. Zweibaum, and D. Louvard. 1985. Can villin be used to identify malignant and undifferentiated normal digestive epithelial cells? *Proc. Natl. Acad. Sci. USA.* 82:8488-8492.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
- Schuger, L., A.P.N. Skubitz, A.D.L. Morenas, and K. Gilbride. 1995. Two separate domains of laminin promote lung organogenesis by different mechanisms of action. *Dev. Biol.* 169:520-532.
- Simo, P., P. Simon-Assmann, F. Bouziges, C. Leberquier, M. Kedinger, P. Eklom, and L. Sorokin. 1991. Changes in the expression of laminin during intestinal development. *Development (Camb.)* 112:477-487.
- Simo, P., F. Bouziges, J.C. Lissitzky, L. Sorokin, M. Kedinger, and P. Simon-Assmann. 1992a. Dual and asynchronous deposition of laminin chains at the epithelial-mesenchymal interface in the gut. *Gastroenterology.* 102:1835-1845.
- Simo, P., P. Simon-Assmann, C. Arnold, and M. Kedinger. 1992b. Mesenchyme-mediated effect of dexamethasone on laminin in cocultures of embryonic gut epithelial cells and mesenchyme-derived cells. *J. Cell Sci.* 101:161-171.
- Simon-Assmann, P., F. Bouziges, C. Arnold, K. Haffen, and M. Kedinger. 1988. Epithelial-mesenchymal interactions in the production of basement membrane components in the gut. *Development (Camb.)* 102:339-347.
- Simon-Assmann, P., F. Bouziges, J.-N. Freund, F. Perrin-Schmitt, and M. Kedinger. 1990. Type IV collagen mRNA accumulates in the mesenchymal compartment at early stages of murine developing intestine. *J. Cell Biol.* 110:849-857.
- Simon-Assmann, P., M. Kedinger, A. De Arcangelis, V. Orian-Rousseau, and P. Simo. 1995. Extracellular matrix components in intestinal development. In *Extracellular Matrix in Animal Development*. P. Eklom, editor. Multi-author review series. *Experientia (Basel)* 51:883-900.
- Sorokin, L.M., S. Conzelmann, P. Eklom, C. Battaglia, M. Aumailley, and R. Timpl. 1992. Monoclonal antibodies against laminin-A chain fragment-E3 and their effects on binding to cells and proteoglycan and on kidney development. *Exp. Cell Res.* 201:137-144.
- Sorokin, L., W. Girg, T. Gopfert, R. Hallmann, and R. Deutzmann. 1994. Ex-

- pression of novel 400-kDa laminin chains by mouse and bovine endothelial cells. *Eur. J. Biochem.* 223:603-610.
- Stieger, B., K. Matter, B. Baur, K. Bucher, M. Hochli, and H.P. Hauri. 1988. Dissection of the asynchronous transport of intestinal microvillar hydrolases to the cell surface. *J. Cell Biol.* 106:1853-1861.
- Streuli, C.H., N. Bailey, and M.J. Bissell. 1991. Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. *J. Cell Biol.* 115:1383-1395.
- Streuli, C.H., C. Schmidhauser, N. Bailey, P. Yurchenco, A.P.N. Skubitz, C. Roskelley, and M.J. Bissell. 1995. Laminin mediates tissue-specific gene expression in mammary epithelia. *J. Cell Biol.* 129:591-603.
- Timpl, R., and J.C. Brown. 1994. The laminins. *Matrix Biol.* 14:275-281.
- Timpl, R., H. Rohde, P. Gehron Robey, S.I. Rennard, J.-M. Foidart, and G.R. Martin. 1979. Laminin. A glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933-9937.
- Timpl, R., M. Dziadek, S. Fujiwara, H. Nowack, and G. Wick. 1983. Nidogen: a new, self-aggregating basement membrane protein. *Eur. J. Biochem.* 137:455-465.
- Utani, A., M. Nomizu, R. Timpl, P.P. Roller, and Y. Yamada. 1994. Laminin chain assembly. Specific sequences at the C terminus of the long arm are required for the formation of specific double- and triple-stranded coiled-coil structures. *J. Biol. Chem.* 269:19167-19175.
- Vachon, P.H., and J.F. Beaulieu. 1995. Extracellular heterotrimeric laminin promotes differentiation in human enterocytes. *Am. J. Physiol.* 268 (*Gastrointest. Liver Physiol.* 31):G857-G867.
- Yurchenco, P.D., and Y.S. Cheng. 1993. Self-assembly and calcium-binding sites in laminin A three-arm interaction model. *J. Biol. Chem.* 268:17286-17299.
- Yurchenco, P.D., and J.C. Schittny. 1990. Molecular architecture of basement membranes. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:1577-1590.
- Yurchenco, P.D., Y.S. Cheng, and H. Colognato. 1992. Laminin forms an independent network in basement membranes. *J. Cell Biol.* 117:1119-1133.
- Yurchenco, P.D., U. Sung, M.D. Ward, Y. Yamada, and J.J. O'Rear. 1993. Recombinant laminin-G domain mediates myoblast adhesion and heparin binding. *J. Biol. Chem.* 268:8356-8365.
- Zweibaum, A., M. Laburthe, E. Grasset, and D. Louvard. 1991. The use of cultured cell lines in studies of intestinal cell differentiation and function. *In* The Gastrointestinal System IV. R. Frizzell, and H. Fields, editors. Handbook of Physiology. Alan Liss, New York. 223-255.