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Basal lamina is not a barrier to neural crest cell emigration: documentation by TEM and by immunofluorescent and immunogold labelling

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Summary

One of the factors proposed to control initiation of migration of neural crest (NC) cells is disruption of the basal lamina (BL) that is presumed to exist over the dorsal portion of the neural tube. Previously, we discovered that, in the mouse embryo, a continuous BL is not deposited over the dorsal portion of the neural tube until emigration of the NC cells is terminated. Here, we show that the pattern of BL deposition in chick embryos is similar, but not identical, to that in the mouse. In particular, (i) patches of BL are deposited on the premigratory NC cells in the chick but not in the mouse and (ii) BL is thicker and more interstitial matrix is deposited at the same stage of development in the chick. In addition, immunofluorescent and immunogold labelling of collagen IV, laminin and fibronectin show that (i) patches of young BL contain all three molecules; (ii) collagen IV and laminin are present in BL throughout neurulation but fibronectin either disappears or becomes masked in more mature BL and (iii) collagen IV and especially fibronectin are present in the interstitial matrix, but the relative abundance of fibronectin changes with time. The simultaneous use of immunolabelling for both light and TEM sections has allowed us to determine unambiguously that presence of a basement membrane (light microscopy) does not necessarily imply presence of basal lamina. We conclude that, as in mouse, the BL cannot be involved in the timing of the initiation of migration of NC cells. Our evidence in both the mouse and the chick, together with work in the axolotl, suggests that the basic pattern of BL deposition during neurulation may be a general phenomenon in embryonic development. Moreover, these results, in conjunction with the work of others, suggest that the critical step for initiation of migration of NC cells may be the loss of adhesions between cells.

Key words: basal lamina, neural crest, chick embryos, mouse embryos, collagen IV, laminin, fibronectin, immunofluorescence, immunogold.

Introduction

Various aspects of migration and differentiation of neural crest (NC) cells have been investigated previously. However, the processes involved in the initiation of migration of these cells have not been elucidated (Newgreen & Gibbins, 1982; Erickson & Weston, 1983; Newgreen & Gooday, 1985). It has been suggested (Newgreen & Gibbins, 1982) that four conditions must be fulfilled in order for NC cells to emigrate from the neural tube: (i) presence of a suitable substratum for migration; (ii) absence of physical barriers to emigration; (iii) intrinsic locomotory competence of the NC cells and (iv) breakage of adhesions between cells. A possible barrier to the initiation of migration of NC cells is the basal lamina (BL) that is presumed to be present over the dorsal portion of the neural tube (Tosney, 1978; Erickson & Weston, 1983; Le Douarin, 1984; Weston, 1984; Löfberg *et al.* 1985) and has been shown to be impenetrable by NC cells (Erickson, 1987). Light-microscope studies investigating the distribution of extracellular matrix in the trunk of chick and mouse embryos using specific stains (Erickson &

Weston, 1983) and immunohistochemistry (Newgreen & Thiery, 1980; Sternberg & Kimber, 1986*a*) have suggested that prior to emigration the NC cells are trapped within the neural tube by a continuous basement membrane (we here define the basement membrane to be BL and associated extracellular molecules; Bluemink, Faber & Lawson, 1984; Laurie & Leblond, 1985). In addition, Newgreen & Gibbins (1982), Erickson & Weston (1983) and Löfberg *et al.* (1985), using the transmission electron microscope (TEM), have shown that in the trunk regions of chick, mouse and salamander, respectively, a discontinuous BL exists on the basal surfaces of the presumptive NC cells at the time of emigration. Consequently, it was thought that the BL must be disrupted in order for emigration to take place. Recently, we have demonstrated that in the trunk of mouse embryos, a continuous BL does not form over the portion of the neural tube containing the NC cells until emigration is terminated. Moreover, BL deposition is delayed on the basal surface of the epidermal ectoderm (EE) that overlies the NC cell region (Martins-Green & Erickson, 1986).

The large majority of NC studies, in particular studies on the initiation of migration, has employed chick embryos. Therefore, it is important to determine if BL deposition is delayed in this organism, as it is in the mouse, and to determine the pattern of deposition of the major components of the BL and of the extracellular matrix (ECM). A number of ultrastructural studies have described in detail the morphological nature of the ECM encountered by migratory avian NC cells (Newgreen *et al.* 1982; Brauer, Bolender & Markwald, 1985) and the distribution of fibronectin in the matrix of the pathways followed by migrating trunk NC cells (Mayer, Hay & Hynes, 1981). However, the ultrastructural distribution and progressive deposition of fibronectin and other matrix components such as collagen IV and laminin have not been studied prior to emigration and during the early stages of migration.

In this study, we first present a detailed ultrastructural elucidation of the progressive deposition of BL and then correlate those observations with the changing distribution of specific ECM molecules using both light microscopy and TEM. We have found that (i) as in mouse, a continuous BL is not deposited over the dorsal portion of the neural tube until emigration is terminated, (ii) collagen IV exists within the BL as well as in the loose interstitial matrix, whereas laminin is found predominantly in the BL and (iii) fibronectin-antibody staining is present in young BL but disappears as development proceeds. We also demonstrate that under some circumstances, observations with the light microscope provide sufficient characterization of the BL, but in most cases the lower-resolution images can be misleading. We conclude that delay of BL deposition until after NC cell emigration is probably a universal phenomenon and that onset of emigration most likely is induced by loss of adhesions of the NC cells.

Materials and methods

White Leghorn chicken eggs were incubated at 37°C and 92% relative humidity and were carefully staged according to the system of Hamburger & Hamilton (1951). Stages 10–15 (9–24 somites) were used; the head and the lateral and posterior blastoderm were trimmed away and the remaining portion of the embryo prepared for histology and TEM.

Specimen preparation for light and electron microscopy

Embryos were fixed for 2 h at room temperature in 2·5% glutaraldehyde and 1% paraformaldehyde in a 0·1 M-sodium cacodylate buffer, pH 7·4 or in a 3% glutaraldehyde solution in 0·05 M-phosphate buffer, pH 6·8. The embryos were washed in buffer, then postfixed for 1 h at room temperature in a 2% aqueous solution of osmium tetroxide and stained in a saturated solution of uranyl acetate in ddH_2O for 2 h at 4°C in the dark. This was followed by dehydration in an ascending acetone series and embedding in Epon–Araldite. Sections 2 μm thick were cut using a Reichert ultramicrotome and stained in 0·1% toluidine blue in 0·1 M-sodium borate for light microscopic observations. Thin sections were cut with the same instrument and stained with a saturated solution of uranyl acetate in 70% ethanol followed by lead citrate (Reynolds, 1963; Venables & Coggeshall, 1965). The ultrastructural observations were made at 80 kV on a JEM 200 or Philips 401 TEM.

Specimen preparation for immunolabelling

Small pieces of neural tube with associated somites or lateral plate mesoderm, about 0·6 mm (approx. 4 somites) long, 0·3 mm wide and 0·2 mm high, were excised and prepared as described in detail in Martins-Green & Tokuyasu (1987). Briefly, the tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature and treated with PBS containing 0·02 M-glycine for 30 min at room temperature to quench aldehyde groups. Incubations with both primary and secondary antibodies were done overnight at 4°C and the washes in between were extensive (6 h at 4°C) with three changes of buffer. Specimens for both light microscopy and TEM were then processed for embedding in Spurr's resin (Spurr, 1969). For light microscopy, specimens were fixed again in 4% paraformaldehyde for 1 h at room temperature, treated with glycine for 30 min, dehydrated in an ascending acetone series, infiltrated in acetone:Spurr's (1:1) for 1 h, then acetone:Spurr's (1:2) overnight and finally 100% Spurr's for 3 h. Specimens were embedded in 100% Spurr's in transparent flat moulds (Pelco) and polymerized at 60°C for 18–20 h. For TEM, specimens were fixed again in 3% glutaraldehyde for 1 h at room temperature, washed three

times in buffer, postfixed in 1% OsO_4 in ddH_2O for 1 h, washed twice in ddH_2O and incubated for 1½ h at 4°C in the dark in saturated aqueous solution of uranyl acetate. Dehydration and embedding were carried out as described for light microscope preparations.

Antibodies

The antibodies to collagen IV and laminin were made in rabbit and were provided to us by Dr H. K. Kleinman of the National Institutes of Health, Bethesda, Maryland (Kleinman *et al.* 1982). The specificity of these antibodies was tested by enzyme-linked immunoassay (ELISA) and immunoblot. Antibodies to fibronectin were purchased from Cappel (Catalogue no. 0201-0831) and were made in goat against human plasma fibronectin. We used rhodamine-conjugated goat anti-rabbit IgG or rhodamine-conjugated rabbit anti-goat IgG (Cappel) as secondary antibodies for light microscopy. For TEM, we used Protein-A gold and rabbit anti-goat gold, 5 or 10 nm in diameter (Janssen).

All antibodies were diluted in PBS containing 0·002% Triton-X 100 to reduce the surface tension and 0·02% sodium azide to prevent bacterial growth. Antibodies were used at the following dilutions: collagen IV, 1:20; laminin, 1:50; fibronectin, 1:100; vimentin, 1:50; rhodamine-conjugated secondaries, 1:50; and gold-conjugated Protein A or secondary, 1:10. In the controls, (i) we preincubated each primary antibody for 1 h at room temperature with its respective antigen (50 $\mu g\,ml^{-1}$) to determine specificity of the antibodies and (ii) we used buffer alone and no primary antibody or used an unrelated antibody (vimentin) to determine the nonspecific binding of the secondary antibody to the tissue components or to the IgGs.

Results

To elucidate the progressive development of BL from early neural fold elevation to NC cell emigration, we have undertaken, using light microscopy and TEM: (i) morphological studies and (ii) immunolabelling studies using antibodies to collagen IV, laminin and fibronectin. We studied the process of BL deposition on the basal surfaces of the neural epithelium (NE), the presumptive NC cells contained within the NE, and the EE that overlies the NE.

Morphological studies

The entire trunk region of each of three 14-somite embryos (Fig. 1A) was serially thick sectioned and selected levels were examined by TEM. First, it was necessary to determine the distribution of BL with TEM since this is the only criterion that unambiguously identifies a BL. The advantage of using 14-somite embryos is that the NC cells can be found at progressively more advanced stages of development from the posterior to the anterior portion of the trunk and therefore the entire spectrum of development can be studied in a single embryo (Tosney, 1978; Bancroft & Bellairs, 1976; Erickson & Weston, 1983; Martins-Green & Erickson, 1986). In addition, we studied this progression of BL development at a single somite level by examining the 13th somite or its equivalent axial level in 9-, 13- and 24-somite embryos (Fig. 1B–D).

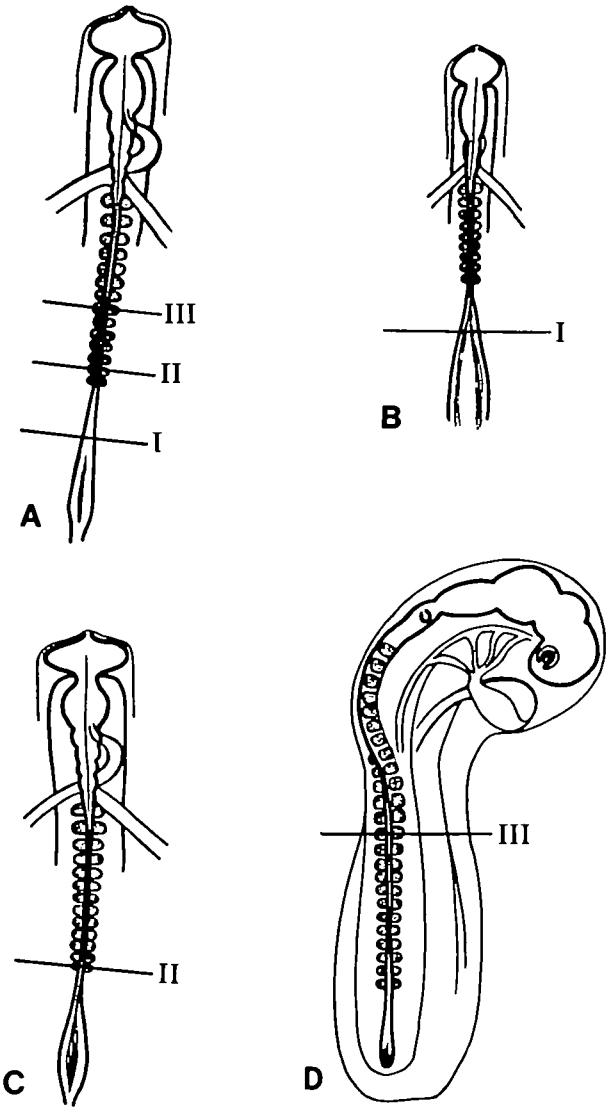


Fig. 1. Drawings of chick embryos at stages 11+ (A), 10− (B), 11 (C) and 14 (D) (Hamburger & Hamilton, 1951). (A) 14-somite embryo. At this stage, a broad spectrum of NC cell morphogenesis can be found from the posterior to the anterior end of the trunk. The lines labelled by Roman numerals indicate levels of development corresponding to the 13th somite (similarly marked) in B, C and D. (B) 9-somite embryo. A cross-section through the future 13th somite is marked (I) and is shown in detail in Fig. 2 (early neural fold stage). (C) 13-somite embryo. A cross-section through the 13th somite is labelled (II) and is represented in detail in Fig. 3 (late premigratory stage). (D) 24-somite embryo. A cross section through the 13th somite is marked (III) and is shown in detail in Fig. 4 (migratory stage).

At early neural fold stages (Fig. 2), in the region of close apposition of the EE and the NE, there is no BL on the presumptive NC cells (Fig. 2C,D) and only very small segments of BL are present at the basal surface of the EE (Fig. 2E). On the basal surfaces of the lateral EE and the lateral and ventral NE, there are large segments of BL. As folding of the NE progresses, traces of BL appear on the basal surface of some of the presumptive NC cells. The segments of BL associated with the basal surface of the EE that overlies the presumptive NC cells become larger and more numerous. The basal laminae of the lateral EE and of the lateral and ventral neural tube become continuous at this stage. As reported previously by Newgreen & Gibbins (1982, in their fig. 2), we found that the BL of the lateral portion of the neural tube is fused with the BL of the lateral EE at the dorsolateral corners of the tube (see also Fig. 5B–E). After fusion of the neural folds, but before emigration of the NC cells begins (late premigratory stages; Fig. 3), the BL associated with the NC cells remains scant and patchy (Fig. 3D). By this stage, the BL has become continuous along the basal surface of the EE overlying the NC cells (Fig. 3E), has extended dorsolaterally on the NE (Fig. 3B, arrows) and is thicker around the lateral and ventral portions of the neural tube. In addition, ECM in the form of flocculent and fibrillar material is present in the space between the NC cells and the overlying EE (Fig. 3E, stars). During migratory stages (Fig. 4), some of the NC cells have segments of BL on their surface (Fig. 4C,D; see also Tosney, 1978) and the regions of continuous BL on the lateral surfaces of the neural tube have now extended more dorsally (Fig. 4B, arrow and Fig. 4E). Finally, when emigration has terminated, a BL identical to the one shown in Fig. 4E forms over the dorsalmost part of the neural tube (not shown).

Immunolabelling studies

In order to determine the composition of the BL and associated ECM at these early embryonic stages, we have examined the same spectrum of development using antibodies to collagen IV, laminin and fibronectin, molecules that are known to be basic components of the BL at more mature stages.

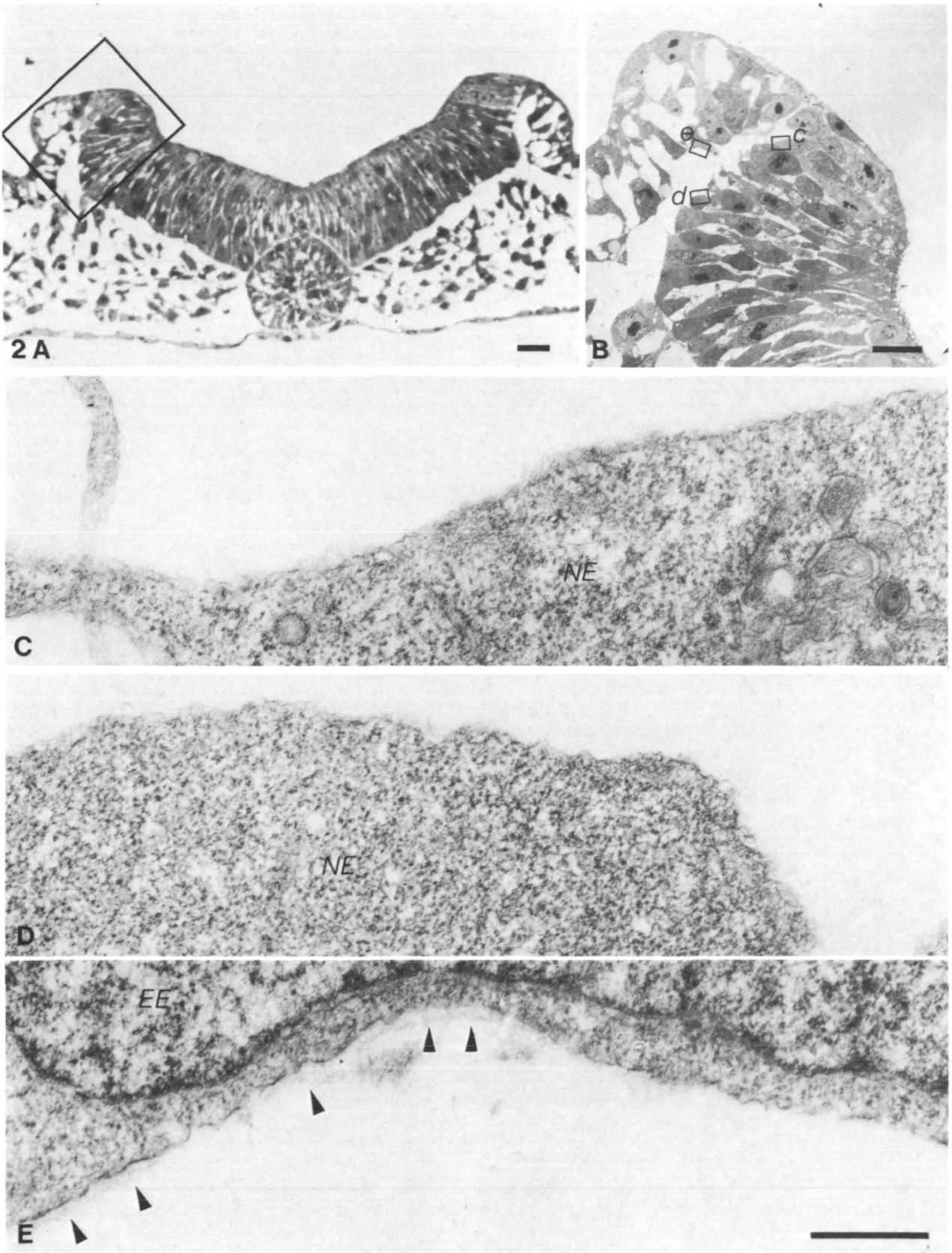
Immunofluorescence

At the light microscope level, the distribution of collagen IV, laminin and fibronectin has been determined by labelling tissues with antibodies to these molecules and examining the embryos in 2·5 μm plastic sections. The progressive deposition of collagen IV from early neural fold elevation to late premigratory stages is illustrated in Fig. 5A–D. Collagen IV is deposited initially on the EE and on the lateral NE; it generally appears as a continuous basement membrane but is intermittent in the region where the EE is apposed to the NE (Fig. 5A). As neural fold elevation continues, the basement membrane containing collagen IV staining extends to the ventralmost portion of the NE and a double basement membrane (intermittent on the NE and at the dorsalmost tip on the EE) extends progressively farther into the area of apposition of the EE and NE (Fig. 5B, arrowheads). In this region, collagen IV also assumes a punctate appearance on and between the two basement membranes (Fig. 5B, arrows). Shortly after fusion of the neural folds (Fig. 5C), collagen IV deposition has progressed dorsally and medially between the EE and NE, but remains incomplete near the site of fusion (double arrowheads); there are two basement membranes lateral to the single arrowhead in Fig. 5C, but only a single one dorsal to the arrowhead. Punctations also continue to appear along the basement membranes (arrows). Just prior to emigration, the single basement membrane illustrated in Fig. 5C has extended to the point of fusion from both sides and becomes continuous (Fig. 5D,E). At the same time, the region of double basement membrane has extended dorsally to the points marked by arrowheads in Fig. 5D,E. The punctate nature of the staining continues to be visible (Fig. 5D,E arrows).

The deposition of laminin throughout these stages of development is nearly identical to collagen IV (Fig. 5F,G). Specifically, laminin staining between the NE and EE progresses dorsally, with the basement membrane on the NE intermittent and lagging behind the one on the EE. One significant difference between collagen IV and laminin deposition is the absence of the punctate staining (compare Fig. 5E with 5G).

The distribution of fibronectin staining is shown in Fig. 5H–J. At early stages of neural fold elevation (Fig. 5H), fibronectin appears primarily as punctations associated with the EE and NE (arrows) but small continuously stained segments of basement membrane also are present (arrowheads). The punctate distribution of fibronectin is much more abundant than for collagen IV but, like collagen IV, it appears within the space between the two epithelia in the region of their apposition as well as associated

Fig. 2. 9-somite embryo. (A) Cross-section through the future 13th somite (early neural fold stage). This level is represented by I in Fig. 1B. Scale bar, 20 μm. (B) TEM enlargement of box in A. Boxes mark areas shown at higher magnification in C–E. Scale bar, 10 μm. (C,D) Details of boxes *c* and *d*; no BL is present on the neural epithelial cells (NE). (E) Detail of box e; small pieces of BL (arrowheads) have been deposited under the epidermal ectoderm (EE) overlying the NC cell region. Scale bar, 0·5 μm.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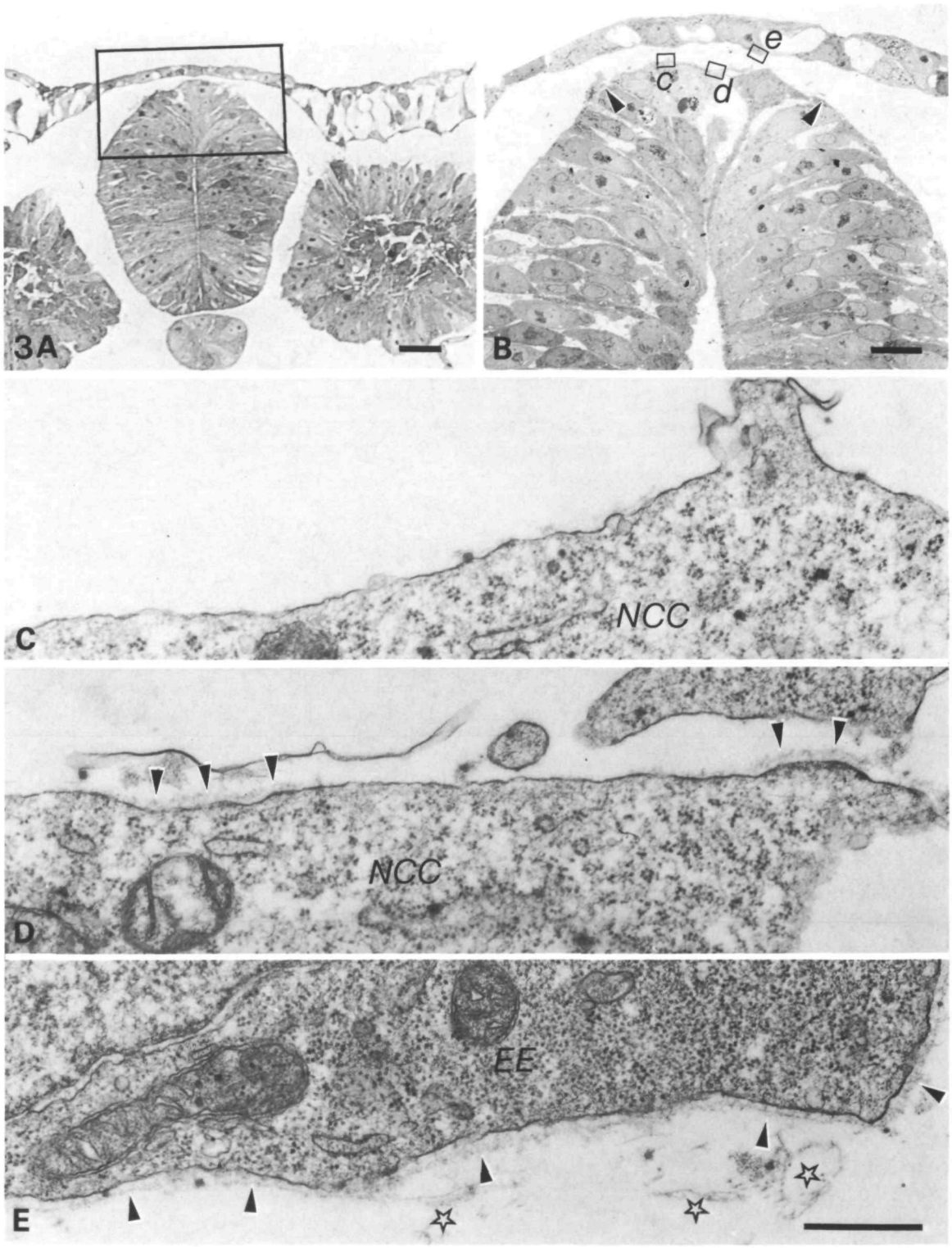


Fig. 3. 13-somite embryo. (A) Cross-section through the 13th somite (late premigratory stage). This level is represented by II in Fig. 1C. Scale bar, 20 μm. (B) TEM enlargement of box in A. Boxes mark areas shown at higher magnification in C–E and arrowheads point to the areas where BL stops on the dorsolateral neural tube. Scale bar, 10 μm. (C) Detail of box *c*; no BL is present on this neural crest cell (*NCC*); only traces of matrix can be seen. (D) Detail of box *d*; small segments of BL (arrowheads) are present on this NCC. (E) Details of box *e*; there is a continuous BL (arrowheads) underlying the EE and ECM components also can be seen (stars). Scale bar, 0·5 μm.

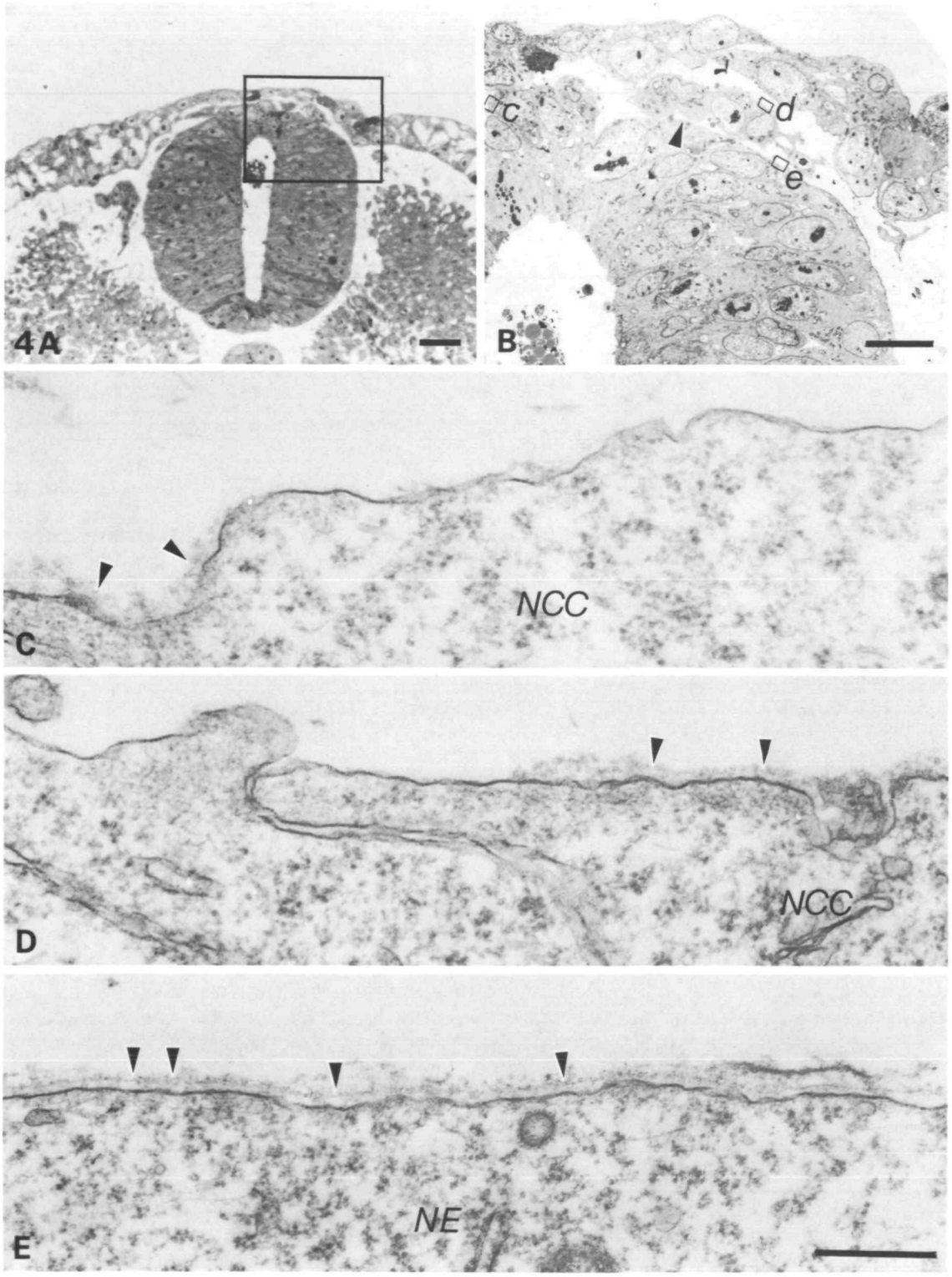
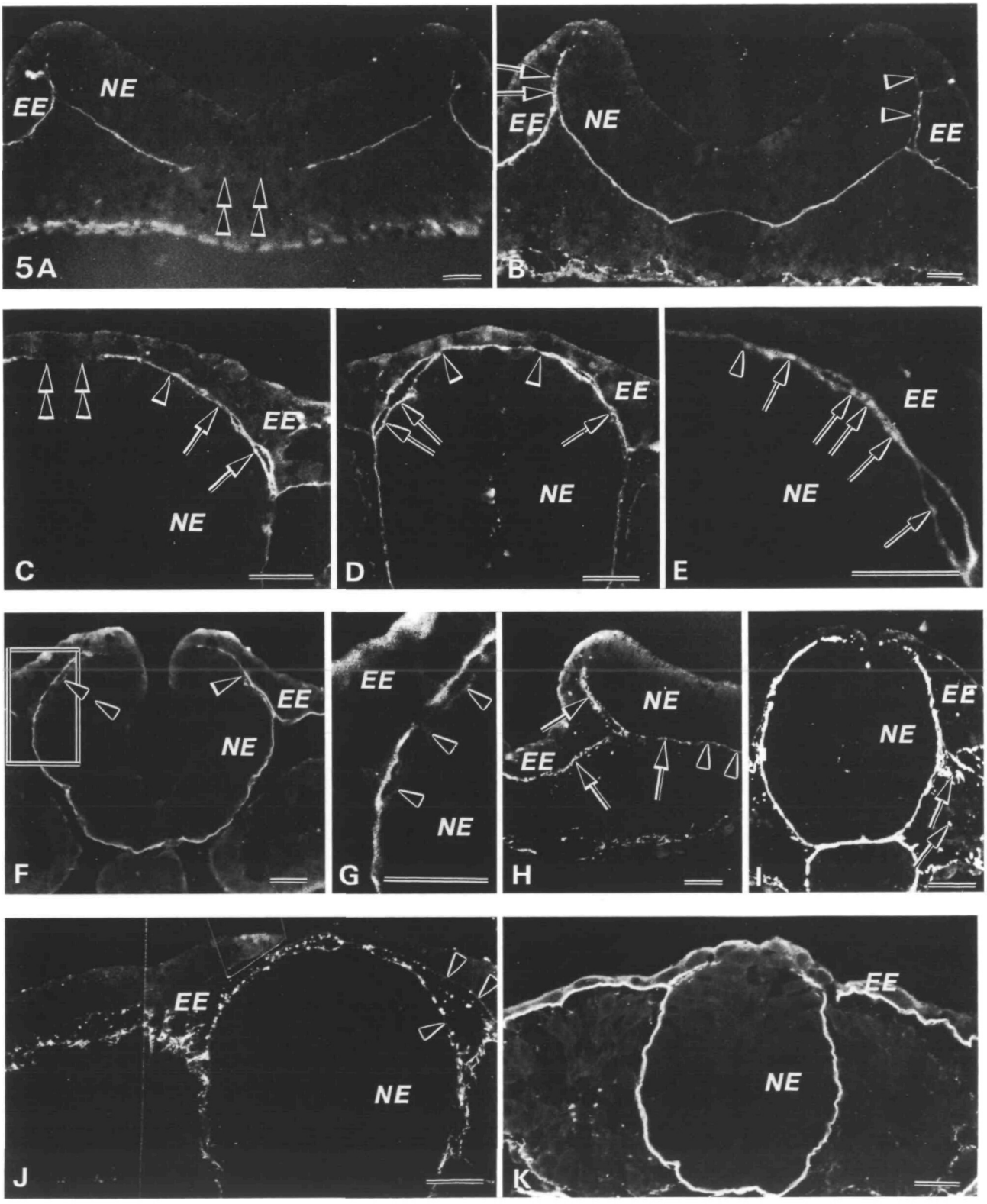


Fig. 4. 24-somite embryo. (A) Cross-section through the 13th somite (migratory stage). This level is represented by III in Fig. 1D. Scale bar, 20 μm. (B) TEM enlargement of box in A. Boxes mark areas shown at higher magnification in C–E and arrowhead points to the area where BL stops on the dorsolateral neural tube. Scale bar, 10 μm. (C) Detail of box *c*; small piece of BL (arrowheads) on neural crest cell (*NCC*) before emigration. (D) Detail of box *d*; pieces of BL (arrowheads) on some of the neural crest cells (NCC) are carried away as the cells leave the neural tube. (E) Detail of box *e*; the BL (arrowheads) is already deposited on the neural epithelium (*NE*) dorsolaterally. Scale bar, 0·5 μm.

with the basement membranes. As folding progresses and fusion occurs (Fig. 5I), fibronectin labelling in the basement membrane becomes more intense around the NE and the notochord and also appears as fibrils and becomes more abundant in the extracellular spaces. After fusion, however, the intensity of labelling around the neural tube decreases markedly (Fig. 5J).

Fig. 5K shows the distribution of collagen IV at the late premigratory stage of *mouse* development (compare with chick, Fig. 5D). The only significant differences between mouse and chick are that



(i) deposition of collagen IV between the dorsal portion of the neural tube and EE is delayed in mouse; in this region, the extent of collagen IV deposition is comparable to that of the intermediate neural fold stage in the chick (Fig. 5B) and (ii) punctate staining is absent in mouse.

Immunogold

The ultrastructural localization of collagen IV, laminin and fibronectin during the initial stages of deposition of these molecules over the dorsal portion of the neural tube is shown in Fig. 6. Fig. 6A shows, at low magnification, the contents of the dorsalmost portion of the extracellular space between the EE and NE just after neural fold fusion (see also Fig. 3A). Pieces of BL (arrowheads), dense interstitial bodies connecting the two epithelia, fibrils and unorganized matrix (double arrowheads) are present in the space. In this region, collagen IV labelling (Fig. 6B) is present in the patchy BL (arrowheads), associated matrix (double arrowheads) and interstitial bodies (see also Fig. 8B). Laminin labelling is also present in the small patches of BL (Fig. 6C, arrowheads), but it is found less often than collagen IV in the unorganized matrix and is not in the interstitial bodies. Fibronectin labelling (Fig. 6D) is present in the patches of BL and associated matrix (Fig. 6D) and also on the surface of interstitial bodies (not shown here, but see Fig. 8A).

The abundance and composition of a more developed BL and closely associated ECM, as exemplified by BL associated with the lateral neural tube just after neural fold fusion, are shown in Fig. 7. In Fig. 7A, a typical region shows a continuous BL (arrowheads), dense bodies, fibrils and unorganized matrix (double arrowheads). Proteoglycan granules (Mayer *et al.* 1981) also are seen (arrows). Gold particles labelling collagen IV (Fig. 7B) are scattered on the BL with no recognizable pattern, on the unorganized matrix adjacent to the BL and in the interstitial bodies (not shown here, but see Fig. 8B). Laminin antibody (Fig. 7C) labels BL in a similar way but it does not label interstitial bodies. Fibronectin staining (Fig. 7D) is sparse on this more mature BL, but is more abundant in the matrix on flocculent matrix closely associated with fibrils and on the surface of interstitial bodies. Although proteoglycan

Fig. 5. Cross-sections of 7- to 14-somite chick embryos (A–J) and a 9-day mouse embryo (K) at different levels along the trunk showing the pattern of deposition of collagen IV (A–E & K), laminin (F, G), and fibronectin (H–J). (A) At early neural fold elevation, collagen IV appears on the basal surfaces of the EE and lateral NE but is not found in the most ventral part of the NE (double arrowheads) where the NE is still in the process of organizing. (B) At intermediate stages of neural fold elevation, when the chordomesoderm starts to condense, collagen IV is deposited on the ventralmost part of the NE. In the regions where the EE and NE are apposed, the pattern of deposition is intermittent (arrowheads) and punctate (arrows). (C) Stage just after fusion of the neural folds. Collagen IV appears now as a continuous basement membrane that extends almost to the point of fusion of the neural folds, where it is absent (double arrowheads). Note, however, that the basement membrane is double ventral to the arrowhead, whereas a single basement membrane continues dorsally. Punctate labelling (arrows) is still present in association with the basement membranes and between them. (D) Section through the penultimate somite; collagen IV labelling of the basement membrane has extended to the point of fusion. The double basement membrane has progressed dorsally (arrowheads), but there is only a single basement membrane between the EE and the dorsalmost portion of the NE. The punctate labelling (arrows) extends across the entire dorsal portion of the neural tube.
(E) Enlargement of another section at the same age and axial level as Fig. 5D. The punctate staining is clearly seen (arrows). Note that the single basement membrane dorsal to the arrowhead appears to be associated with the EE. (F) Laminin staining at an intermediate stage of neural fold elevation; the pattern of laminin labelling is identical to that of collagen IV except that there is no punctate staining. In the region of apposition of EE and NE, the basement membrane associated with the EE is continuous but is intermittent on the NE (arrowheads). (G) Enlargement of the boxed area in F demonstrating the lack of punctate labelling of laminin (compare with the collagen IV labelling indicated by arrows in Fig. 5E); (H) At early neural fold stage, punctate labelling of fibronectin (arrows) appears in all regions where basement membrane is forming and, in addition, appears in the space between the EE and NE. Small segments of continuous labelling occur at the basal surface of the ventral NE (arrowheads). (I) As development proceeds, fibronectin deposition increases. By the time of fusion of the neural folds, fibronectin is found in a very thick basement membrane around the NE and notochord and also appears as fibrils and punctations in the extracellular spaces (arrows). (J) After fusion of the neural folds, fibronectin labelling of the basement membrane decreases rapidly; just prior to emigration, fibonectin labelling is very faint in the basement membrane (arrowheads), but staining is prevalent in abundant punctations over the dorsal portion of the neural tube and in the extracellular spaces. At this stage, the labelling around the notochord remains strong (not shown); (K) Collagen IV labelling of a section of a mouse embryo at the same stage as shown in D for chick. Note absence of labelling over the dorsal portion of the neural tube, confirming lack of BL in this region (compare with fig. 7 of Martins-Green and Erickson, 1986). Scale bars, 15 μm.

granules are present in the ECM (arrows), fibronectin does not appear to be closely associated with them. Fig. 7E shows the area between the ventral neural tube and the notochord, confirming the abundance of fibronectin seen with immunofluorescence (see also Fig. 5G).

At still later stages of BL development, fibronectin staining (Fig. 8A) disappears from the BL but continues to appear throughout the matrix and on the surface of interstitial bodies. Both 5 and 10 nm gold particles that mark antibody distribution are restricted to peripheral positions on the interstitial bodies.

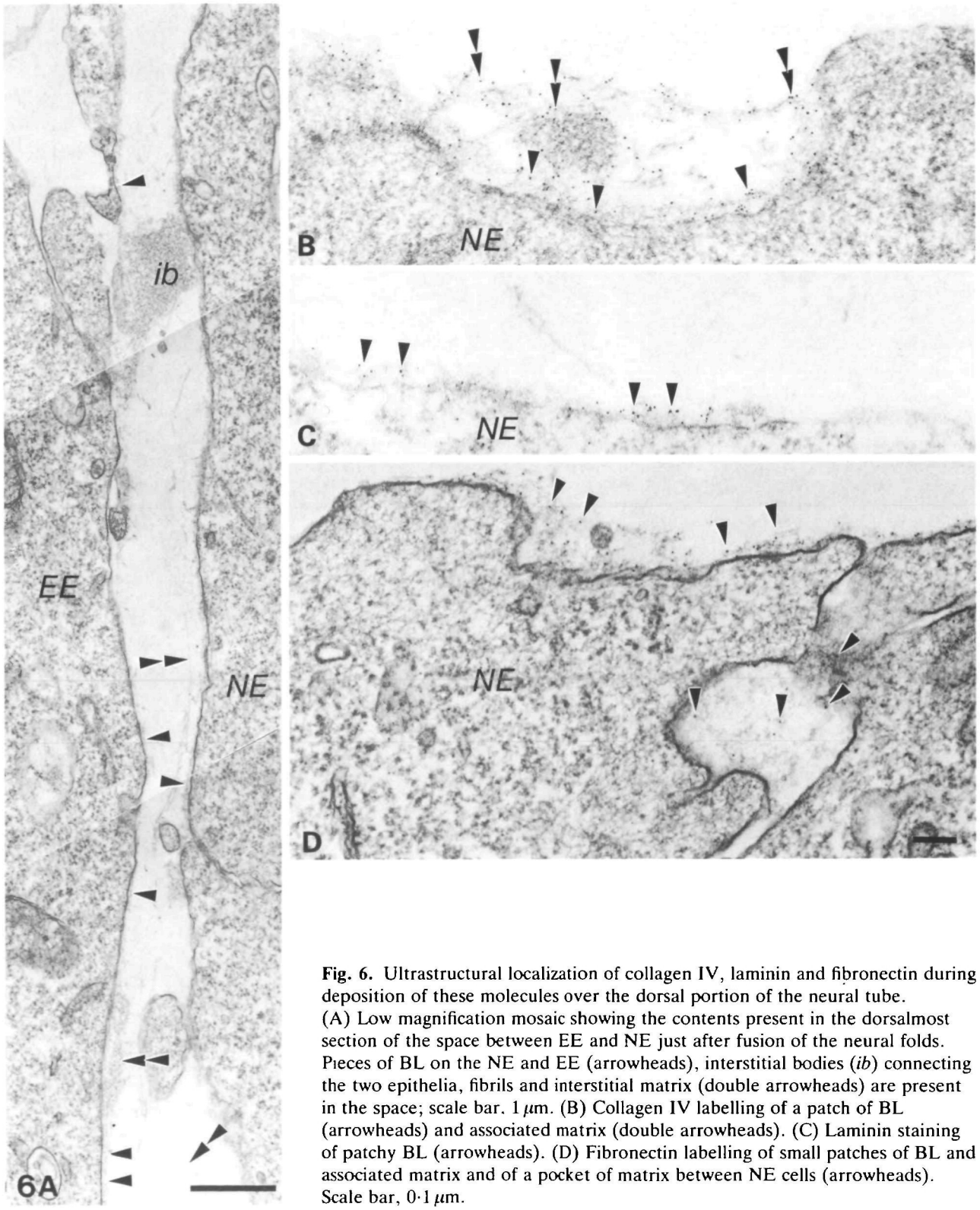


Fig. 6. Ultrastructural localization of collagen IV, laminin and fibronectin during deposition of these molecules over the dorsal portion of the neural tube. (A) Low magnification mosaic showing the contents present in the dorsalmost section of the space between EE and NE just after fusion of the neural folds. Pieces of BL on the NE and EE (arrowheads), interstitial bodies (*ib*) connecting the two epithelia, fibrils and interstitial matrix (double arrowheads) are present in the space; scale bar. 1 μm. (B) Collagen IV labelling of a patch of BL (arrowheads) and associated matrix (double arrowheads). (C) Laminin staining of patchy BL (arrowheads). (D) Fibronectin labelling of small patches of BL and associated matrix and of a pocket of matrix between NE cells (arrowheads). Scale bar, 0·1 μm.

By contrast, collagen IV staining occurs throughout the interstitial bodies at all of these stages of development (Fig. 8B) and is also present in matrix distant from, and apparently unassociated with, BL (Fig. 8C). Laminin labelling continues to be restricted principally to BL (not shown).

To test the specificity of the antibodies and to determine the extent of nonspecific binding, we performed three controls. Specificity was determined by preincubating the antibodies with their respective antigens before incubation with the tissue. Fig. 9A shows such a control for collagen IV antibody; only a few gold particles can be seen. The controls for laminin and fibronectin antibodies showed a similar lack of labelling (not shown). Nonspecific binding was evaluated (Fig. 9B) by (i) incubating the tissue with an antibody against vimentin, a molecule known not to be present in the BL or ECM (e.g. Lazarides, 1980) and (ii) incubation of the tissue with buffer alone before incubation with Protein A–gold or rabbit anti-goat gold (Fig. 9C). Very few gold particles were found (arrowheads).

Discussion

In our ultrastructural study of the mouse (Martins-Green & Erickson, 1986), we published micrographs of serially sectioned embryos as well as micrographs of a time sequence at a single-somite level and demonstrated the equivalence of the two approaches. Therefore, in this report, we have illustrated the morphology of BL development only at the 13th somite level in progressively older embryos and indicated the locations of the corresponding stages in a 14-somite embryo in Fig. 1A. The observations of immunolabelled sections were made on similar stages of embryos with 7–14 somites.

BL development in the chick can be summarized as follows. (i) around the lateral and ventral portions of the neural tube at late neural fold stages, BL is continuous; (ii) the BL associated with the basal surface of the EE overlying the NC cell region becomes continuous shortly after fusion of the neural folds; (iii) before emigration, some NC cells have small segments of BL on their basal surfaces (never more than 50 %) that may be carried away with them as they leave the neural tube and (iv) a continuous BL does not form over the dorsal portion of the neural tube until NC cell emigration terminates.

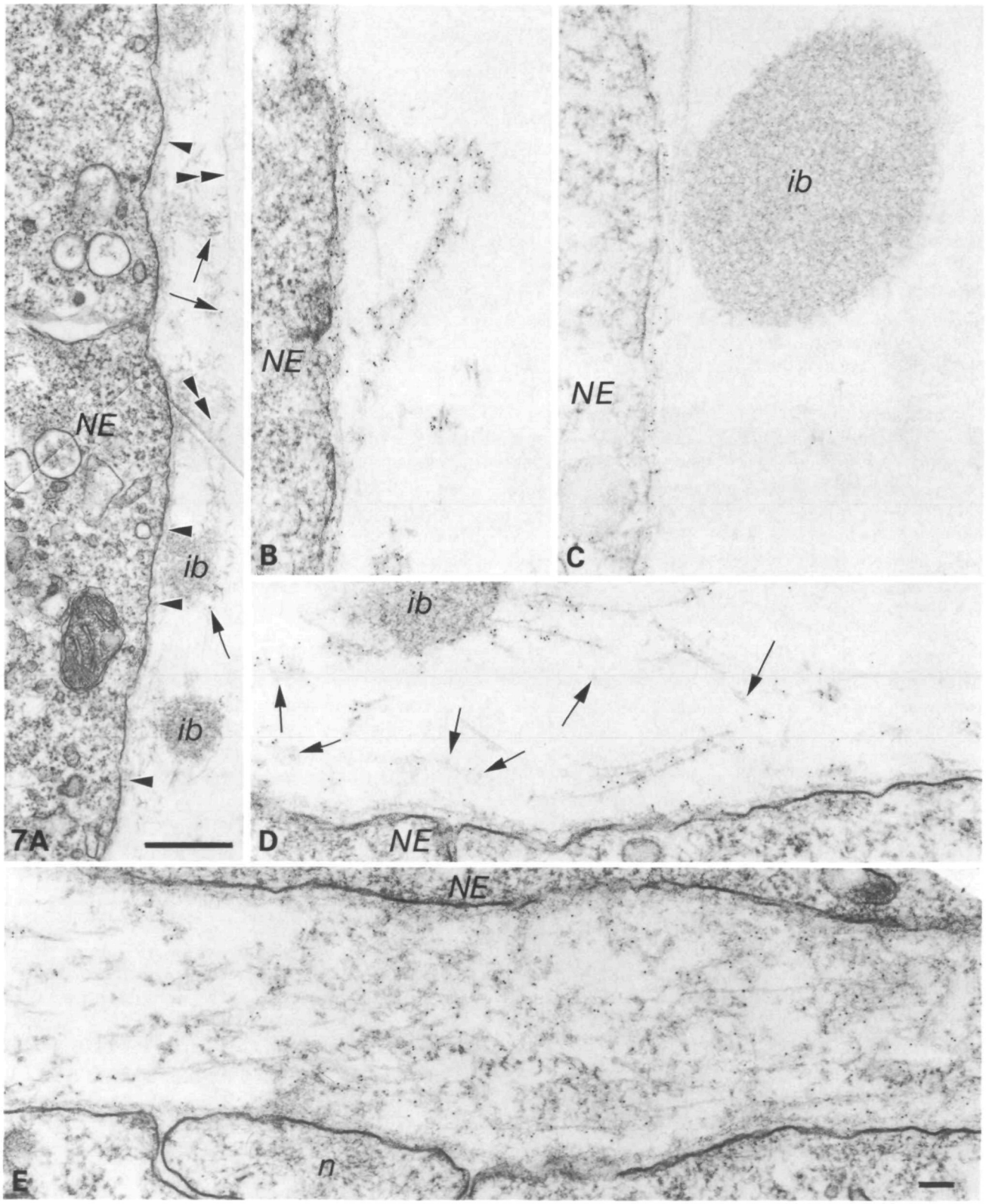
The immunolabelling data can be summarized as follows (i) the patches of BL over the NC cell region contain at least collagen IV, laminin and fibronectin; (ii) the more developed BL contains collagen IV and laminin distributed through the whole structure; (iii) fibronectin is present in young BL but either disappears or becomes masked as the BL matures; (iv) collagen IV and fibronectin (and to a lesser extent laminin) are present in the ECM in association with 10 nm fibrils and (v) interstitial bodies contain collagen IV throughout, fibronectin on the periphery, and do not contain laminin.

Even though our conclusions concerning the presence or absence of BL prior to NC emigration differ from those of previous workers (discussed in the Introduction) our observations are not in disagreement with previous electron microscopy studies. The difference between this study and previous work is that we have examined the process of BL deposition at much earlier stages of NC morphogenesis and have observed the full spectrum of development. As a consequence, we discovered that the small patches of BL associated with the NC cells before their emigration (see also Tosney, 1978; Newgreen & Gibbins, 1982) are not vestiges of a once-continuous BL, but represent the earliest stages of BL deposition.

An additional factor that mistakenly led previous workers to suggest that the BL is initially complete over the premigratory NC cells is that ECM components such as laminin, fibronectin and entactin, when identified by immunofluorescence, appear to form a continuous basement membrane over the NC cell region at premigratory stages (Newgreen & Thiery, 1980; Sternberg & Kimber, 1986*a*). Therefore, in this study we have examined very thin, well-oriented, optical sections (1–2·5 μm) to differentiate between a patchy and a continuous distribution of BL components (Fig. 5) and we have confirmed our light microscope observations by determining the distribution of the same molecules in the TEM. We have found that the distribution of laminin at these stages of development (Figs 5F,G, 6C and 7C) is largely restricted to the BL and, not surprisingly, it is continuous where there is continuous BL and intermittent where the BL is patchy (Figs 5F,G and 6C). The pattern of immunofluorescent labelling of collagen IV (Fig. 5A–E) is similarly coincident with BL distribution, but in addition shows a punctate distribution of fluorescence that the immunogold labelling identifies as dense interstitial bodies in the ECM (Fig. 8B). Collagen IV labelling is found in the interior of these interstitial bodies as well as on their surfaces (Fig. 8B) and also occurs in the flocculent matrix associated with 10 nm fibrils (Fig. 8C). Thus, some BL components are present in areas where structural BL is absent, indicating that they may play a variety of roles in addition to being structural components of BL (Mardi, Pratt, Yurchenco & Furthmayr, 1984).

In order to determine in detail the progressive development of BL over the dorsal portion of the neural tube, it is particularly instructive to follow the progressive labelling of collagen IV and laminin in

this region. In very thin cross-sections, oriented precisely normal to the axis of the neural tube (Fig. 5A–G), the fluorescence from the two basement membranes (one on the EE and the other on the NE) can be distinguished. Before fusion of the neural folds, the intermittent labelling and punctation seen in this region at the level of the light microscope (Fig. 5A,B,F,G) are due primarily to patchy BL (Fig. 6B,C) and interstitial bodies (Fig. 6B and 8B), respectively, as seen in the TEM. Only one of these basement membranes becomes continuous after fusion of the neural folds (Fig. 5C–E); sections



examined in the TEM (Fig. 3D) show that this is due to the completion of BL deposition on the EE while BL on the NE remains sparse. We had found previously in mouse (Martins-Green & Erickson, 1986) that completion of BL on the EE does not occur until near the end of emigration and that BL is absent over the dorsal neural tube before emigration. A section of mouse at a stage comparable to Fig. 5D shows that collagen IV labelling is absent over the dorsal portion of the neural tube and beneath the overlying EE (Fig. 5K), confirming our previous findings (Sternberg & Kimber, 1986*b* also have recently reported lack of collagen IV in this region of the mouse). Our observation that laminin is largely restricted to the BL at these early stages of neurulation makes interpretation of fluorescent micrographs of this antibody straight-forward. The existence of collagen IV in the ECM associated with BL as well as in the BL itself, however, makes ultrastructural studies necessary to supplement immunofluorescent observations.

The combination of immunofluorescent and immunogold labelling is especially powerful for understanding fibronectin distribution. In young BL (Figs 5H, 6D), fibronectin labelling is common, but in progressively more mature (and more organized) BL (Fig. 7D,E), fibronectin labelling decreases and eventually is no longer found (Fig. 8A); this may reflect disappearance of fibronectin from the BL during maturation or it may be caused by masking of the antigenic determinants labelled by the antibodies we used (Little & Chen, 1981). At the same time that fibronectin labelling is decreasing in the BL, there is a corresponding increase of fibronectin labelling in the matrix close to the BL, especially in association with 10 nm fibrils (Fig. 7D,E) and on the surface of the interstitial bodies (Fig. 8A). Fluorescent labelling of the matrix adjacent to the BL produces the appearance on the optical scale of a thick basement membrane (Fig. 5I) even though there is, in fact, very little labelling of BL when resolved by the TEM (Fig. 7D,E).

The presence of fibronectin on the surface of interstitial bodies (Mayer *et al.* 1981) and in association with 10 nm fibrils has been reported previously (Furcht *et al.* 1980; Mayer *et al.* 1981). The former authors showed the codistribution of fibronectin and procollagen labelling on 10 nm, nonstriated fibrils in human fibroblasts. In our specimens, fibronectin staining appears on flocculent material in close association with the fibrils but not on the fibrils themselves; when fibrils are devoid of this material, no labelling is seen (Fig. 7D,E; see also Mayer *et al.* 1981).

A number of factors could contribute to the absence of a continuous BL over the NC cells. One possibility is that NC cells produce proteases that degrade BL components, thereby preventing assembly of the BL over the dorsal portion of the neural tube. Plasminogen activator, which is known to degrade ECM components (Laug, DeClerck & Jones, 1983; Sheela & Barrett, 1982; Fairbairn *et al.* 1985) and is produced by NC cells from both the head (Valinsky & Le Douarin, 1985) and the trunk (Erickson & Isseroff, 1986), could play such a role. Other possibilities are that the NC cells have a low number of surface receptors for BL components and, therefore, cannot organize a BL, or that one or more components critical for BL assembly are in short supply. In this regard, Laurie (1985) showed that heparan sulphate proteoglycan, which is known to bind to collagen IV, laminin and fibronectin and may serve as a receptor molecule for these and other matrix components, is absent from the discontinuous and irregular basement membranes of spongiotrophoblast cells, even though components such as collagen IV, laminin, fibronectin and entactin are present.

Although our observations are fundamentally the same for mouse and chick, we did find differences in detail between the two species. The principal differences are (i) in the chick, the BL on the lateral and ventral portions of the neural tube becomes continuous by late neural fold stage, whereas in the mouse, it becomes complete only at early migratory stages; (ii) the BL beneath the EE is continuous at premigratory stages in the chick, whereas in the mouse it is not continuous until terminal migratory stages; (iii) pieces of BL are deposited upon premigratory NC cells in the chick but not in the mouse. Because the processes of BL deposition during neurulation are so similar in the trunk of the two species, we believe that the differences in detail probably reflect differences in the degree or timing of the same operative

Fig. 7. Ultrastructural localization of collagen IV, laminin and fibronectin on the BL and associated matrix of the lateral neural tube just after fusion of the neural folds. This area contains more mature ECM and BL. (A) Low-magnification mosaic showing the presence of a continuous BL on the NE (arrowheads), interstitial bodies (*ib*), fibrils, unorganized matrix (double arrowheads) and proteoglycan granules (arrows); scale bar, 1 μm. (B) Collagen IV distribution in the BL and associated matrix. The 5 nm gold particles are scattered over the BL and ECM; no specific pattern of staining was observed. (C) Laminin is present in the BL but not in the interstitial bodies (*ib*); the gold particles are scattered over the BL with no evident pattern. (D) Fibronectin staining is abundant in flocculant material associated with 10 nm fibrils and on interstitial bodies (*ib*); there is very little labelling of BL. Proteoglycan granules can be seen in the matrix (arrows). (E) In the region between the NE and notochord (*n*), ECM is especially abundant; fibronectin labelling is very strong in the matrix but rare on BL. Scale bar, 0·1 μm.

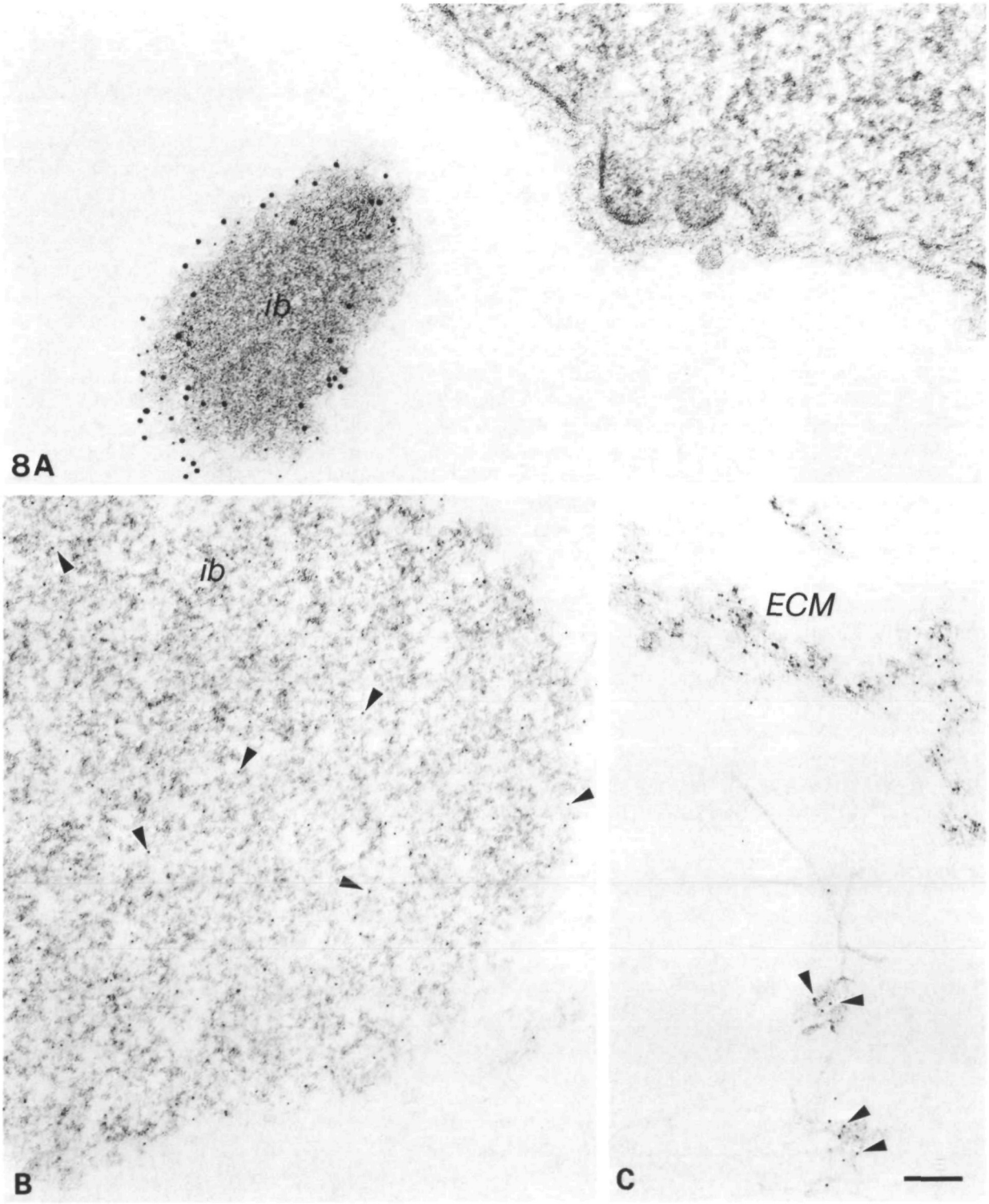


Fig. 8. Ultrastructural localization of fibronectin and collagen IV in the interstitial spaces at early migratory stages. (A) Fibronectin labelling is no longer detectable on the BL of the NE but is abundant on the surface of the interstitial bodies (*ib*); in this case both 5 and 10 nm gold particles were used to see if the smaller particles would penetrate further into the interstitial bodies; the restriction of particles of both sizes to superficial areas suggests that fibronectin is absent from the interior of these bodies. (B) Part of a large interstitial body labelled with antibodies to collagen IV (arrowheads); the 5 nm gold particles show that this molecule is present throughout these bodies. (C) Collagen IV labelling also is present in the interstitial matrix and in flocculant matrix associated with fibrils (arrowheads). Scale bar, 0·1 μm.

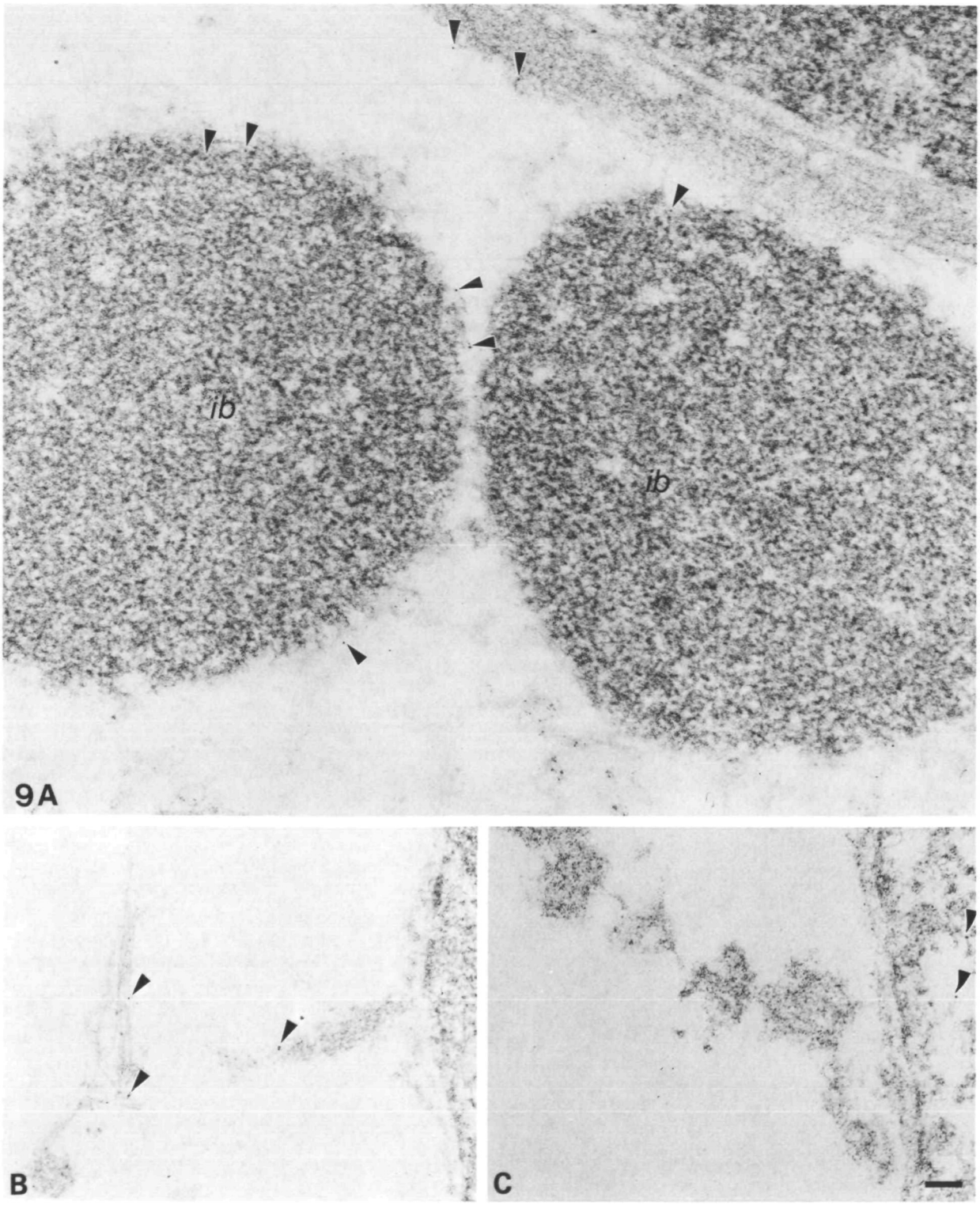


Fig. 9. Controls for immunogold labelling. (A) Tissue incubated with anti-collagen IV that had been preincubated with collagen IV: specificity of the antibodies to collagen IV is high; only a few gold particles appear on BL, unorganized matrix and interstitial bodies (arrowheads). Controls for laminin and fibronectin showed similarly high specificity; (B) Tissue incubated with antibodies against vimentin (a molecule not present in BL and ECM). The nonspecific binding of IgG molecules to ECM was very low (arrowheads); (C) Tissue incubated with buffer in place of antibodies. The nonspecific binding of protein A to the tissues was virtually nonexistent; a few gold particles inside the cell are shown by arrowheads. Scale bar, 1 μm.

mechanisms. In spite of the differences between the two species, the pattern of BL deposition is remarkably similar in chick and mouse. Moreover, Löfberg *et al.* (1985) also have reported that BL deposition in axolotl embryos is delayed over the dorsal surface of the neural tube and beneath the EE that overlies it. It would appear, therefore, that in the trunk region, the basic pattern of BL development described here and the mechanism(s) controlling it probably are universal. However, in the head region, the events immediately preceding emigration of NC cells may be different (Tosney, 1982; Nichols, 1985).

We have shown here in the chick and previously in the mouse that BL does not form a barrier to emigration of trunk NC cells. Therefore, the BL cannot be involved in the timing of the initiation of migration of these cells. Removal of any physical barrier was one of the four conditions listed by Newgreen & Gibbins (1982) as necessary to allow NC cell emigration (see Introduction). Our demonstration that there is no physical barrier to emigration reinforces the previous arguments of Newgreen & Gibbins (1982) that, with the exception of the breaking of adhesions between NC cells and NE cells, all of these conditions are fulfilled well before emigration.

Newgreen & Gooday (1985) have determined that either reduction of environmental calcium or the application of calcium blockers stimulates premature NC emigration from quail neural tubes in culture. They argue that calcium deprivation breaks cell–cell adhesions and migration ensues. It is not clear, however, what could be the mechanism of local calcium deprivation *in vivo*. Nevertheless, of the four criteria proposed by Newgreen & Gibbins (1982), the breaking of adhesions appears to be the most likely candidate for triggering NC cell emigration. The mechanism of that de-adhesion *in vivo* and whether or not it involves calcium deprivation remain to be determined.

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