Original Article

Cellular origin of the basement membrane in embryonic chicken/quail chimeras

FERNAND HARRISSON*

University Center of Antwerp, Department of Morphology, Laboratory of Human Anatomy and Embryology, Antwerp, Belgium

ABSTRACT The cellular origin of the components of the basal lamina of an epithelium, the chicken epiblast, has been investigated in a model system resulting from the transplantation of metabolically labeled quail hypoblast (an epithelium without basal lamina), associated or not with individual mesoblast cells, into an unlabeled chicken blastoderm deprived of its own hypoblast. The ability to discriminate chicken from quail cells after nuclear staining, combined with autoradiographic labeling of the basal lamina components, made it possible to determine the origin of the cells and labeled compounds in tissue sections of the chimeras. The transplantation of ^{3}H-glucosamine or ^{3}H-fucose-labeled quail hypoblast into a chicken host embryo, and subsequent culture of the chimeric embryo for 5 h, led to the transfer of labeled macromolecules from the quail graft to the chicken basal lamina. Pre-treatment of sections with several glycosaminoglycan-degrading enzymes with different substrate specificities suggested that the ^{3}H-glucosamine-labeled compounds that are deposited in the basal lamina are glycoproteins and/or heparan sulfate proteoglycan. However, in view of our current knowledge of the cellular origin of the latter compound, this molecule probably originates from the epithelium itself. Transfer of ^{3}H-proline and ^{3}H-hydroxyproline-containing molecules (mainly collagens) from the graft to the host basal lamina was not observed. Chasing the labeled compounds with unlabeled precursor during culture of the chimeras did not influence the final autoradiographic pattern. It is concluded that the basal lamina of the epiblast has a dual epithelial origin, resulting from the interaction of epiblast-derived materials and non-collagenous glycoproteins synthesized by the hypoblast. Evidence supporting the case of a non-epithelial, mesoblastic origin of non-collagenous glycoproteins was not found. An extensive review of literature on the epithelial vs non-epithelial origin of basement membrane components, mainly in mammalian species, is also provided.

KEY WORDS: *basal lamina, basement membrane, extracellular matrix, chicken/quail chimera, gastrulation*

Introduction

The classic concept of basement membranes, based upon light microscopy, has been that a basement membrane is a homogeneous hyaline band that separates an epithelium or an endothelium from connective tissue, and that takes silver stains for reticulin and is PAS-positive (Lillie, 1952). Gersh and Catchpole (1949) have postulated that they are formed by polymerization of glycoproteins of ground substance and thus are of connective tissue origin.

Using the electron microscope, Weiss and Ferris (1954) described an orthogonal system of fibrils in the basement «lamella» that underlies the epidermis of amphibian larvae. The collagenous nature of this lamella and the well-developed endoplasmic reticulum of the associated fibroblasts, together with evidence that fibroblasts can secrete collagen, prompted Weiss and Ferris to conclude that it is «the epidermal underside which serves as foundation for the

architectural development of the basement lamella». Contrasting with the views of Gersh and Catchpole (1949) and Weiss and Ferris (1954), Pierce et al. (1962) observed that parietal yolk cells are able to secrete the thick Reichert's basement membrane upon which they rest, and they can secrete in total absence of connective tissue. Moreover, they also found that basement membranes of some epithelia of mouse originate in the endoplasmic reticulum of the epithelial cells, and this material differs antigenically from collagen, reticulin, and basement membranes of blood vessels (Pierce et al., 1963). Although they still believed that basement

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*Address for reprints: University Center of Antwerp (RUCA), Laboratory of Human Anatomy and Embryology, 171 Groenenborgerlaan, B-2020 Antwerpen, Belgium. FAX: 32-2-218.0398.
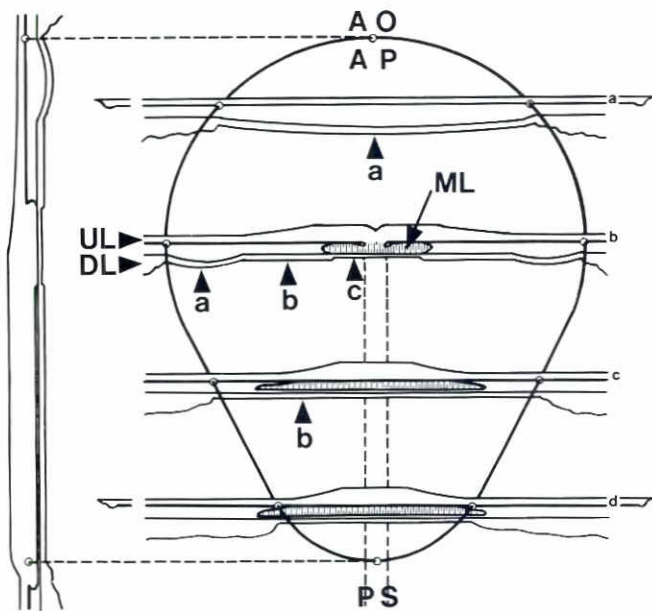
Fig. 1. Schematic representation of a stage-4 blastoderm (Vakaet, 1970), showing transverse sections and a mediosagittal section through the area pellucida. *The arrowheads indicate the different areas of the deep layer, i.e., endophyll (a), hypoblast (b) and definitive endoblast (c).*

membranes of blood vessels are of mesenchymal origin, they concluded that «it is probable that all epithelial cells secrete their own basement membranes». These contradictory views of the cellular origin of basement membrane components — non-epithelial vs epithelial origin — were unified by Hay and Revel (1963), who showed, using tritiated proline for the autoradiographic labeling of regenerating salamander limbs, that «collagen of basement lamella, and other prolin-rich proteins, are secreted by associated fibroblasts *and* by the epidermis, and accumulate at the epidermal-lamellar junction». In other words, both underlying fibroblasts and epidermis contribute to the basal lamina and striated collagen fibers of the basement membrane. Also emphasizing a non-epithelial contribution to the assembly of basement membranes, Kallman and Grobstein (1965) observed that «embryonic salivary mesenchyme initially labeled with tritiated proline or glycine, transmits a labelable material across a filter membrane between the mesenchyme and salivary epithelium», suggesting that basement membrane formation involves transfer of soluble tropocollagen from the mesenchyme, where it is synthesized, to the epithelial surface, where it is polymerized.

Notwithstanding the clear evidence for, at least, a non-epithelial contribution to the assembly of basement membranes, the idea that, unlike the striated collagen fibrils, epithelial cells themselves are the predominant source of basal lamina became strongest (Pierce et al., 1964; Pierce, 1965, 1966; Dodson and Hay, 1971; Hay and Dodson, 1973; Trelstad et al., 1974; Meier and Hay, 1975; Minor et al., 1976a,b; Banerjee et al., 1977). Lipton (1977), using cultures of embryonic quail myoblasts, was probably first to provide evidence for a dual origin of the basal lamina. Indeed, he observed that a distinct basal lamina and a fibrous extracellular matrix

develops in myoblast cultures only after the addition of muscle fibroblasts, suggesting a contribution of fibroblasts to the formation of basement membranes in skeletal muscle. However, although no extracellular matrix was observed in absence of fibroblasts, a soluble collagenous protein could be recovered from the culture medium. These preliminary observations were confirmed by several authors, who showed that both myogenic and fibrogenic cells of mouse and quail skeletal muscles synthesize and secrete types I, III and V collagen of the fibrous extracellular matrix (Sasse et al., 1981), as well as type IV collagen and laminin of the basal lamina (Kühl et al., 1982). In a further series of experiments, Kühl et al. (1984) and Sanderson et al. (1986) also added to the concept that type IV collagen synthesized by muscle fibroblasts is incorporated into the basal lamina of myotubes. Finally, in *Drosophila*, the αIV collagen chain is synthesized by individual mesoblast cells and deposited in basement membranes of skeletal and visceral muscles (Le Parco et al., 1989).

Since the early eighties, several other systems have been the object of studies on the origin of basement membrane components. Studies in the developing kidney have clearly indicated that the glomerular basement membrane originates from both endothelial, visceral epithelial and mesangial cells (Thorning and Vracko, 1977; Nayyar et al., 1980; Abrahamson, 1985; for review see Sariola, 1985). Especially the work of Sariola et al. using interspecies hybrid glomeruli and species-specific antibodies, deserves special attention. These authors explanted 11-day-old mouse embryonic kidney rudiments on chicken chorioallantoic membrane. During incubation, the chicken vessels invade the mouse tissue, forming hybrid glomeruli composed of mouse epithelium and chicken endothelium. After immunostaining with species-specific antibodies, their results have shown that type IV collagen has a dual origin, namely the podocytes and the endothelial cells (Sariola, 1984; Sariola et al., 1984a), while fibronectin is not synthesized by glomerular epithelial cells, the main endogenous cellular sources for fibronectin in the embryonic kidney being interstitial and vascular cells (Sariola et al., 1984b).

In normal and fibrotic adult livers, several cell types participate in the elaboration of the connective biomatrix. Hepatocytes are capable of producing laminin, fibronectin and types I and IV collagen; fat-storing cells synthesize types III and IV collagen and, to a lesser extent, fibronectin and type I collagen; endothelial cells produce small amounts of all components (Foidart et al., 1980; Diegelmann et al., 1983; Clément et al., 1984, 1986, 1988; Geerts et al., 1986). This mixed origin of the extracellular matrix has been confirmed by Rescan et al. (1989) in developing human and rat livers.

In the intestine, the basal lamina molecules deposited at the epithelial-mesenchymal interface are synthesized by both mesenchymal and endodermal cells (Weiser et al., 1989, 1990; Simon-Assmann et al., 1988, 1990; Simo et al., 1992). Heparan sulfate proteoglycan is produced exclusively by the epithelial cell population or enterocytes (Simon-Assmann et al., 1989). Using embryonic rat/chicken and mouse/chicken interspecies hybrid intestines, Simo et al. (1992) were able to demonstrate that laminin originates from both mesenchymal and endodermal cells, but that the deposition of its A and B1/B2 chains by the mesenchyme occurs asynchronously.

The mammary epithelial cells synthesize fibronectin, laminin, type IV collagen, proteoglycans and hyaluronate (David and Bernfield, 1979; Liotta et al., 1979; Gordon and Bernfield, 1980; Warburton
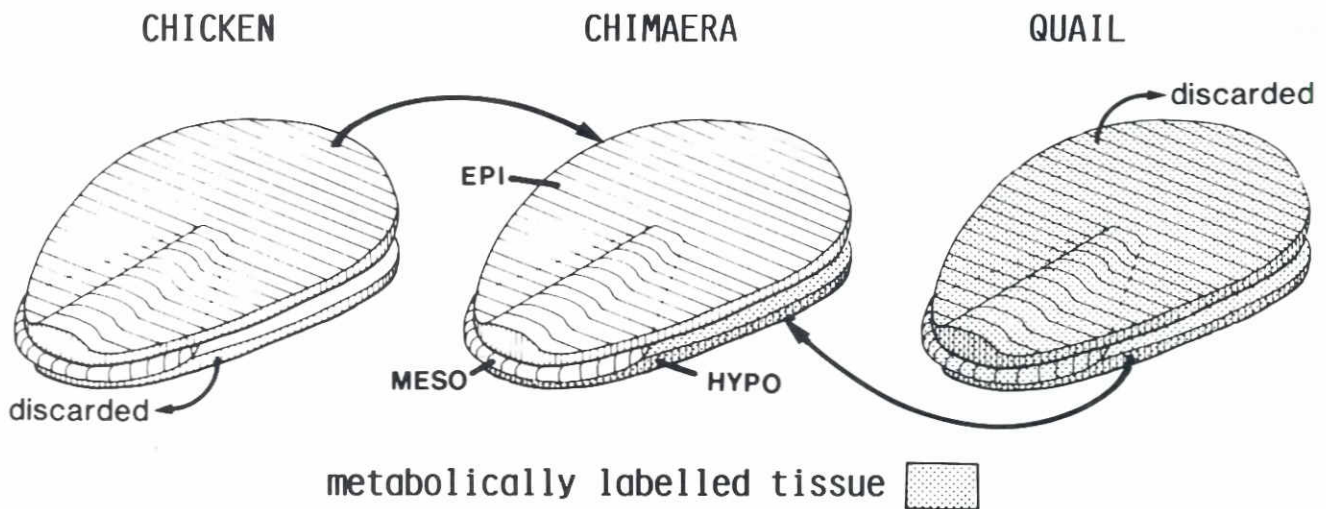
Fig. 2. Schematic representation of the experimental procedure used for the preparation of metabolically labeled chimeras. *A labeled quail deep layer (mainly hypoblast) is transplanted into an unlabeled chicken blastoderm deprived of its own deep layer.*

et al., 1981; Silberstein and Daniel, 1982; David and Van den Berghe, 1983, 1985; Ormerod *et al.*, 1983; Rapraeger and Bernfield, 1983). However, two different mesenchymes also appear to participate in the synthesis of basement membrane components during initiation of ductal branching of the epithelium of mouse mammary gland: while fibroblastic mesenchymal cells synthesize fibronectin at all times, the fat pad precursor cells start synthesizing laminin and heparan sulfate proteoglycan only from the 16th day of gestation on (Kimata *et al.*, 1985). Moreover, interstitial type I collagen may stabilize the basal lamina proteoglycans by reducing their degradation (David and Bernfield, 1981). Entactin, which is produced by fibroblastic cells in culture, but not by mammary epithelial and myoepithelial cells, is present in the lamina lucida and lamina densa, and is associated with interstitial collagen fibers surrounding the secretory alveoli in lactating rats. In virgin rats, entactin is present only in vascular basement membranes and in interstitial connective tissue of the mammary gland (Warburton *et al.*, 1984).

The basement membrane interfacing the inner dental epithelium and the dental papilla has been considered to be epithelially derived, since tritiated glucosamine is not stored by EDTA-isolated dental papillae (Frank *et al.*, 1979) and since trypsin-isolated enamel organs cultured on top of plasma coagulum are able to deposit a new basement membrane (Osman and Ruch, 1980, 1981). The latter argument has, however, been contradicted by Brownell *et al.* (1981), who pointed out that a basal lamina is reconstituted only in the presence of substrata containing fibronectin. Brownell *et al.* (1981) also demonstrated that fibronectin, which is produced by the dental papilla mesenchyme, can be isolated from the basal lamina of enamel organ epithelial explants. Since fibronectin is not produced by isolated epithelia, but exclusively by the differentiating mesenchymal cells (Hurmerinta *et al.*, 1986), the basal lamina results from a supramolecular interaction between epithelium-derived macromolecules, such as type IV collagen, glycosaminoglycans (GAGs), proteoglycans and laminin, and mesenchyme-derived fibronectin. Similar observations have been made in rat parietal yolk sacs, which synthesize and secrete

laminin, type IV procollagen and entactin in Reichert's membrane, but do not synthesize any detectable fibronectin (Amenta *et al.*, 1983). Tenascin appears to be synthesized by the epithelium, at least in growing brochi (Koch *et al.*, 1991).

Summarizing, in spite of a large variability in structure and chemical composition of basement membranes, a dual cellular origin (epithelial/mesenchymal or epithelial/endothelial) of the basal lamina as well as of the interstitial reticular layer (pars fibroreticularis) has been demonstrated in several systems. However, difficulties in interpreting results obtained by light microscopy often stem from the facts that it is difficult to discern the basal lamina from the basement membrane, and that it is not always clear whether fibronectin has to be considered as an integral component of the basal lamina, as a molecule associated with the interstitial fibrous matrix, or merely as an exogenous component trapped from body fluids. The stage-4 (H&H st. 2) chicken blastoderm (Fig. 1), therefore, appears to be the material of choice for the study of the cellular origin of basal lamina components. Indeed, the basal lamina at the stage mentioned above is not yet associated with a reticular layer, while fibronectin is already an integral component of the basal lamina (Sanders, 1982; Harrisson *et al.*, 1985c). Blood circulation is not yet established at the used stage, a feature that eliminates the possibility of trapping of exogenous components. Metabolically labeled quail deep layers, associated or not with quail middle-layer cells, were transplanted into unlabeled chicken blastoderms (Fig. 2) to visualize a possible participation of the deep layer (composed of endophyll, hypoblast and definitive endoblast) or of the middle layer (mesoblast) in the synthesis and assembly of upper layer (epiblast) basement membrane — the term basement membrane being used whenever the chicken blastoderm is concerned, merely because the results were obtained by light microscopy, but bearing in mind that the basement membrane of the chicken blastoderm is composed, at the ultrastructural level, only of basal lamina (lamina densa plus lamina lucida, without pars fibroreticularis). The ability to distinguish chicken from quail cells after nuclear staining (Le Douarin, 1973), combined with autoradiographic labeling of the extracellular matrix, made it possible to determine the origin

of the cells and labeled matrices in the chimeras. The precursors used for metabolic labeling of the extracellular matrix were ^{3}H-glucosamine, which incorporates into GAGs and (collagenous and non-collagenous) glycoproteins; ^{3}H-fucose, which specifically labels glycoproteins; and ^{3}H-proline, which incorporates in large amounts into the primary amino acid sequence of collagen precursors and becomes hydroxylated within the cisternae of the rough endoplasmic reticulum without loss of label. Glycolipids are not preserved in our experimental procedure.

Results

After transplantation of a ^{3}H-glucosamine-labeled quail deep layer into an unlabeled chicken blastoderm deprived of its own deep layer and after 5 h culture, the autoradiographs not only demonstrated silver grains over the quail tissue, as expected, but also along the basal surface of the chicken epiblast (Figs. 3-5), where electron microscope studies have localized a basal lamina (lamina lucida plus lamina densa, without pars fibroreticularis). The labeling was low in the vicinity of the primitive streak and higher towards the lateral borders of the embryonic area. This labeling was restricted to the region of the basement membrane situated immediately above labeled quail deep layer, and did not occur where it was underlain by unlabeled chicken deep layer. Labeling at the basal side of the epiblast was also interrupted at the level of the primitive streak, where a basement membrane is not present. The quail cells could easily be recognized by their Feulgen-positive nucleolus. The chicken epiblast cells did not show silver grains above background values over their cytoplasm.

Culture of the chimeras for 5 h on a medium containing unlabeled glucosamine influenced neither the final autoradiographic pattern nor the density of silver grains in the basement membrane (Fig. 6). The labeling of the graft was, however, somewhat less pronounced.

Treatment of the sections with chondroitinase ABC (Fig. 7), testicular hyaluronidase (Fig. 8), or *Streptomyces* hyaluronidase (not shown) suggested that the labeled compounds that are deposited in the basement membrane are glycoproteins and/or heparan sulfate proteoglycan, since the final autoradiographic labeling remained unchanged after digestion of hyaluronate and chondroitin sulfate proteoglycans. The effective digestion of these GAGs was controlled using alcian blue staining, which was negative after enzymatic treatment (not shown).

The use of ^{3}H-fucose also resulted in labeling of the chicken basement membrane according to a gradient increasing from the primitive streak towards the lateral and anterior edges of the area pellucida (Figs. 9-11). Labeling of the chicken basement membrane was most pronounced at the level of the endophyllic crescent, where mesoblast cells are not present (Fig. 9). The labeling of the

basement membrane region at the epithelial-mesenchymal interface appeared to be somewhat less pronounced than when using ^{3}H-glucosamine. Chase with cold fucose did not influence the labeling pattern in the basement membrane (not shown).

Tritiated proline did not appear to participate in the transfer of macromolecules from the quail deep layer to the chicken basement membrane, since labeling occurred only in quail tissue, and never at the level of the basement membrane (Figs. 12-14). The addition of unlabeled proline to the culture medium of the chimeras had no clear effect (not shown). ^{3}H-4-hydroxyproline could not be incorporated in the quail embryo.

The presence of labeled quail mesoblast cells (Figs. 8,11,14) or of unlabeled chicken mesoblast cells (Figs. 6,10,13), as well as the absence of mesoblast cells (Figs. 3,9,12), between the chicken basement membrane and the labeled quail deep layer did not interfere with the final result, whatever the label used, suggesting that mesoblast cells had no particular role in the deposition of labeled macromolecules from the graft to the host.

Discussion

The choice of label

Metabolic labeling with labeled glucosamine, fucose and proline, among other precursors, followed by autoradiographic localization of the label, became a very popular method in the early seventies. ^{3}H-glucosamine incorporates into a variety of macromolecules, including GAGs and (collagenous and non-collagenous) glycoproteins (Warren, 1972). This method, combined with enzymatic degradation of particular carbohydrate moieties, permitted the identification and autoradiographic localization of GAGs in the early chicken embryo (for references see Vanroelen *et al.*, 1980a,b).

^{3}H-fucose proved to be an excellent precursor for the study of newly-synthesized glycoproteins (for review see Bennett and Leblond, 1977), because it is not readily catabolized or converted to other sugars (Sear *et al.*, 1977). Consequently, ^{3}H-fucose is incorporated as such, predominantly in glycoproteins and, to a much lesser extent, in glycolipids. The latter compounds, as well as fucose-1-P and GDP-fucose are, however, not preserved during routine histological procedures. Therefore, it is believed that the autoradiographic reaction observed in the sections indicates the presence of glycoproteins, where it occupies non-reducing positions in the carbohydrate side chains (for references see Severson, 1984).

^{3}H-proline has often been used for the autoradiographic labeling of collagenous glycoproteins in the extracellular matrix and in basement membranes (for references see Weinstock and Leblond, 1977; Minor *et al.*, 1976a; Romen *et al.*, 1976; Johnson, 1986; Fatemi, 1987), because collagens contain high amounts of 4-hydroxyproline (for review see Timpl and Martin, 1982). Indeed, ^{3}H-

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Figs. 3-14. Photomicrographs of transverse sections of stage-4 chicken/quail chimeras in which the quail deep layer has been labeled previous to transplantation in a chicken host blastoderm. *Magnifications: x600. For each experimental series, the pictures are from the same blastoderm. The pictures have been focused on the silver grains. Consequently, fine structural details, such as quail nucleoli, are not clear in all cells. The drawings show the place where the photomicrographs were taken. The dashed area indicates the location of the mesoblast.* **(3-8) Xenographs composed of unlabeled chicken tissue and ^{3}H-glucosamine-labeled quail deep layer associated or not with labeled quail middle layer. (3, 4 and 5)** *Show labeling in the quail tissue and in the basement membrane (arrowheads) of the chicken epiblast.* **(6)** *Shows a chimera cultured on a medium containing unlabeled glucosamine (chase experiment). The autoradiographic labeling is essentially the same as in* **(5)**, *suggesting that unprocessed ^{3}H-glucosamine is not deposited in the basement membrane.* **(7 and 8)** *Show sections of chimeras treated with chondroitinase ABC and testicular hyaluronidase, respectively, before Feulgen staining and dipping. The results suggest that the labeling in the basement membrane is due to the presence of glycoproteins and/or heparan sulfate proteoglycan.*
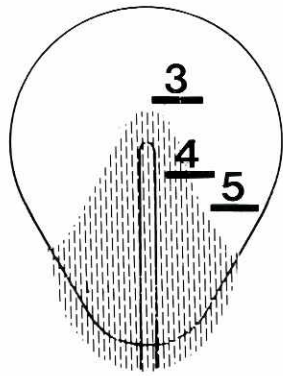
D-GlcN
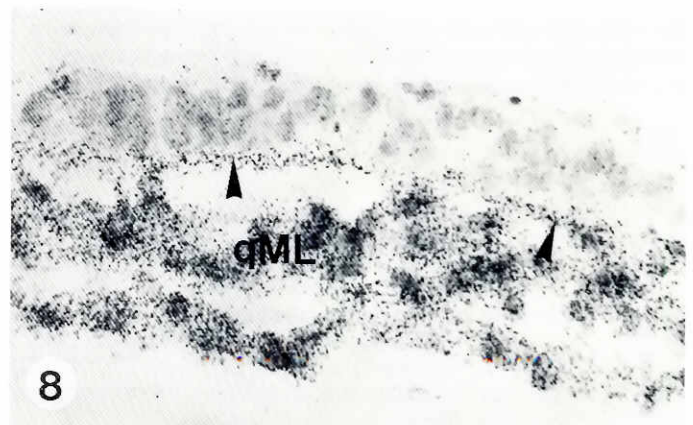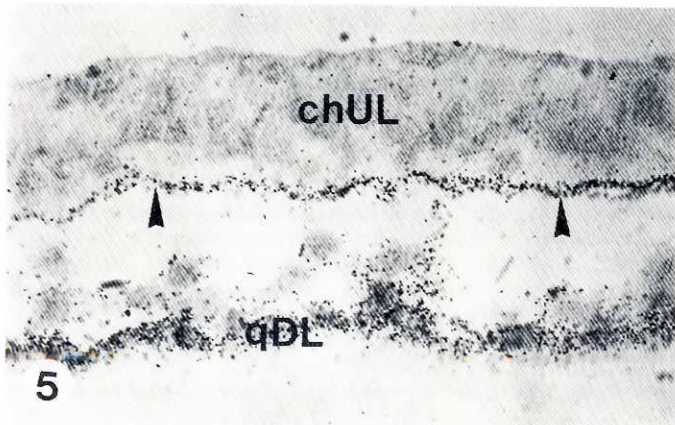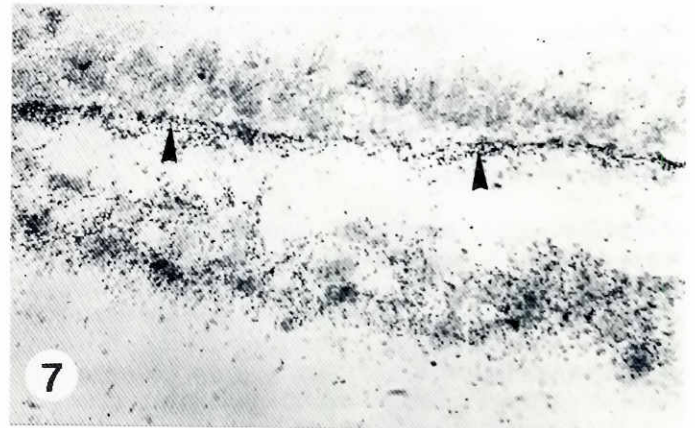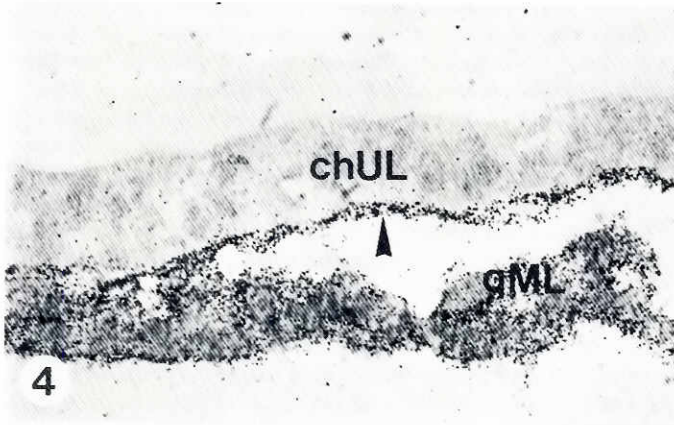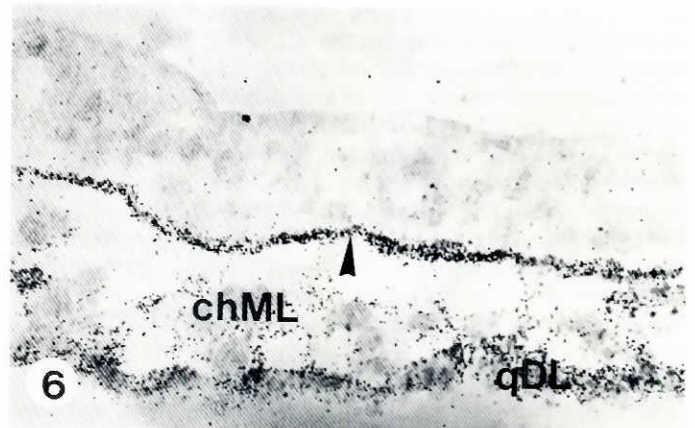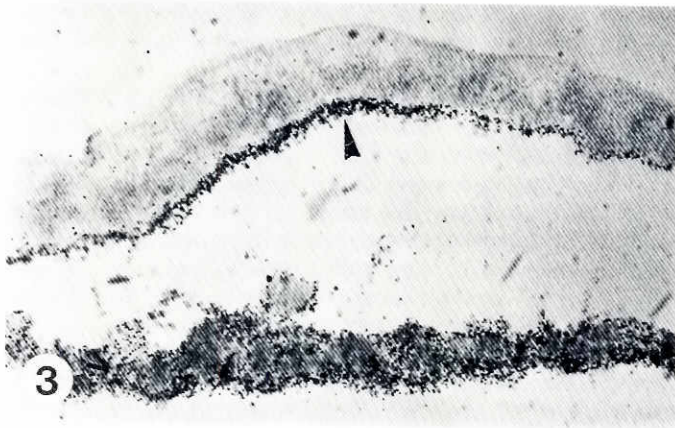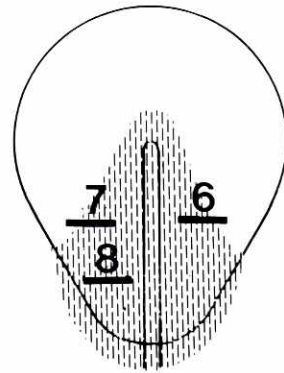
proline applied in culture becomes incorporated into the primary amino acid sequence of collagen precursors and becomes hydroxylated within the cisternae of the rough endoplasmic reticulum without loss of label (Prockop et al., 1979a,b; for review see Fessler et al., 1985). Therefore, isotopic labeling of ^{3}H-proline, followed by autoradiographic localization of the label, indicates the presence of both proline and hydroxyproline. Most studies of collagen have indicated that it is either metabolically inert or has extremely low rates of synthesis and degradation (Nissen et al., 1978).

The chicken/quail system

The results reported here show that culture of chimeric avian blastoderms has many attractive features for studying the cellular origin of basal lamina components. First, the ability to distinguish chicken from quail cells after nuclear staining (for review see Le Douarin, 1973), combined with autoradiographic labeling of the extracellular matrix, makes it possible to determine both the origin of cells and of labeled matrices in the chimeras.

Second, at the time of gastrulation, the basement membrane is composed only of a basal lamina (lamina densa plus lamina lucida, without reticular layer; for review see Sanders, 1983), fibronectin is an integral component of the basal lamina (Sanders, 1982; Harrisson et al., 1985c), and trapping of exogenous components from the blood circulation is excluded. These features make it possible to investigate, at the light-microscope level, the cellular origin of components in an epithelial basal lamina.

Finally, the method has the advantage of associating in culture an epithelium and its basal lamina (the epiblast) with another epithelium (mainly hypoblast, without own basal lamina) and, eventually, with mesenchymal cells (the mesoblast cells). In addition to a cellular origin of basal lamina components from the epithelium upon which the basal lamina rests, this experimental procedure makes it possible to investigate whether the basal lamina has a dual epithelial origin (epiblast and hypoblast), and whether the basal lamina has an additional non-epithelial origin (epiblast and mesoblast cells).

Dual epithelial origin of basal lamina components

The results demonstrated that ^{3}H-glucosamine-containing macromolecules, i.e. GAGs and/or glycoproteins, but not unprocessed ^{3}H-glucosamine, are synthesized by the deep layer and deposited in the basal lamina of the epiblast. Enzymatic digestion of hyaluronate and chondroitin sulfates A, B, and C, using several GAG-degrading enzymes with different substrate specificities, suggested that the labeled compounds that are deposited in the basal lamina are glycoproteins and/or heparan sulfate proteoglycan. Indeed, analyses of the chemical composition of GAGs in the chicken blastoderm revealed that, among the 10% of the GAGs that are sulfated (84% of the total GAGs represents hyaluronate), 55% are sensitive to

testicular hyaluronidase (the chondroitin sulfates) and 36-39% are resistant to enzyme degradation, but sensitive to nitrous acid (heparan sulfate). Thus heparan sulfate represents about 4% of the total amount of GAGs (Solursh, 1976). Autoradiographically, Vanroelen et al. (1980a) has also demonstrated the presence of a minor testicular hyaluronidase-resistant fraction of ^{3}H-glucosamine-containing molecules in the basal lamina and in the extracellular matrix of cells of the primitive streak and of the deep layer. Although we can not exclude the possibility of a transfer of heparan sulfate proteoglycan from the deep layer to the basal lamina of the epiblast, we suggest that, as in the inner dental epithelium (Frank et al., 1979; Brownell et al., 1981), the mammary gland (David and Bernfield, 1979; David and Van den Berghe, 1983), and the intestine (Simon-Assmann et al., 1989), this sulfated proteoglycan may originate from the epithelium upon which rests the basal lamina.

The use of ^{3}H-fucose, which specifically labels glycoproteins, confirmed the transfer of, at least, glycoproteins from one epithelium to the basal lamina of another. Conspicuously, the labeling of ^{3}H-fucose-containing compounds in the basal lamina was highest in the endophyllic crescent. It is worth noting (i) that this pattern of autoradiographic labeling strongly recalled the gradient of fibronectin immunoreactivity, increasing from medial to lateral in the area pellucida, which was previously observed in the basal lamina (Harrisson, 1989), and (ii) that carbohydrate analyses of the collagen component of various basement membranes did not reveal the presence of fucose (Kefalides et al., 1979). This suggests that the ^{3}H-fucose-labeled compounds are mainly non-collagenous glycoproteins.

In order to discriminate between non-collagenous glycoproteins and collagens, ^{3}H-proline-labeled deep layer has been transplanted in chicken host embryos. Although type IV collagen has been localized to the basal lamina of the epiblast (Harrisson et al., 1991), the deep layer does not appear to participate in the synthesis and assembly of basal lamina components, since labeled proline- and hydroxyproline-containing molecules were never found in the basal lamina. It is concluded that the basal lamina of the epiblast results from the interaction between epiblast-derived compounds and, at least, a class of non-collagenous glycoproteins synthesized by the deep layer. Our light-microscope observations do not make it possible to discriminate between endophyll, hypoblast and definitive endoblast, but we are convinced that the area of the deep layer of stage-3/4 blastoderms that we have used for transplantation is composed almost exclusively of hypoblast. In this respect, it is worth mentioning that the formation of the basal lamina correlates with the completion of the hypoblast in early gastrulation (Harrisson et al., 1991). So far, we have no arguments supporting the case of a non-epithelial, mesoblastic origin of non-collagenous glycoproteins, and we have no reasons to believe that mesoblast cells play a

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Figs. 3-14. Photomicrographs of transverse sections of stage-4 chicken/quail chimeras in which the quail deep layer has been labeled previous to transplantation in a chicken host blastoderm. Magnifications: x600. For each experimental series, the pictures are from the same blastoderm. The pictures have been focused on the silver grains. Consequently, fine structural details, such as quail nucleoli, are not clear in all cells. The drawings show the place where the photomicrographs were taken. The dashed area indicates the location of the mesoblast. **(9-14) Xenografts composed of unlabeled chicken tissue and ^{3}H-fucose labeled, (9-11)** ^{3}H-proline labeled **(12-14)** quail deep layer, associated or not with labeled quail middle layer. Using ^{3}H-fucose, the chicken basement membrane (arrowheads) became labeled, confirming that at least glycoproteins are deposited in the basement membrane. Deposition of ^{3}H-proline/hydroxyproline-containing macromolecules could never be observed (the arrowheads indicate unlabeled basement membrane), suggesting that the deep layer (probably hypoblast) participates in the assembly of the basement membrane by the synthesis and deposition of non-collagenous glycoproteins.
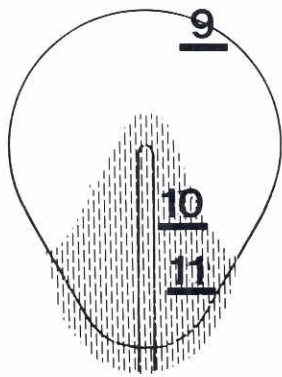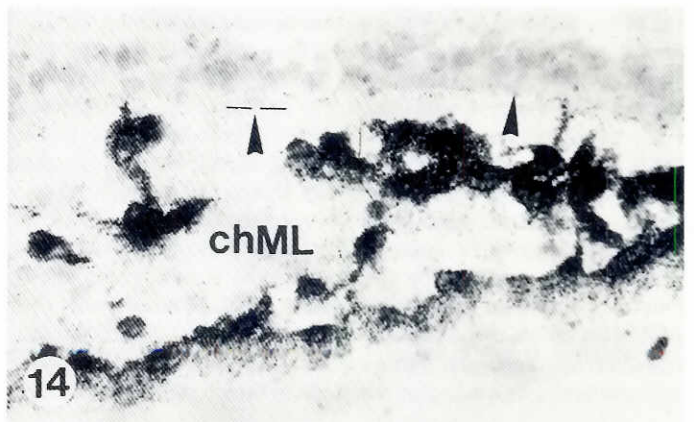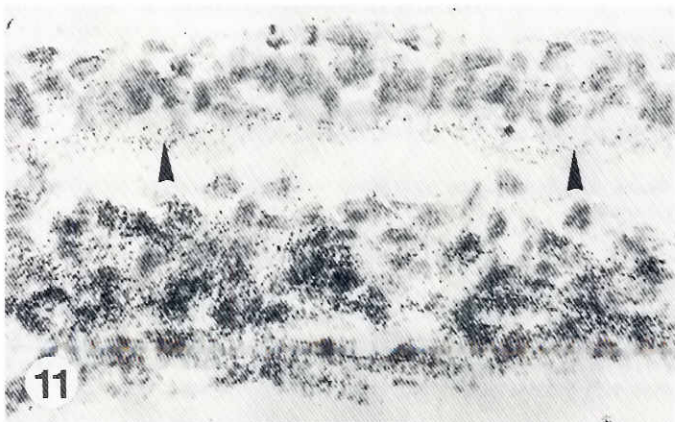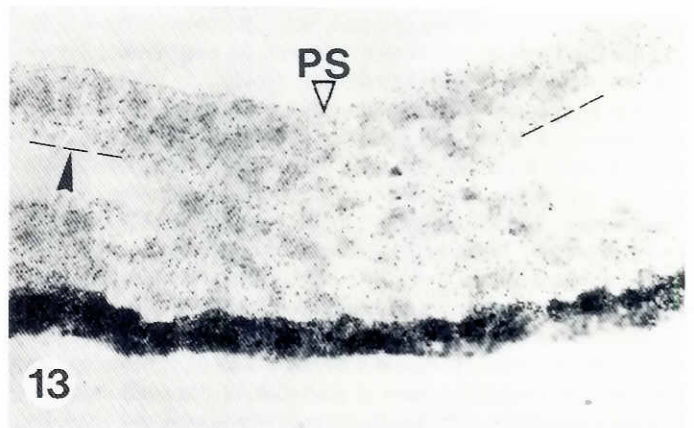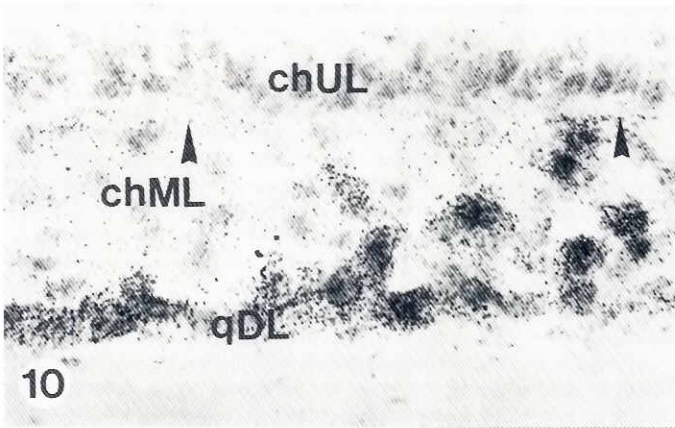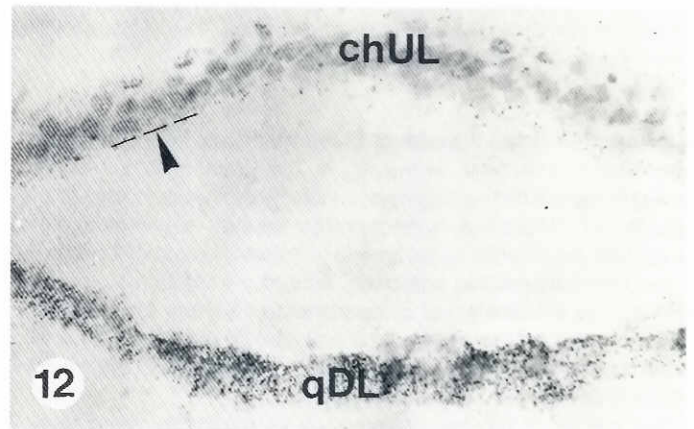
L-Fuc   L-Pro
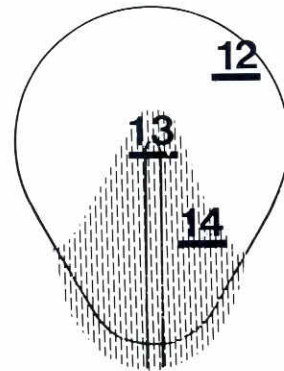
OVERVIEW OF THE PROCEDURE USED FOR ISOTOPIC LABELING OF EXTRACELLULAR MATRIX COMPONENTS

Radiochemical (incorporation for 2h)	Quail embryos			Chimeras		
	dose (in µCi/ml)	spec. act. (in Ci/mmol)	fixative (after 5h culture)	n (normal medium)	n (chase medium)
D-[6-³H]-glucosamine	24	34.6	AFA	29	8
	24	40	FoCa/CPC	12	-
L-[6-³H]-fucose	42	20	AFA	7	7
L—[5-³H]-proline	56	15	AFA	13	20
L—[4-³H]-hydroxyproline	40	5.9	AFA	7	-

AFA stands for Alcohol-Formaldehyde-Acetic acid; FoCa/CPC for Formaldehyde-Calcium chloride/CetylPyridinium Chloride. The radiochemicals were obtained from the Radiochemical Centre (Amersham, UK).

particular role in the transfer of these materials from the labeled hypoblast to the host. However, in the case of basal lamina collagen, we can not exclude the possibility that visualization of the transfer of ³H-proline/hydroxyproline-labeled molecules from mesoblast cells to the basal lamina is below the limit of sensitivity of our autoradiographic approach. Indeed, in addition to the epithelium, the mesenchymal compartment of several systems contributes to the synthesis of type IV collagen (in muscle: Kühl et al., 1982, 1984; Sanderson et al., 1986; Le Parco et al., 1989; in the intestine Simon-Assmann et al., 1988).

The nature of the non-collagenous glycoproteins must be investigated further, but it is pertinent to mention (i) that both the chicken hypoblast and the epiblast, but not the mesoblast cells, have the capability to synthesize, at least, fibronectin and laminin in culture (Sanders, 1980; Mitrani and Eyal-Giladi, 1982), and (ii) that both glycoproteins have been localized to the basal lamina of the epiblast (for review see Harrisson, 1989). However, if we refer to the results of Brownell et al. (1981) and Hurmerinta et al. (1986), we note (i) that fibronectin, which is produced exclusively by the differentiating papilla mesenchyme, can be isolated from the basal lamina of enamel organ epithelial explants, which do not produce fibronectin themselves, and (ii) that the basal lamina of trypsin-isolated enamel organs is reconstituted only in the presence of substrata containing fibronectin. Finally, the case of a non-epithelial origin of fibronectin is supported by Sariola et al. (1984a,b), who demonstrated that fibronectin in glomeruli is not synthesized by glomerular epithelial cells, but by interstitial and vascular cells, and by Kimata et al. (1985), who reported that fibroblastic mesenchymal cells in the developing mammary gland synthesize fibronectin at all times. Laminin is usually considered to be derived from epithelial cells (see Introduction). However, several exceptions have been reported. EHS sarcoma cells (Klebe, 1974; Timpl et al., 1979; Foidart et al., 1982), fibrogenic cells of skeletal muscles (Khül et al., 1982), Schwann cells (Cornbrooks et al., 1983), astrocytes (Liesi et al., 1983), fat pad precursor cells of developing mammary gland (Kimata et al., 1985), and intestinal mesenchymal cells (Simo et al., 1992) synthesize laminin. Entactin, a laminin-associated glycoprotein, is produced by fibroblastic cells in culture, but not by mammary epithelial and myoepithelial cells (Warburton et al., 1984). This review of the literature demonstrates that the synthesis, deposition and assembly into a macromolecular scaffold known as the basal lamina is a complex mechanism, which is probably unique for each system, depending on the developmental fate, the physiological or pathological state of the system, and the environmental conditions (in vivo vs in vitro, for example). In our experimental conditions, we have no evidence for the participation of the mesenchyme in the organization of fibronectin and laminin in the basal lamina. Instead, evidence for a dual epithelial origin of these major non-collagenous glycoproteins of the basal lamina is provided.

Materials and Methods

Preparation of chimeric embryos

Fertilized chicken eggs (Warren SSL strain from commercial stock) and quail eggs (Coturnix coturnix japonica from a laboratory breed) were incubated for 8-12 h at 38°C, to obtain stage-3/4 blastoderms (Fig. 1; Vakaet, 1970, in the chicken; for review see Harrisson et al., 1988), corresponding to stage 2 of Hamburger and Hamilton (1951). The stages of development of quail embryos were determined by analogy with the chicken. The embryos were explanted with their vitelline membrane on a glass ring (New, 1955; for details see Harrisson and Vakaet, 1989). The quail embryos were cultured for 2 h on a medium containing 0.5 ml egg white and 0.5 ml Ringer solution mixed with 3 mg agar and supplemented with one of the radiochemicals listed in Table 1.

Chicken/quail xenografts (n= 103) were obtained by transplanting labeled quail deep layers, associated or not with labeled quail mesoblast cells, into unlabeled chicken blastoderms deprived of their own deep layer (Fig. 2; for details see Harrisson et al., 1985b). To obtain such chimeras, deep layers of unlabeled chicken blastoderms mounted with their vitelline membrane on a glass ring and labeled quail blastoderms cultured for 2 h on a radioactive medium, were removed using 0.04 mm diameter watchmaker needles, and the quail deep layers were transplanted into the previously operated chicken embryos. The chimeras were further cultured for 5 h on the above medium without tritiated precursor. The development of the chimeras in culture was normal; healing of the wound occurred within a few hours, provided that the polarity of the transplanted deep layers had been respected.

Radioactive precursors and chase experiments

The radioactive compounds that have been used to label the extracellular matrix, their specific activity, the dose applied in the culture medium, and the number of embryos used in each experimental series are listed in Table 1. D-[6-³H]-glucosamine has been used for the metabolic labeling of GAGs and (collagenous and non-collagenous) glycoproteins. L-[6-³H]-fucose is a glycoprotein-specific marker. Labeling with L-[5-³H]-proline, which becomes hydroxylated, exploits the fact that hydroxyproline is present in large amounts and almost uniquely in collagens. L-[4-³H(G)]-hydroxyproline, which is never incorporated as such in collagens, was used as a control.

In order to determine autoradiographically whether there was a redistribution or reutilization of the label, we performed chase experiments by adding an excess of unlabeled D-glucosamine (0.8 mM), L-fucose (12.2 mM), or L-proline (2.3 and 4.9 mM) to the culture medium of the chimeras. Although there was no reutilization of the hydroxyproline, the reutilization of proline before its post-translational hydroxylation is generally considered as a potential source of error (Jackson and Heininger, 1975). Therefore, it is essential to eliminate such proline reutilization by chasing with large amounts of unlabeled proline subsequent to administering the radioactive amino acid (Nissen et al., 1978; Laurent, 1982).

Tissue preparation and treatment with glycosaminoglycan-degrading enzymes

After culture for 5 h, the chimeric embryos were fixed overnight, either in a mixture of 72% ethanol, 8% formaldehyde and 5% acetic acid, or in a solution containing 10% formaldehyde, 2% calcium chloride and 0.5% cetylpyridinium chloride. The addition of cetylpyridinium chloride to aqueous fixatives is a long-standing method, which is commonly used to ensure the preservation of GAGs in tissues (Scott, 1955). After fixation, the chimeras were dehydrated in an ascending series of ethanol concentrations, cleared

in xylene, and embedded in Paraplast Plus embedding medium. A nuclear staining according to Feulgen and Rossenbeck (1924) was performed on rehydrated, 6-μm-thick sections before processing for autoradiography. The advantage of this nuclear staining is that it makes it possible to distinguish chicken from quail cells on the basis of their different nuclear characteristics during interphase (for review see Le Douarin, 1973).

Several studies have shown that treatment of tissue sections with GAG-degrading enzymes makes it possible to discriminate between ^{3}H-glucosamine-labeled GAGs and glycoproteins, the former being digested during the enzymatic treatment (in the early chicken embryo: for references see Vanroelen *et al.*, 1980a,b; Harrisson *et al.*, 1984, 1985a). Therefore, serial sections of ^{3}H-glucosamine-labeled chimeras were incubated, before Feulgen staining, in one of the following GAG-degrading enzyme preparations:

(1)   for 4 h at 37°C in 0.1% bovine testicular hyaluronidase type I-S (300 NF units/mg solid; Sigma, St. Louis, USA) dissolved in 0.1 M phosphate buffer at pH 5. This enzyme degrades hyaluronate and chondroitin sulfate A/C proteoglycans (Leppi and Stoward, 1965).

(2)   for 2 h at 37°C in *Streptomyces* hyaluronidase (100 turbidity-reducing units/ml buffer; Calbiochem-Behring Corporation, La Jolla, CA, USA) dissolved in 0.1 M phosphate buffer at pH 5. This enzyme exhibits an absolute substrate specificity for hyaluronate (Yamada, 1973).

(3)   for 2 h at 37°C in chondroitinase ABC from *Proteus vulgaris* (1 unit/ml buffer; Sigma, St. Louis, USA) dissolved in enriched Tris-buffer at pH 8.6. This enzyme degrades the corresponding chondroitin sulfate proteoglycans (Derby and Pintar, 1978).

Controls included incubations of serial sections with buffer solution, and staining of GAGs with alcian blue (for details see Harrisson *et al.*, 1985a).

Preparation of autoradiographs

Rehydrated and stained sections, some of them after enzymatic treatment, were dipped at 45°C in darkroom conditions in L4 nuclear emulsion (Ilford Ltd., Basildon Essex, UK), diluted in an equal volume of distilled water. The air-dried slides were exposed for 8-10 weeks at 4°C in light-tight black boxes, developed in Kodak D-19, and mounted for observation in DePeX mounting medium (BDH Gurr, Poole, UK).

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