Anat Embryol (1989) 180:65–69

Anatomy and Embryology

Immunohistochemistry of laminin in early chicken and quail blastoderms

H. Bortier[1], G. De Bruyne[2], M. Espeel[1], and L. Vakaet[1]

[1] Laboratory of Anatomy and Embryology, State University Gent, Godshuizenlaan 4, B-9000 Gent, Belgium
[2] Laboratory of Experimental Cancerology, Department of Radiotherapy and Nuclear medicine, University Hospital, De Pintelaan, 185, B-9000 Gent, Belgium

Summary. We have used immunohistochemical techniques to study laminin in quail blastoderms milked from the oviduct and the distribution of laminin in laid chicken and quail blastoderms. Laminin is a constituent of the basement membrane in both chicken and quail blastoderms. It is found at the ventral side of the upper layer cells. Laminin is first observed under individual upper layer cells in prelaid quail blastoderms 15 h post-ovulation, but is absent at the ingression site of endophyll cells. The presence of a continuous laminin layer coincides with the epithelialization of the epiblast after 5–10 h incubation. The laminin layer is discontinuous at the primitive streak and at Hensen's node. It is thinner and partly discontinuous at the median part of the neural plate. By induction, either of an ectopic primitive streak or a neural plate, we have demonstrated, using the chicken-quail nucleolar marker technique, that at these sites the laminin layer is interrupted. A laminin layer might confer rigidity onto the epiblast, whereas disruption of a laminin layer seems to be correlated with ingression of cells or bending of the neural plate.

Key words: Laminin – Basement membrane – Avian embryos – Immunohistochemistry

Introduction

Laminin is the most abundant glycoprotein in basement membranes and seems to be specific for this structure. So far every basement membrane examined seems to contain laminin. In normal tissues extracellular laminin has been found only in basement membranes. The biological functions ascribed to laminin are numerous and sometimes contradictory (Kleinman et al. 1985; Martin and Timpl 1987).

Low (1967) has studied with transmission electron microscopy the boundary (basement) membrane in the chicken blastoderm (from 0 to 36 h of incubation). He observed primordia of this structure in the freshly laid egg along the ventral surface of the epiblast. After 24 h of incubation he found a basal lamina with a lamina lucida.

Laminin and fibronectin distributions have been studied by Mitrani (1982) in early gastrulating chicken blastoderms. Prior to stage XIII (Eyal-Giladi and Kochav 1976), corresponding to stage 3 of Vakaet (1970), laminin was only occasionally detected by Mitrani as "faint, very short interrupted lines scattered on the lower side of the upper epiblastic layer".

We studied immunohistochemically the presence of laminin in quail blastoderms milked from the oviduct and the distribution of laminin in laid chicken and quail blastoderms. We compared the laminin in the epiblast of the proamnion during normal development with its distribution after experimental induction of either a neural plate or primitive streak (Vakaet et al. 1980; Vakaet 1984). These inductions are accompanied by a disruption of the thick laminin layer, normally present at the site of the inductions. The distribution of laminin is then similar to that seen in a normal primitive streak and neural plate.

Offprint requests to: H. Bortier

Materials and methods

Materials

- Quail (*Coturnix coturnix japonica*) blastoderms, milked from the oviduct ($n=22$).
- Laid, but unincubated chicken (*White Plymouth Rock*) ($n=4$) blastoderms and quail blastoderms ($n=15$).
- Chicken ($n=24$) and quail blastoderms ($n=4$), incubated at 37.5 °C to the desired stage (Vakaet 1970).

The youngest quail blastoderm was 10 h post-ovulation. The age was determined from the time of laying of the previous egg and compared to the table of Woodard and Mather (1964). The oldest chicken blastoderm was at stage 9.

Silver enhanced immuno-gold staining of laminin

Blastoderms were fixed for 2 h in Bouin-Hollande's solution containing 10% (v/v) of a saturated aqueous solution of mercuric chloride (Romeis 1968). They were dehydrated, embedded in paraffin and serially sectioned at 10 µm. The consecutive series of deparaffinized sections were either stained with 1% (w/v) toluidine blue in distilled water or immunostained with affinity purified rabbit antibodies against laminin from EHS mouse sarcoma (diluted 1:2000, gift to M. Mareel of L. Liotta NIH, Bethesda, MD, USA). For immunostaining rehydrated sections were treated with Lugol's iodine for 5 min and subsequently dipped in sodium thiosulphate (5% w/v in distilled water) for 3 min. Then the sections were washed extensively in distilled water. In order to reveal extracellular laminin (Albrechtsen et al. 1981; De Bruyne et al. 1988), the sections were incubated

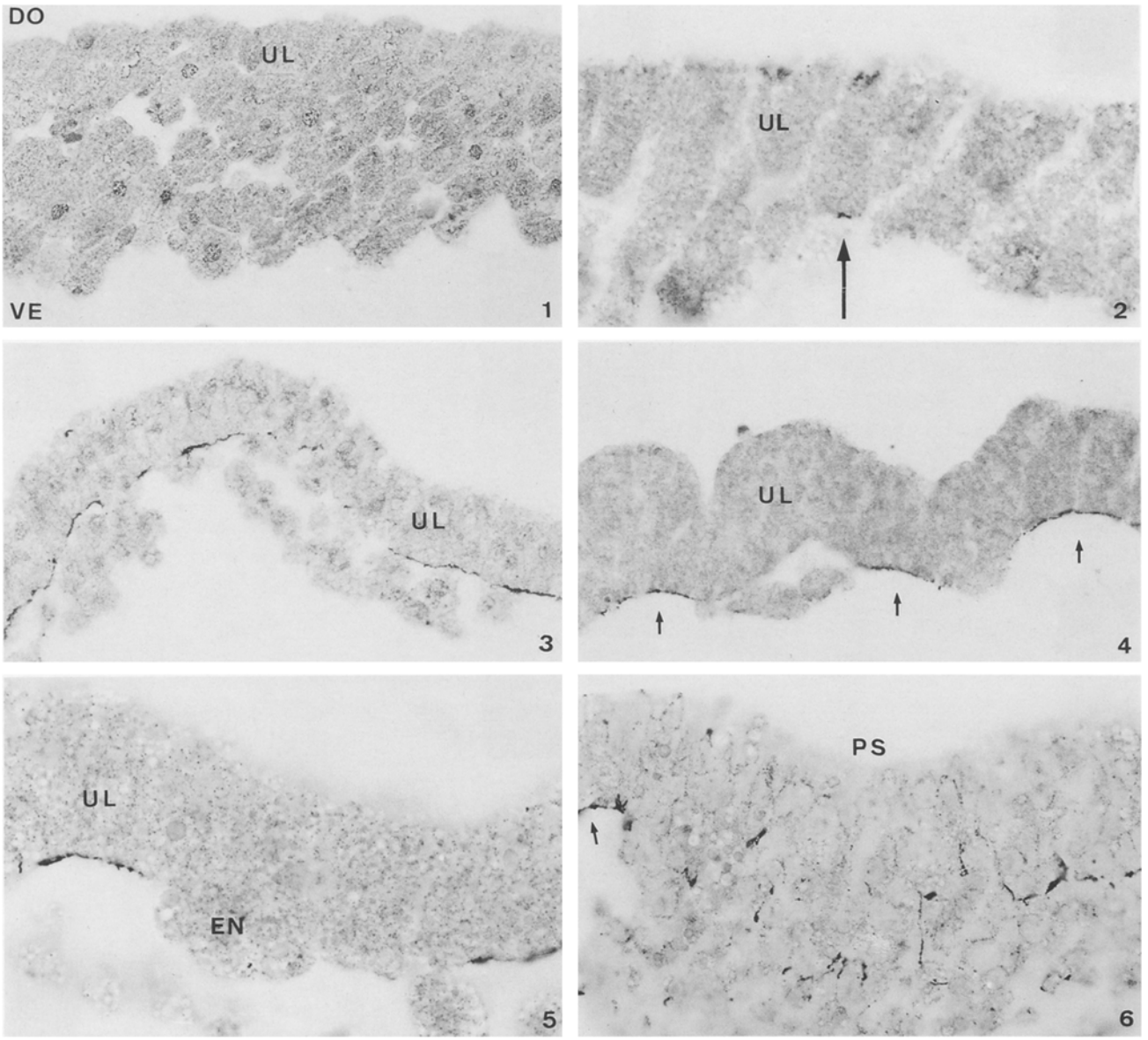

Fig. 1. Milked quail blastoderm (10 h post-ovulation): no laminin. No compaction of the upper layer (*UL*). Dorsal (*DO*), ventral (*VE*) aspect of the blastoderm. Obj. Leitz Pl Apo 63/1.40 oil

Fig. 2. Milked quail blastoderm (15 h post-ovulation): laminin under individual upper layer (*UL*) cell (*arrow*). Obj. Leitz Pl Apo 63/1.40 oil

Fig. 3. Unincubated laid chicken blastoderm: patchy discontinuous laminin layer. Upper layer (*UL*). Obj. Leitz Pl Apo 25/0.65

Fig. 4. Chicken blastoderm (6 h incubation): laminin in the ventral concavities of the folds described by Merbach (*arrows*). Wrinkled upper layer (*UL*). Obj. Leitz Pl Apo 25/0.65

Fig. 5. Chicken blastoderm (6 h incubation): laminin is absent at the leading edge of ingressing endophyll (*EN*) cells. Upper layer (*UL*). Obj. Leitz Pl Apo 63/1.40 oil

Fig. 6. Chicken blastoderm, stage 3: continuous laminin layer (arrow) interrupted at the primitive streak (*PS*). The ingressing cells show short segments of laminin staining. Obj. Leitz Pl Apo 63/1.40 oil

at 37° C in a 0.04% (w/v) solution of crystalline pepsin (BDH Chemicals Ltd., Poole, England) in 0.01 M HCl, for 5 min. Enzymic treatment was stopped by dipping the sections into cold running tap water. They were subsequently washed in distilled water and then in 0.1% BSA-Tris buffer (20 mM Tris-base; 0.9% (w/v) NaCl), adjusted to pH 8.2 with 1 M HCl and containing 0.1% (w/v) Bovine Serum Albumin (BSA) and 20 mM sodium azide). In order to block nonspecific staining the sections were incubated for 45 min in 5% (w/v) BSA in 0.1% BSA-Tris buffer. After overnight incubation with the antibodies against laminin, the sections were extensively washed in 0.1% BSA-Tris buffer. The second antibody goat anti-rabbit (GAR) conjugated with gold (AuroProbe LM GAR, Janssen Life

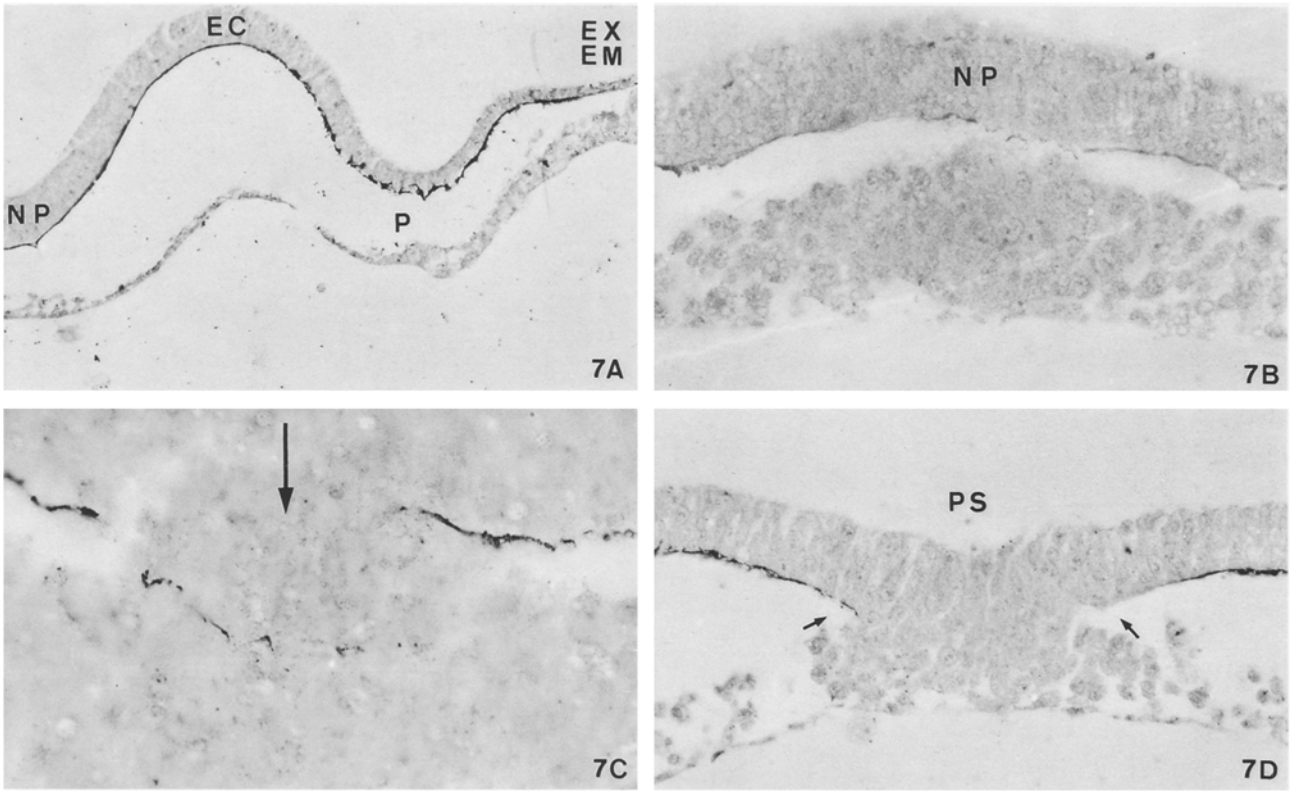

Fig. 7A–D. Chicken blastoderm, stage 6. **A** In a section just anterior to the primitive streak, most laminin is found in the proamnion (*P*). Less laminin is found in the extraembryonic area (*EXEM*) and in the future ectoblast area (*EC*). Obj. Leitz Pl Apo 16/0.45. **B** Only a thin, partly interrupted laminin layer is found in the median part of the neural plate (*NP*). Obj. Leitz Pl Apo 63/1.40 oil. **C** At Hensen's node: a discontinuous laminin layer (*arrow*). Obj. Leitz Pl Apo 63/1.40 oil. **D** Lateral to the primitive streak (*PS*), in the pre-ingressing area, the laminin layer is thinner (*arrows*). Obj. Leitz Pl Apo 25/0.65

Sciences Products, Olen, Belgium), dilution 1:80 in 0.1% BSA-Tris buffer was applied for 2 h and silver enhanced (IntenSE II™, Janssen Life Sciences Products, Olen, Belgium) under microscopic control for about 7 min. Finally, the sections were washed in running tap water, dehydrated and mounted with Merckoglass (Merck, Darmstadt, FRG).

Omission of the primary or secondary antibody respectively gave no laminin staining. We did not observe laminin, either cytoplasmic or extracellular, without pepsin treatment. In preliminary tests the pattern of laminin staining was similar between 5–30 min of pepsin treatment. We used 5 min of pepsin treatment to minimize digestion of cells.

Inductions of neural plate and primitive streak

We induced experimentally either a secondary neural plate or a primitive streak as described by Vakaet et al. (1980), Vakaet (1984). For neural induction experiments ($n=6$), a Hensen's node from a stage 5 quail blastoderm was inserted into the proamnion of a stage 5 chicken blastoderm. For primitive streak induction experiments ($n=5$) a quail nodus posterior was inserted into the proamnion of a stage 5 chicken blastoderm. The cultures were reincubated for 6–10 h, the time required to show the earliest morphological reactions. The sections were stained after Feulgen-Rossenbeck (Lison 1960) to distinguish between chicken and quail nuclei (Le Douarin 1973). One series of sections was processed for immunohistochemistry as described above.

Results

Normal blastoderms

Laminin was found at the ventral side of the upper layer.

Quail blastoderms milked from the oviduct showed laminin from 15 h post-ovulation on. We saw the first laminin in milked quail blastoderms under individual upper layer cells (Figs. 1, 2).

Unincubated laid chicken blastoderms showed a patchy discontinuous laminin layer (Fig. 3). After 6 h of incubation laminin was found in the ventral concavities of the folds described by Merbach (1935). The laminin was discontinuous at the rim of these folds where the endophyll was formed by polyingression (Fig. 4). Laminin was absent at the leading edge of ingressing endophyll cells (Fig. 5). The upper layer was wrinkled at this stage.

At stage 3 (Vakaet 1970), corresponding to stage XIII of Eyal-Giladi and Kochav (1976), laminin formed a continuous, even layer under the compacted epiblast. The epiblast was interrupted under the primitive streak. There, the ingressing cells showed short laminin segments at their leading edge (Fig. 6). At stage 6–7, anterior to the primitive streak, the thickest laminin layer was found in the proamnion (Fig. 7A). A thinner laminin layer was found in the extraembryonic area and in the future ectoblast area. Only a thin and partly interrupted laminin layer was found in the median part of the neural plate (Fig. 7B). At Hensen's node we saw a discontinuous laminin layer. Laminin was seen in the node as short lines (Fig. 7C). Just lateral to

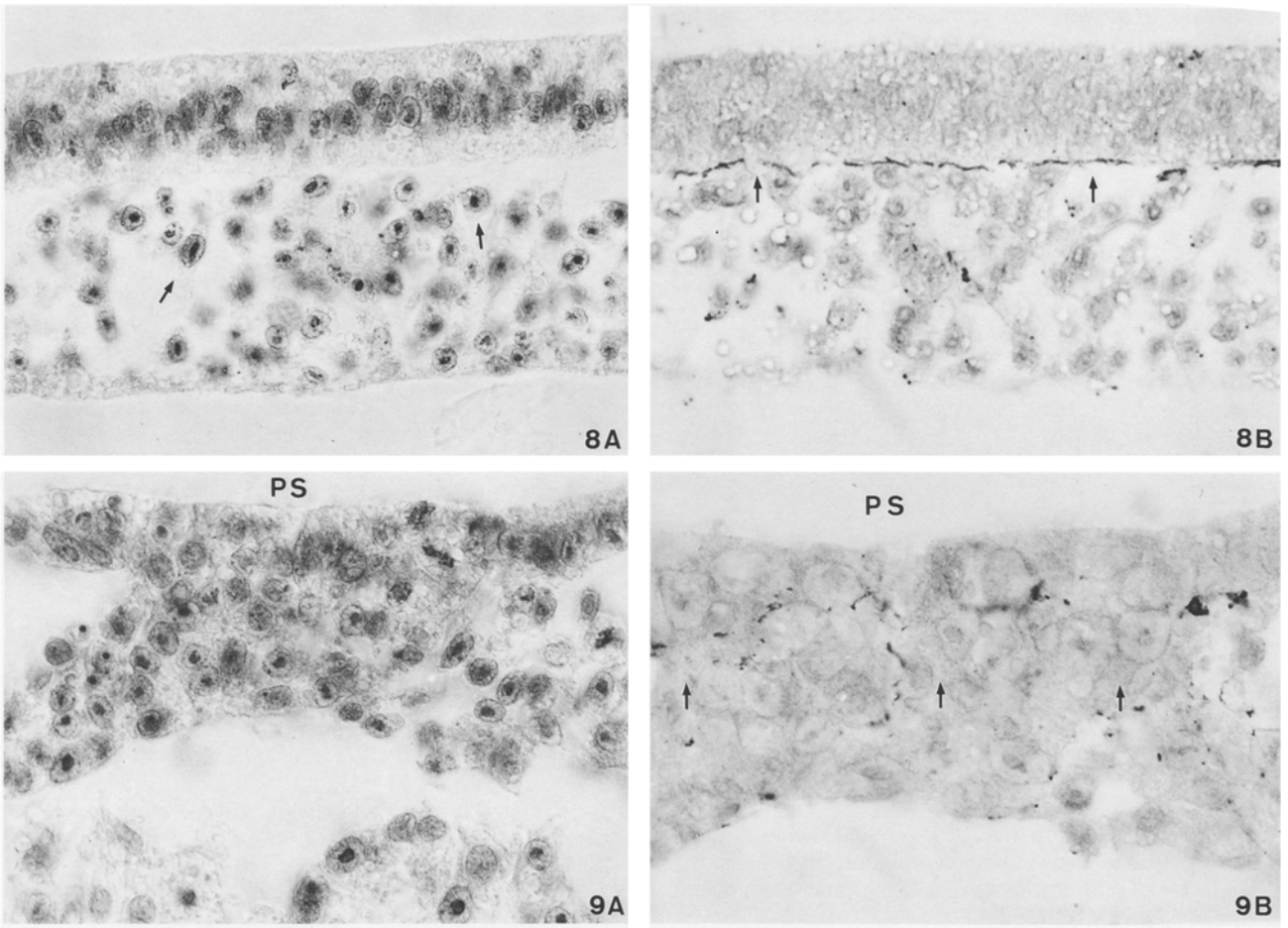

Fig. 8A, B. Neural induction experiment: a Hensen's node from a stage 5 quail blastoderm was inserted into the proamnion of a stage 5 chicken blastoderm. **A**, **B** Consecutive sections. **A** Neural induction by the quail cells (*arrows*) in the chicken epiblast. Feulgen staining. Obj. Leitz Pl Apo 63/1.40 oil. **B** After 6 h the laminin layer between evoking axial quail mesoblast and neural reacting chicken epiblast is thinner and focally interrupted (*arrows*). Obj. Leitz Pl Apo 63/1.40 oil

Fig. 9A, B. Primitive streak induction experiment: a quail nodus posterior was inserted into the proamnion of a stage 5 chicken blastoderm. **A**, **B** Consecutive sections. **A** Initial stage of primitive streak (*PS*) induction. Feulgen staining. Obj. Leitz Pl Apo 63/1.40 oil. **B** After 10 h the laminin layer is interrupted at the site of induction (*arrows*). Obj. Leitz Pl Apo 63/1.40 oil

the primitive streak, in the pre-ingressing area, the laminin layer was thinner and at some sites discontinuous (Fig. 7D).

Laminin was not found at the dorsal side of the embryonic and extraembryonic epiblast nor in the deep layer, consisting of endophyll, hypoblast, definitive endoblast and yolk endoderm; nor in the middle layer; nor in the margin of overgrowth; nor in the vitelline membrane.

Inductions of neural plate and primitive streak

In the 6 neural induction experiments, we compared consecutive Feulgen and immunohistochemically stained sections. We found that the laminin between evoking axial quail mesoblast and neural reacting epiblast of the chicken proamnion consisted of a thin layer with interruptions similar to the disposition of the laminin layer in normal neurulation (Fig. 8A, B). In the contralateral proamnion, the upper layer still possessed its thick laminin layer (see Fig. 7A, site of P).

In 4 of the 5 (one was lost) primitive streak induction experiments, ingression had started. The laminin distribution was similar to that in normal primitive streak stages 3–4. The original thick layer of laminin in the proamnion was interrupted and parts of it were found at the leading edge of ingressing cells (Fig. 9A, B).

Discussion

The results show that extracellular laminin in the milked quail blastoderm 15 h post-ovulation appears at the ventral side of individual upper layer cells. At that stage, no other cell layers are present. This means that in the prelaid quail blastoderm the future epithelial cells produce the laminin of their basement membrane. The endophyll cells do not show extracellular laminin.

The morphology of the upper layer seems to be correlated with the presence or absence of laminin. In blastoderms where a continuous laminin layer is not yet present, we find a wrinkled upper layer (Fig. 4). Low (1967) states that at that time it is not called an epithelium. When the laminin layer gradually forms, the upper layer flattens. To-

gether with the appearance of strips of laminin, the upper layer cells show a smooth ventral aspect and appear as a partly compacted epithelium. This epithelialization resembles compaction in mammals, in which "the first distinct laminin (appears) in the 16 cell compacted mouse embryo" (Leivo et al. 1980). Duband and Thiery (1987) claim that laminin in the avian embryo occurs at the onset of gastrulation but they did not study pre-gastrulation blastoderms.

Interruption of a laminin layer occurs during early primitive streak formation (stages 3–4). These results confirm the observations of Mitrani (1982) who studied the distribution of fibronectin and laminin in these stages with immunofluorescence. Our observations suggest that the ingressing cells carry away parts of the laminin layer. The experiments in which a preexisting laminin layer is disrupted during primitive streak induction are consistent with observations on the normal primitive streak.

In stages 5–8 the laminin layer alongside the primitive streak is thinner and may be discontinuous. It seems to thin out progressively below the future mesoblast cells that are going to deepithelialize and ingress. The ingressing cells interrupt this thinned layer in an irregular way.

This interruption of the laminin layer corresponds to the remnants of basal lamina in front of ingressing cells described by Vakaet et al. (1980) using transmission electron microscopy.

Below the median part of the neural plate the laminin layer is thinner and shows interruptions. This is consistent with neural induction experiments. The thinning and the interruptions of the laminin layer may diminish the rigidity of the basement membrane. This may facilitate the bending of the neural plate at the median hinge point described by Smith and Schoenwolf (1987).

The role of the changes in the laminin layer during normal and experimental inductions remains speculative. Duband and Thiery (1982) and Sanders and Prasad (1986) tried to interpret the changes in fibronectin distribution, although fibronectin is less specifically localized than is laminin. The parallel changes in the distribution of both glycoproteins as described by Mitrani (1982), suggest to us that they might be epiphenomena of the induction processes.

In conclusion we propose:

The observation that laminin appears first under individual cells of the monolayered upper layer, provides evidence that laminin is produced by these upper layer cells.

The presence of a continuous laminin layer is correlated with the epithelialization of the upper layer cells, which resembles compaction in early mammalian embryos.

Laminin is discontinuous at ingression sites and under the median part of the neural plate. This suggests that a continuous laminin layer confers rigidity onto the epiblast, whereas disruption of a laminin layer seems to be correlated with ingression of cells or bending of the neural plate.

Acknowledgements. We thank Guido Van Limbergen for invaluable technical assistance and the staff of the laboratory of Experimental Cancerology of Gent (Prof. M. Mareel) for reviewing the paper.

This study was supported by Grant No. 3.9001.87 of the Belgian National Fund for Medical Scientific Research.

References

Albrechtsen R, Nielsen M, Wewer U, Engvall E, Ruoslahti E (1981) Basement membrane changes in breast cancer detected by immunohistochemical staining for laminin. Cancer Res 41:5076–5081

De Bruyne GK, Bracke ME, Plessers L, Mareel MM (1988) Invasiveness in vitro of mixed aggregates composed of two human mammary cell lines MCF-7 and HBL-100. Invasion Metastasis 8:253–265

Duband J-L, Thiery JP (1982) Appearance and distribution of fibronectin during chick embryo gastrulation and neurulation. Dev Biol 94:337–350

Duband J-L, Thiery JP (1987) Distribution of laminin and collagens during avian neural crest development. Development 101:461–478

Eyal-Giladi H, Kochav S (1976) From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. Dev Biol 49:321–337

Hamburger V, Hamilton H (1951) A series of normal stages in the development of the chick embryo. J Morphol 88:49–92

Kleinman HK, Cannon FB, Laurie GW, Hassell JR, Aumailley M, Terranova VP, Martin GR, DuBois-Dalcq M (1985) Biological activities of laminin. J Cell Biochem 27:317–325

Le Douarin NM (1973) A Feulgen-positive nucleolus. Exp Cell Res 77:459–468

Leivo I, Vaheri A, Timpl R, Wartiovaara J (1980) Appearance and distribution of collagens and laminin in the early mouse embryo. Dev Biol 76:100–114

Lison L (1960) Histochimie et cytochimie animales, principes et méthodes, vol II, 3 edn. Gauthier-Villars, Paris, pp 398–842

Low FN (1967) Developing boundary (basement) membranes in the chick embryo. Anat Rec 159:231–238

Martin GR, Timpl R (1987) Laminin and other basement membrane components. Ann Rev Cell Biol 3:57–85

Merbach H (1935) Beobachtungen an der Keimscheibe des Hühnchens vor dem Erscheinen des Primitivstreifens. Z Anat Entwicklungsgesch 104:635–652

Mitrani E (1982) Primitive streak-forming cells of the chick invaginate through a basement membrane. Wilhelm Roux's Arch 191:320–324

Romeis B (1968) Mikroskopische Technik, 16 edn, R. Oldenbourg Verlag, München Wien, 757

Sanders EJ, Prasad S (1986) Epithelial and basement membrane responses to chick embryo primitive streak grafts. Cell Differ 18:233–242

Smith JL, Schoenwolf GC (1987) Cell cycle and neuroepithelial cell shape during bending of the chick neural plate. Anat Rec 218:196–206

Timpl R, Rohde H, Robey P, Rennard S, Foidart J-M, Martin G (1979) Laminin-a glycoprotein from basement membranes. J Biol Chem 254 19:9933–9937

Vakaet L (1970) Cinephotomicrographic investigations of gastrulation in the chick blastoderm. Arch Biol (Liège) 81:387–426

Vakaet L (1984) The initiation of gastrular ingression in the chick blastoderm. Am Zool 24:555–562

Vakaet L, Vanroelen Chr, Andries L (1980) An embryological model of non-malignant invasion or ingression. In: De Brabander M, is De Brabander M, Mareel M, De Ridder L (eds) Cell movement and neoplasia. Pergamon Press, Oxford New York, pp 65–75

Woodard AE, Mather FB (1964) The timing of ovulation, movement of the ovum through the oviduct, pigmentation and shell deposition in the Japanese quail (Coturnix coturnix japonica). Poultry Sci 43(6):1427–1432

Accepted January 25, 1989