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Labelling of basement membrane constituents in the living chick embryo during gastrulation

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SUMMARY

The basement membrane of the living chick embryo epiblast has been labelled with ultrastructural markers in order to study the movement and turnover of this structure during gastrulation. Two problems were addressed in these experiments. Firstly, to what extent does the basement membrane move medially with the epiblast during morphogenesis? Secondly, what is the relationship to the basement membrane of the so-called interstitial bodies? The ultrastructural markers used were concanavalin A conjugated to ferritin and fibronectin antibodies conjugated to peroxidase. Embryos were cultured using the technique of New, and the label was applied to the periphery of the basal surface of the epiblast through a hole in the endoblast at the early primitive streak stage of development. The embryos were then allowed to develop to the full primitive streak stage in the presence of the label. When the position of the label was determined after incubation, it was found to have accumulated in large amounts at the edge of the primitive streak at the point where the basement membrane is disrupted. This indicates that constituents of the basement membrane are transported medially with the epiblast cells and are sloughed off as the latter pass through the primitive streak. This movement of basement membrane constituents is counter to the direction of migration of the underlying mesoderm cells. When embryos are exposed to label for only 1 h, then washed and incubated for a further three hours, the marker was found in the interstitial bodies and not distributed throughout the basement membrane itself. This suggests that the interstitial bodies, which have been implicated in influencing the migration of the mesoderm cells, are turnover products of the basement membrane to which they are attached.

INTRODUCTION

During gastrulation in the chick embryo, cells of the upper epithelial layer (epiblast) move towards the mid-line of the blastoderm and migrate through the primitive streak. The majority of the invaginated cells emerge as the mesoderm, some cells of which may then use the basement membrane of the now overlying epiblast as a substratum for outward migration (see Bellairs, 1982, for a recent discussion). The structure and composition of this basement membrane has been examined during various stages of early development, and both fibronectin and laminin are present prior to the formation of the primitive streak (Sanders, 1979, 1982; Mitrani & Farberov, 1982; Mitrani, 1982). The presence of these substances and the current widespread perception of the significance of extracellular

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matrix materials to morphogenesis (Hay, 1981*a*,*b*; 1982), has lead to speculation that this basement membrane might influence the migratory behaviour of the mesoderm (Sanders, 1983).

The basement membrane of the epiblast thus represents the interface between the medially migrating epiblast cells and the outwardly migrating mesoderm cells. It is not known to what extent the basement membrane itself moves during these morphogenic events. If it is moving with the epiblast, then clearly it is moving against the flow of the underlying mesoderm cells. In such a situation its putative influence on the mesoderm would be more complex than appears at first sight. If, on the other hand, the basement membrane is stationary with respect to the epiblast and mesoderm then this would raise questions regarding the mechanism of movement of an epithelium over its own basement membrane. These considerations have never been addressed.

The question of movement of the basement membrane during gastrulation was the primary concern in the present work. It was approached by using a labelling technique for basement membrane constituents on the living embryo. The label was followed at the ultrastructural level and it was found that some constituents moved towards the primitive streak during gastrulation. In addition, this technique provided information on basement membrane turnover and the formation of interstitial bodies.

MATERIALS AND METHODS

Chick embryos at stage 3 or 3^+ of Hamburger & Hamilton (1951) were explanted on glass rings according to the technique of New (1955). The lower layer of the embryo (hypoblast) is uppermost in this culture system and is therefore accessible for dissection. Using sharpened tungsten needles, a hole approximately 250 μm diameter was made in the hypoblast of the area pellucida, thus exposing the ventral surface of the epiblast with its covering of basement membrane. The hole was positioned in the area pellucida such that it was level with Hensen's node and centred approximately 500 μm to one side of it. The presence of this hole had no effect on subsequent development, and as described previously (Mareel & Vakaet, 1977; Vanroelen, Verplanken & Vakaet, 1982) it usually healed over during the course of the experiment leaving no trace. The diameter of the area pellucida at this time is approximately 3 mm (see Fig. 1).

The ultrastructural label was delivered to the hole as a drop with an approximate volume of 0·5 μl from a finely pulled Pasteur pipette. The cultures were then returned to a 37 °C incubator for periods up to 5 h, by which time the embryos appeared to be at stage 4 or 5 of normal development. In some cases embryos were given a one hour pulse of label, after which time the excess was washed off with saline and the cultures returned to the incubator for a further 3 h.

Embryos were then fixed on the glass ring with 2·5 % or 0·5 % glutaraldehyde in 0·1 M-phosphate buffer, processed at room temperature for the peroxidase

reaction if necessary (see below), and postfixed with 1·0 % buffered osmium tetroxide for 1 h. After dehydration with ethanol and propylene oxide, the tissue was embedded in araldite and sectioned for transmission electron microscopy.

Ultrastructural markers

Two different methods of labelling basement membrane constituents were used:

a) Concanavalin A–ferritin conjugate (Sigma Chemical Co., St. Louis, U.S.A.). This was effective because it has been determined previously (Hook & Sanders, 1977) that the basement membrane shows the highest affinity for concanavalin A of any tissue surface at this stage of development. The concanavalin A–ferritin conjugate was applied to the exposed basement membrane at an initial concentration of 500 μg/ml in Pannett and Compton's saline. After incubation, the embryos were fixed for 4 h with 2·5 % glutaraldehyde, post-fixed, dehydrated, embedded and examined without staining. Tissue incubated with the conjugate in the presence of 0·5 M α-methyl-D-mannoside showed no labelling, indicating that the concanavalin A binding was specific for mannose residues.

b) Peroxidase conjugated to rabbit immunoglobulins to human fibronectin (Dakopatts, Copenhagen, Denmark). The antiserum to human fibronectin cross-reacts with chick fibronectin in the basement membrane (Sanders, 1982) and was used at a 1: 100 dilution in phosphate-buffered saline (PBS). Following incubation the embryos were rinsed in PBS and fixed on the glass ring with 0·5 % glutaraldehyde for 10 min. The electron-dense deposit was generated by incubating the embryos for 15 min in a mixture of diaminobenzidine (0·5 mg/ml in PBS) and 0·01 % hydrogen peroxide. After a 30 min rinse in PBS the tissue was post-fixed, dehydrated, embedded and examined without further staining. Embryos treated as above but without exposure to the labelled antibody showed no dense deposit.

The presence of the concanavalin A or the antiserum had no noticeable effect on development during the time period studied. The results are based on the ultrastructural examination of approximately thirty embryos for each labelling technique in addition to a similar number of appropriate controls.

RESULTS

a) *Concanavalin A–ferritin conjugate*

When the basement membrane was examined unstained by electron microscopy after 5 h incubation with the label, ferritin was observed bound not only in the area to which it was applied but also towards, and into, the primitive streak at the same level in the embryo (Fig. 1). Ferritin was attached both to the basement membrane proper and also to the associated clumps of extracellular

material or interstitial bodies (Figs 2 and 3). Label could be found up to the point at which the basement membrane fragmented in the primitive streak region. In this area large masses of labelled basement membrane material had accumulated (Figs 4 and 5), indicating that labelled constituents had been carried into the streak and had there been sloughed off. These disrupted masses of labelled basement mebrane were frequently associated with blebbing of the ventral surface of the epiblast, Fig. 4 (see also Vakaet, Vanroelen & Andries, 1980). Although particles were observed in extracellular spaces among the invaginating cells deep within the primitive streak, they were never seen attached to the surfaces of cells in transit through the primitive streak, having apparently all been removed and deposited at the site of accumulation. As the basement membrane was followed into the primitive streak, the density of ferritin particles decreased, presumably by dilution, until the point of accumulation was reached. Ferritin was not found in regions anterior or posterior to the site of original application or peripherally towards the area opaca. This suggested that the movement of the particles into the primitive streak was associated with the movement of the epiblast and not the result of diffusion. Some indication of the fate of the labelled accumulation of basement membrane material was obtained, since particles were seen to have been carried down to the dorsal surface of the endoblast (Fig. 6). These particles were always associated with extracellular matrix resembling interstitial bodies. Ferritin was also observed in association with the extracellular fibrillar elements, Fig. 8, which occur in the immediate vicinity of the primitive streak (Sanders, 1979). The concanavalin A–ferritin conjugate did not appear to be endocytosed appreciably during the experiment, nor was there significant binding to the mesoderm cell surfaces.

In order to further determine whether the ferritin particles were carried to the primitive streak in association with morphogenetic movements, or merely as a result of diffusion, the experiment was repeated using an incubation temperature of 4 °C. Under these conditions ferritin was found only in the region overlying the hole in the lower layer. At this temperature the wound in the hypoblast did not heal.

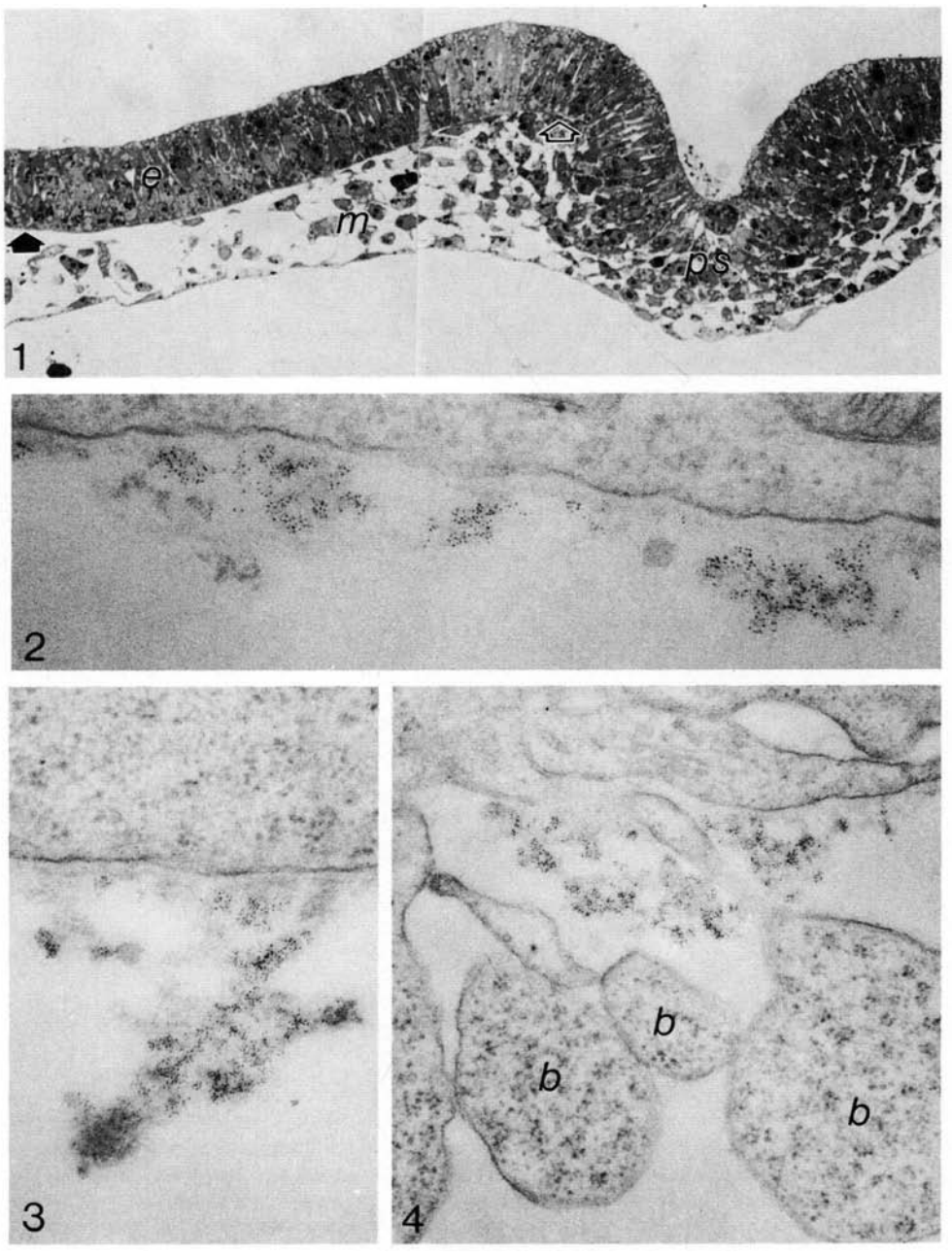
Fig. 1. Light micrograph of a section through a stage-5 chick embryo. The basement membrane of the epiblast (e) was labelled at stage 3 at the periphery of the area pellucida (solid arrowhead). By stage 5, label had moved into the primitive streak region (ps) and had accumulated at the point of basement membrane fragmentation (open arrowhead). The outwardly migrating mesoderm cells (m) are seen beneath the basement membrane. ×215.

Fig. 2. Concanavalin A – ferritin conjugate (Con A–Fe) bound to the basement membrane and interstitial bodies after 5 h incubation. ×29 220.

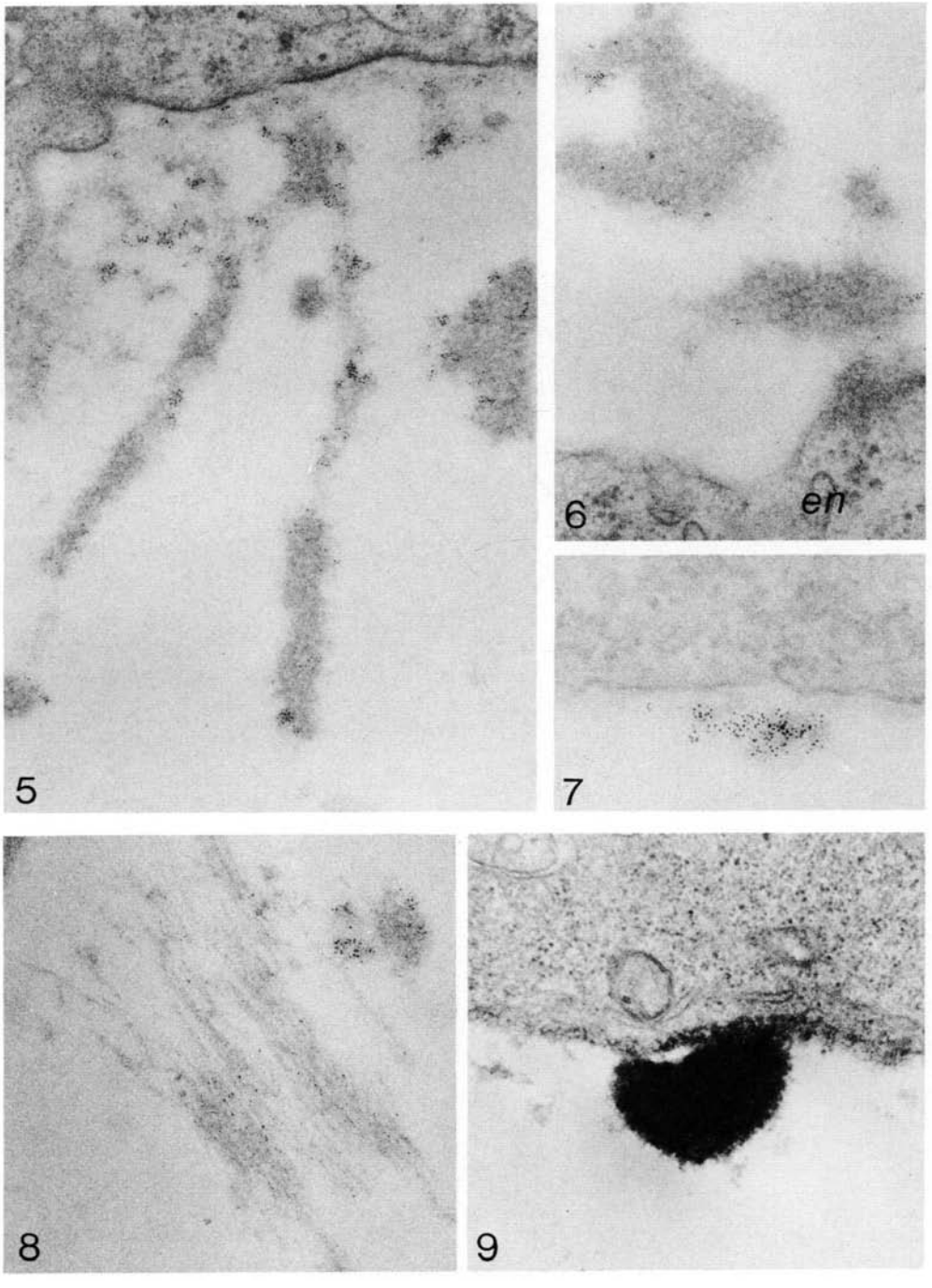
Fig. 3. After 5 h incubation in the con A–Fe conjugate, label tended to accumulate in the interstitial bodies as shown here. ×29 220.

Fig. 4. Label accumulated at the edge of the primitive streak at the point of disruption of the basement membrane. The ventral surface of the epiblast is actively blebbing (b) in this region. ×37 100.

When embryos were exposed to a pulse of label for 1 h, followed by 3 h incubation in the absence of label, the ferritin was observed to have accumulated in the interstitial bodies and was largely absent from the basement membrane itself (Fig. 7).



Figs 1–4



Figs 5–9

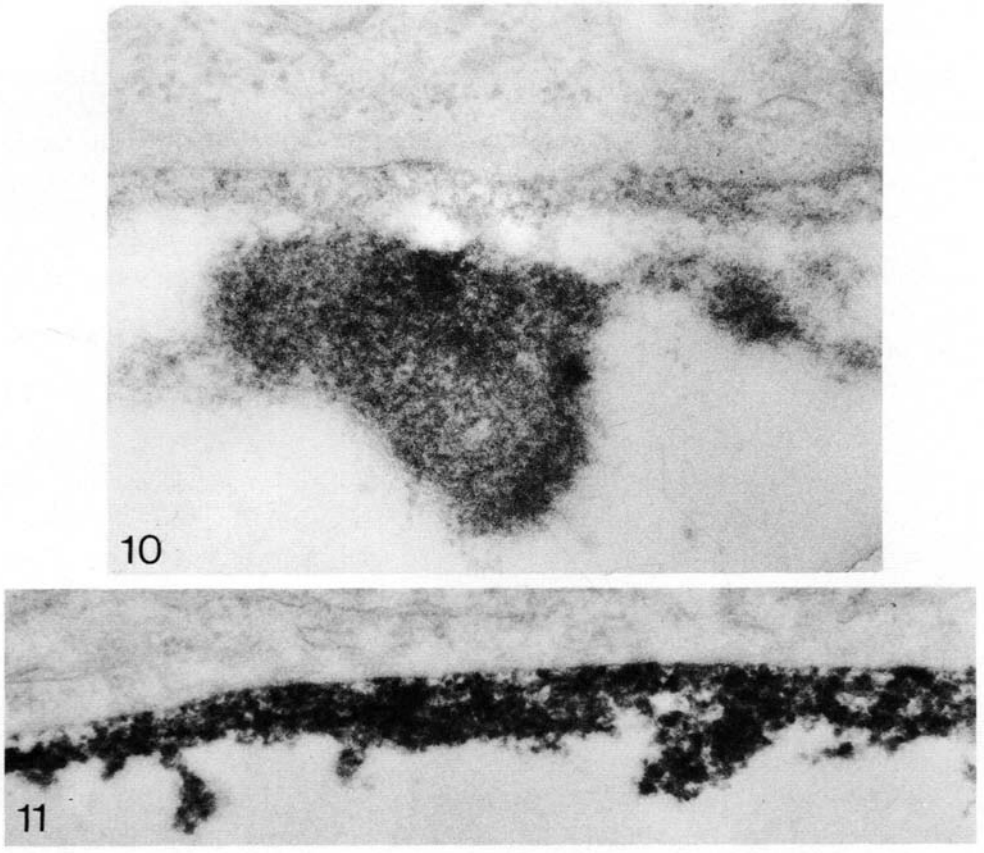


Fig. 11. Tissue incubated for only 20 min with the antibody-peroxidase conjugate showing the uniform labelling of the basement membrane after this time. ×37 100.

b) *Fibronectin antibody – peroxidase conjugate*

Five hours incubation with the antibody – peroxidase conjugate resulted in some deposit appearing in the primitive streak region, but with insufficient density to make it clearly distinguishable. This technique was therefore of limited

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Fig 5. Masses of labelled basement membrane material sloughed off as epiblast cells enter the primitive streak. The primitive streak is to the right of this picture. ×46 100.

Fig. 6. Labelled extracellular matrix resembling an interstitial body, which has been carried ventrally to the region of the endoblast (en). ×46 100.

Fig. 7. Embryos labelled with a 1 h pulse of con A–Fe and incubated for a further 3 h in the absence of label accumulated the marker in interstitial bodies as shown here. Basement membrane to either side is unlabelled. ×55 700.

Fig. 8. Con A–Fe associated with fibrillar extracellular matrix which is present exclusively in the primitive streak region at this stage of development. ×55 700.

Figs 9 and 10. Interstitial bodies from embryos labelled for 5 h with anti-fibronectin antibodies conjugated to peroxidase. The interstitial bodies are labelled but the basement membrane is not. ×37 100 and 55 700 respectively.

use in detecting the movement of materials to the streak owing to dilution of the label. Peripherally, however, dense deposits were observed in a non-uniform distribution on the epiblast basal surface. The staining was poor on the basement membrane proper, but intense on the interstitial bodies (Figs 9, 10). Embryos pulsed with label for 1 h followed by 3 h further incubation also showed label only on the interstitial bodies, suggesting that the interstitial bodies accumulated the fibronectin-bound antibodies from basement membrane during the incubation period. In contrast to this result, incubation with the conjugate for only 20 min produced widespread uniform staining of the basement membrane itself (Fig. 11). As shown previously (Sanders, 1982), the mesoderm cell surfaces reacted very poorly for the presence of fibronectin.

DISCUSSION

The experiments described here appear to represent the first demonstration of the movement of basement membrane components, as distinct from epithelial movement, during morphogenesis. The results thus indicate that the migrating mesoderm cells are moving against the flow of at least some basement membrane constituents and that the interface of the counterflow is at the outer surface of the basement membrane. It is not possible to say whether the entire basement membrane is moving in the direction of the primitive streak since concanavalin A will only label glycoproteins containing mannose and glucose residues, with which the basement membrane is rich (Hook & Sanders, 1977). The interstitial bodies which accumulated label have been shown to contain the glycoprotein fibronectin at this time in development (Sanders, 1982), a substance which has been implicated in the regulation of cell movement on the basement membrane (Critchley, England, Wakely & Hynes, 1979; Wakely & England, 1979; Sanders, 1980). In the case of primary mesoderm migration, it appears therefore that the overlying fibronectin, at least in the interstitial bodies, may itself be moving, but in the opposite direction to the mesoderm cells.

The possibility that the label was simply diffusing from the site of application was considered highly unlikely. The label did not move in the absence of morphogenetic migrations when experiments were conducted at 4 °C and the movement at 37 °C was only in a direction corresponding to epiblast migration. Diffusion of the label could be expected to result in a radial spread of label from the wound, but this clearly did not occur. Efforts to localize the relatively small labelled regions of basement membrane during thin sectioning showed that the label remained discrete within the area described and was not radially diffuse. Ferritin particles were never observed freely distributed in the space between the upper and lower layers, where they might have been expected to occur as a result of diffusion.

Vanroelen *et al.* (1982) have recently shown that partial removal of the hypoblast may result in some changes in the light microscope staining properties

of the epiblast basement membrane. The nature of these alterations has not been defined and at the ultrastructural level they appeared minimal. The changed staining properties were detectable during the period from approximately 1·5 to 3 h following hypoblast removal and thereafter returned to normal with hypoblast regeneration. Whether such changes are significant for the present experiments is not known since the basement membrane appeared ultrastructurally normal after the 20 min and 5 h incubations used here. The wound made in the experiments described here was very small (250 μm diameter).

Attachment of an epithelium to its basement membrane is thought to be accomplished with the participation of the glycoproteins laminin (Terranova, Rohrbach & Martin, 1980; Vlodavsky & Gospodarowicz, 1981; Leivo, 1983) or entactin (Carlin, Jaffe, Bender & Chung, 1981). The present results suggest that this is a relatively stable link, and to what extend the epiblast is able to migrate on its own basement membrane (Wakely & Badley, 1982) is unclear. Disruption of the fibronectin and laminin organization presumably occurs as the epiblast cells invaginate down through the primitive streak (Vakaet, Vanroelen & Andries, 1980; Sanders, 1982; Mitrani, 1982). It must be considered that this basement membrane disruption could be an important factor in precipitating invagination. The mechanism by which the epiblast sloughs off its basement membrane in the streak is unknown but it clearly results from a highly localized and precisely time digestion. This is similar in many ways to the delicate balance between disruption and remodelling of the basement membrane during epithelial morphogenesis in the salivary gland (Bernfield & Banerjee, 1982) and to the local dissolution of basement membrane during tumour invasion (Liotta *et al.* 1980). Since in the chick embryo no specific sites of basement membrane assembly have yet been recognized, it must be assumed that production of the structure is by widespread interpolation of constituents to counterbalance the mid-line disruption.

Although the peroxidase-conjugated anti-fibronectin antibody was found to have moved to the primitive streak region, it produced insufficient electron-dense deposit in this area to be useful as a reliable tracer of movement. This is probably mainly attributable to dilution of the basement membrane-attached antibody as it is transported towards the streak. However, previous results (Sanders, 1982) have indicated that the basement membrane appears fibronectin-poor as it approaches the primitive streak. The present observations are consistent with, though not necessarily attributable to, this situation.

To what extent the basement membrane influences the migration of the mesoderm is still an open question. The movement of components of the basement membrane might explain why it has been difficult to detect aligned extracellular fibrils which could direct mesoderm cells by contact guidance in this situation (Sanders, 1983). Although fibronectin is present in the basement membrane and influences mesoderm cell movement *in vitro* (Sanders, 1980, 1982), its role *in vivo* is equivocal since most mesoderm cells appear not to contact the

basement membrane at all, and the present result shows an unfavourable relationship between it and the movement of the underlying mesoderm cells. The view that the interstitial bodies influence mesoderm migration is still unsubstantiated.

The interstitial bodies appear as clumps of extracellular material attached to the outer face of the basement membrane (Low, 1970). Although their rich fibronectin content has suggested a role for them in controlling the migratory behaviour of the underlying mesoderm (Mayer, Hay & Hynes, 1981; Sanders, 1982), their structural relationship with the basement membrane has never been clear. It appears from the present results that these bodies may represent turnover products of the basement membrane, perhaps as a result of the movements of the epiblast. During the 4 to 5 h incubation, the labelled fibronectin was cleared from the basement membrane and accumulated in the interstitial bodies. This contrasts with the appearance after 20 min incubation or when the antibody is applied after fixation (Sanders, 1982). Under these circumstances, both the basement membrane and the interstitial bodies are labelled. This rapid turnover of fibronectin is similar to that for glycosaminoglycans in the basement membrane of embryonic submandibular epithelium (Bernfield & Banerjee, 1982). Basement membrane (type IV) collagen, however, appears to show a very much slower rate of turnover (Lauri & Leblond, 1983).

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