SEM localization of cell-surface-associated fibronectin in the cranium of chick embryos utilizing immunolatex microspheres

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SUMMARY

Fibronectin has been localized to basement membranes and cell surfaces with the light microscope by fluorescent staining of thick sections, and with the TEM by immunoperoxidase reaction. However, these methods are limited because it is difficult to appreciate the patterned distribution of fibronectin from sectioned material. We have developed a probe for fibronectin that facilitates its identification with the SEM. Our probe consists of two parts; the first component is a derivatized methacrylate microsphere 90 nm in diameter, linked to purified sheep anti-rabbit IgG. The second component is anti-fibronectin IgG raised in rabbits. Stage-3 to -12 chick embryos were fixed and the ectoderm covering the cranial mesoderm was removed. Embryos were treated with testicular hyaluronidase, exposed to rabbit anti-fibronectin IgG and finally to sheep anti-rabbit IgG conjugated microspheres. As expected, the basal lamina of surface and neural ectoderm as well as the remaining fibrous ECM were heavily decorated with microspheres, whereas control embryos treated with preimmune serum were beadless. Fibronectin was localized on the cell soma and processes of primary mesenchyme as early as stage 3. In addition, it was possible to decorate to various extents, populations of prosencephalic, mesencephalic, and rhombencephalic cranial neural crest cells. Our studies suggest that fibronectin is present in the cranium of chick embryos at earlier times than heretofore realized, and that fibronectin accumulates in a cranial to caudal gradient that reflects the sequential differentiation of the embryonic axis.

INTRODUCTION

Fibronectin is a large, extracellular glycoprotein that binds to a variety of macromolecules including fibrin, collagen, and glycosaminoglycans and to cell surfaces as well (for review see Ruoslahti, Engvall & Hayman, 1981). Studies utilizing proteases that cleave fibronectin into fragments with one or more of these binding activities have shown that specific molecular domains are involved, and monoclonal antibodies are currently being used to determine the sequential ordering of active sites in the molecule (Yamada *et al.* 1981; Rouslahti, Pierschbacher, Hayman & Engvall, 1982). Presumably, fibronectin interacts with cell surfaces via receptor molecules anchored in the plasmalemma. The interaction of fibronectin with cells, as evidenced by the attachment and spread

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of cells to surfaces covered with insoluble fibronectin (Klebe, 1974; Turner *et al.* 1983), has contributed to the hypothesis that the molecule serves to link the cell membrane to the extracellular matrix.

Fibronectin has been localized in young embryonic tissues, especially at sites where cell movement occurs. For instance, Mitrani & Farberov (1982) have shown fibronectin to be present during hypoblast formation and Critchley, England, Wakely & Hynes (1979) and Duband & Thiery (1982*a*) have shown fibronectin is present at the primitive streak during chick embryo gastrulation. Katow, Yamada & Solursh (1982) have demonstrated that fibronectin is associated with the primary mesenchyme cell surface during gastrulation in the sea urchin embryo and fibronectin has been localized in matrices that lie along the path of cranial (Duband & Thiery, 1982*b*) and caudal neural crest migrations in developing chick embryos (Newgreen & Thiery, 1980; Mayer, Hay & Hynes, 1981; Thiery, Duband & Delourée, 1982). In addition to its role in facilitating cell movement, fibronectin promotes the morphogenesis of embryonic tissues such as the corneal epithelium (Sugrue & Hay, 1982) and peripheral neurons (Sieber-Blum & Yamada, 1981; Rogers *et al.* 1983). These observations have led to the suggestion that cellular responses to fibronectin are related to the presence or absence of this glycoprotein in localized extracellular environments during specific stages of development.

Until recently, it was thought that the cranial end of the embryonic axis was not as regularly segmented as the rest of the body, and the number of segments comprising the heads of vertebrate embryos is still an unsettled matter (for review see Goodrich, 1958). However, in 1979, Meier, using stereo SEM, discovered a segmental pattern in the cranial paraxial mesoderm of bird embryos based on the tandem accumulation of units termed somitomeres. During gastrulation, pairs of somitomeres are added to the axis (Meier & Jacobson, 1982). In birds, the first seven pairs remain contiguous in the paraxial mesoderm to form the cranial region (Meier, 1981), whereas pairs of somitomeres added to the axis posterior to the cranium eventually separate from one another as they develop into somites (Meier, 1979; Solursh, Fisher, Meier & Singley, 1979; Packard & Meier, 1983). The morphogenesis of cranial somitomeres continues adjacent to specific neuromeres of the brain and it is likely that the initial emigration of cranial neural crest cells is influenced by the somitomeric pattern in the mesoderm as well (Anderson & Meier, 1981; Meier & Packard, 1983). Therefore, even though fibronectin has been localized in the developing chick cranium, the occurrence of fibronectin with regard to the basic metameric pattern of tissues has not been reported.

Since cranial somitomeres are obvious only in stereo SEM, a labelling technique was needed to permit identification of fibronectin with the SEM. To that end, we have combined an immunocytological approach with methacrylate microspheres to generate a probe for fibronectin that is visible with the SEM. In the first part of the report we establish the efficacy of our immunolatex probe.

In the second part, we demonstrate quantitative differences in fibronectin localized on mesodermal cells, neural crest populations, and in various basal laminae of the embryonic chick cranium.

MATERIALS AND METHODS

Antibody preparation

The plasma of White Leghorn chickens was utilized as a source of fibronectin. Chicken blood was collected in a beaker containing 0·75 % NaCl, 5 mM-EDTA and 1 mM-phenylmethylsulfonyl fluoride (PSF). Fibronectin was purified from the plasma fraction by sepharose gelatin affinity chromatography as described by Engvall & Ruoslahti (1977). Our fibronectin preparation yielded a single band at a relative molecular mass of 220000 as determined by SDS Page gel electrophoresis (Laemmli, 1970) and a single line of precipitation resulted from ouchterlony immunodiffusion tests utilizing our antigen against commercially purchased rabbit anti-fibronectins (Calbiochem).

Rabbits were initially given a subcutaneous injection of fibronectin in complete Fruends followed by two injections of fibronectin in incomplete adjuvent. After three weeks, rabbit serum was purified and the IgG fraction was precipitated with ammonium sulfate. Antibodies to fibronectin were analysed by ouchterlony double immunodiffusion against chick plasma, affinity purified chicken fibronectin, and commercially obtained bovine fibronectin (Calbio). All the fibronectin antigens yielded single lines of identity with our antibody preparation. Also, our antibody preparation yielded a single arc of precipitation against quail plasma proteins that were separated electrophoretically in 1 % agar in barbitone at 5 mA for 2 h, indicating cross reactivity with quail fibronectin. Finally, our antibody preparation was assessed by Western gel electrophoresis. Briefly, stage-4 chick embryos, freed from the area opaquea were homogenized in phosphate-buffered saline. The homogenate was electrophoresed on a 6 % SLAB SDS/PAGE gel and proteins were transferred electrophoretically from the SDS-PAGE gel to a sheet of nitrocellulose and incubated with our antibody preparation according to the method of Romani, Vidal, Tahourdin & Bustin (1980). Antibody–protein complexes were reacted with radioactive iodinated protein A, a generous gift of a colleague, Dr R. Daniels. Reacted nitrocellulose sheets were exposed to X-ray film (XRP-1, Kodak) and autoradiograms were developed. Densitometer readings indicated that 82 % of the labelled IgG was concentrated in a band corresponding to the fibronectin control. Overexposure of the gel showed the remaining label concentrated in the low relative molecular mass front (10 %), and two minor bands (6 % and 2 %).

Preparation of microspheres

Derivatized microspheres were synthesized by the copolymerization of methacrylates according to a modification of the method of Molday, Dreyer,

Rembaum & Yen (1975), reported earlier (Meier & Drake, 1982). Derivatized spheres were cross linked by glutaraldehyde (Otto, Takamiya & Vogt, 1973) to antibodies directed against various immunoglobulins. In most cases, spheres were linked to sheep anti-rabbit IgG creating a probe that could react with any IgG made in a rabbit.

Labelling technique

Cell cultures were established from the skin of 10-day-old chick embryos. Sections of skin were washed in phosphate-buffered saline (PBS), pH 7·2, minced, and transferred to culture dishes containing Hams F-12 medium supplemented with 10 % foetal calf serum and 1 % antibiotic-antimycotic (GIBCo). After 24 h, the larger explants were removed, leaving behind mostly dermal fibroblasts which had emigrated onto the dish. Cultures were grown for 1–7 days and switched for their final 24 h *in vitro* to serum-free medium. At the end of the culture period, medium was withdrawn and cells were washed several times in PBS and fixed for 30 min at room temperature in half-strength Karnovsky's (Karnovsky, 1965).

Chick embryos from stages 3–12 (Hamburger & Hamilton, 1951) were fixed for 30 min at room temperature in half-strength Karnovsky's. Younger embryos were fixed in rings placed on the blastoderm, following the sub-blastodisc injection method of Meier & Jacobson (1982). Older embryos were snipped from the blastoderm, washed in PBS and fixed in a culture dish. Any dissections were performed immediately after aldehyde fixation.

Cell cultures or embryos were placed in 0·01 % bovine serum albumin in 0·15 M-Tris buffer (pH 7·4) overnight and washed in PBS (pH 7·2). Those samples receiving hyaluronidase treatment were washed in PBS (pH 5·6) and incubated for 1 h at 37 °C with 4000 NF Units/ml testicular hyaluronidase (Sigma, Bovine Type VI-S) in PBS (pH 5·6). After enzymatic digestion, specimens were rinsed and returned to PBS (pH 7·2). Samples were placed in microwells and rabbit pre-immune serum or rabbit anti-chick fibronectin antibody was added. After 45 min, samples were washed three times with PBS and exposed an additional 45 min to sheep anti-rabbit IgG conjugated microspheres. Samples were again washed three times with PBS, rinsed once in 0·1 M-cacodylate buffer (pH 7·4), and transferred to 1 % osmium tetroxide for 45 min. Samples were

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Fig. 1. SEM of cultured chick embryo fibroblasts. (A) Cells treated with anti-fibronectin antibody are fibronectin positive because they are decorated with microspheres. (B) Control cultures, treated with preimmune rabbit serum are beadless, indicating that there is little non-specific IgG in rabbit serum that adheres to chick embryo fibroblasts. (A) ×5650, (B) ×5935.

Fig. 2. Higher magnification of cultured fibroblast treated with the fibronectin probe. Microspheres are attached individually to the cell surface and are aligned in rows in some areas. ×13335.
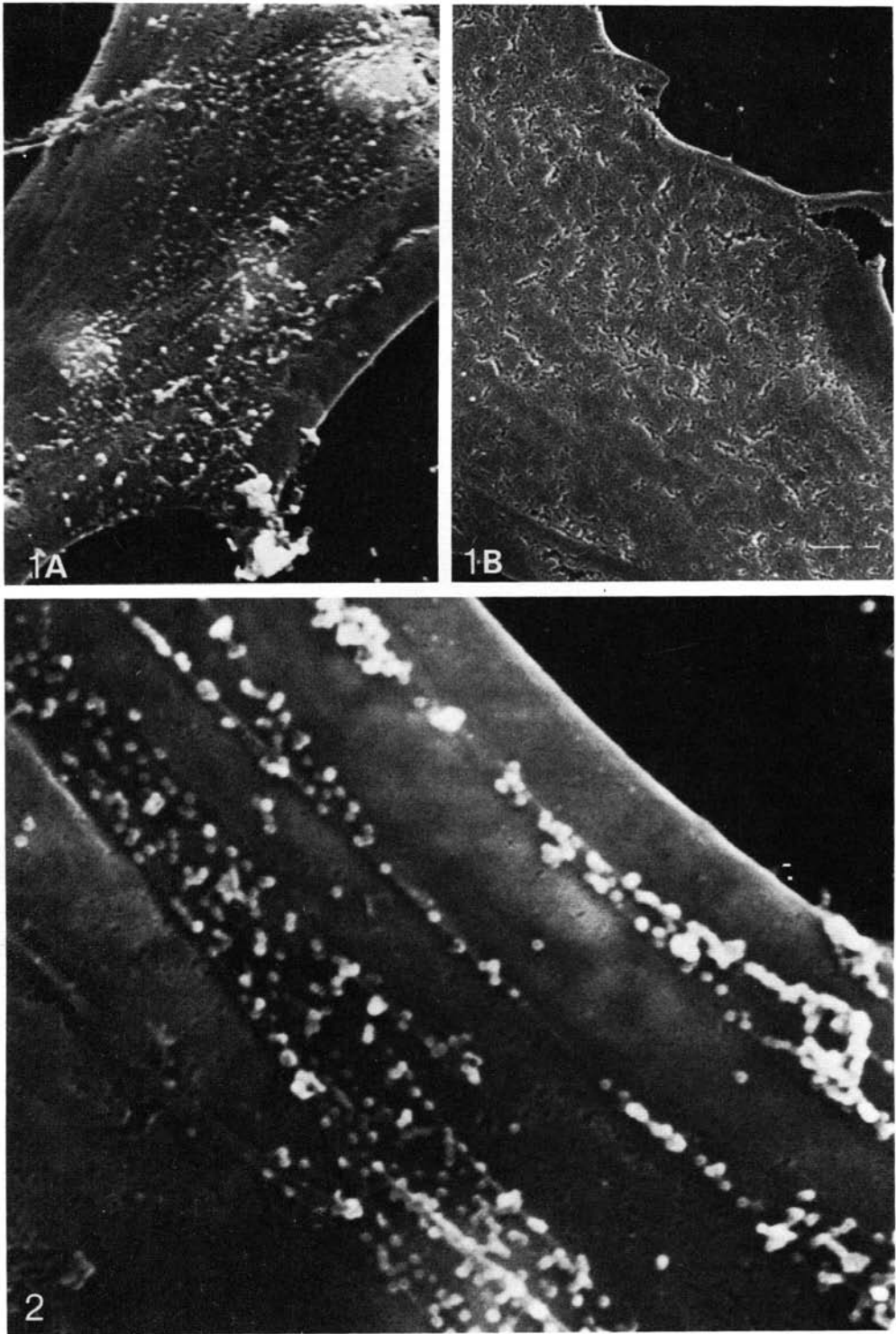
Figs. 1–2
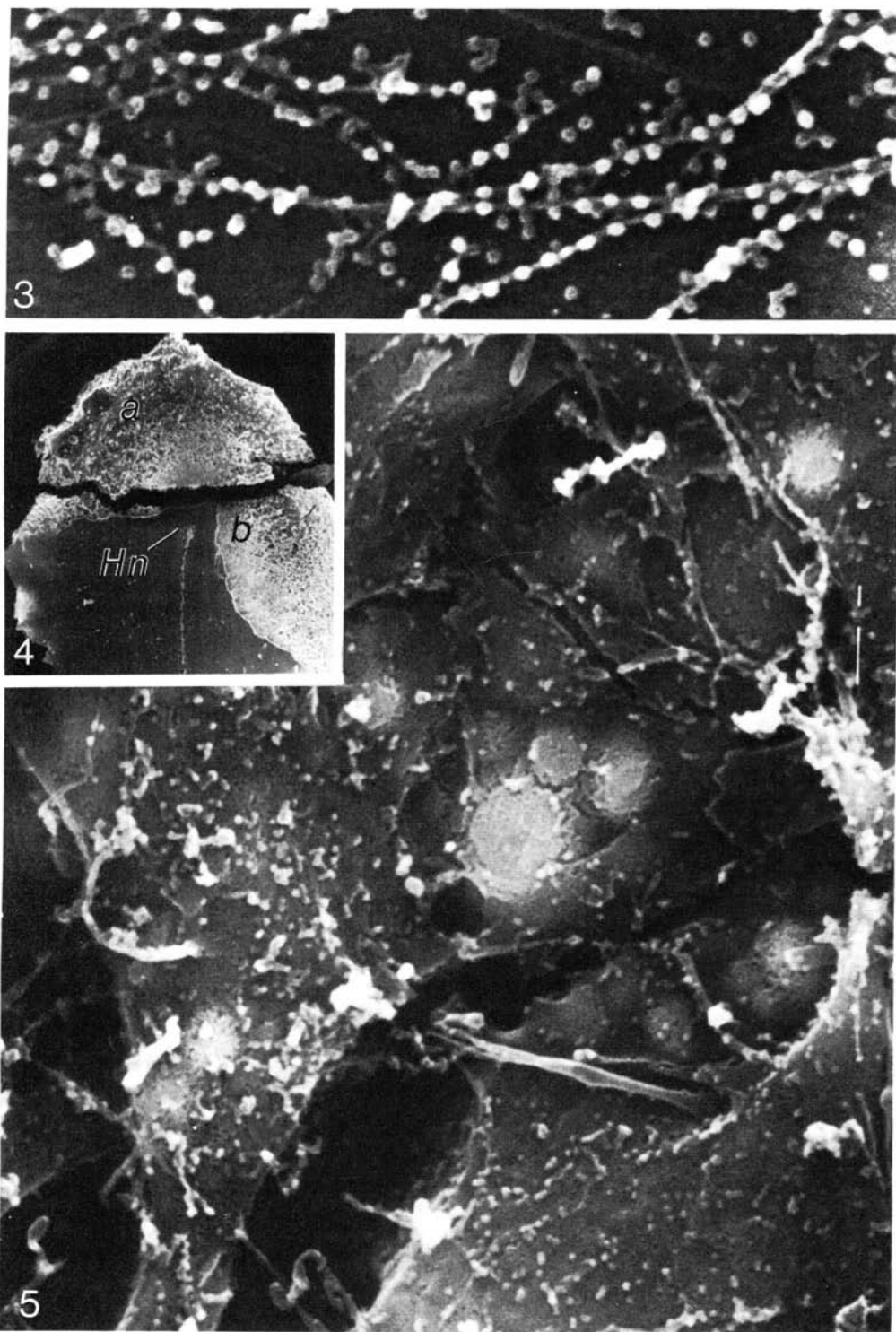
Figs. 3–5

rinsed in cacodylate buffer, dehydrated through graded alcohols, and critical-point dried with CO_2 as the exchange fluid.

Dried specimens were mounted on aluminum stubs covered with double-stick tape and sputter-coated with 6–7 nm of gold–palladium alloy. Samples were examined in an ISI Super IIIA scanning electron microscope.

RESULTS

In order to test our probe, we first chose to label cells that were known producers of fibronectin. Cultured chick fibroblasts have been shown to synthesize fibronectin (Ruoslahti, Vaheri, Kuusela & Linder, 1973) and fibronectin has been localized by immunofluorescence in the extracellular matrix (ECM) produced by fibroblasts *in vitro* (Yamada, 1978). Therefore, primary cell cultures of 10-day-old chick fibroblasts were seeded onto plastic dishes and grown for 24 h *in vitro*. Culture medium was removed and the cells were fixed and treated with either rabbit anti-chicken fibronectin IgG or pre-immune rabbit IgG. After treatment with one of the antibodies, cells were exposed to sheep anti-rabbit IgG conjugated microspheres and processed for SEM. As seen in Fig. 1, the surface of fibroblasts treated with pre-immune serum was beadless, whereas cells treated with rabbit anti-chicken fibronectin were decorated with microspheres. Fibronectin was localized on the fibroblast cell surface, both on the cell soma and on cell processes. All of the microspheres exhibited punctate binding, appearing as single entities on the cell surface. However, there was often an alignment of microspheres into lines and rows along the cell surface (Fig. 2), suggesting that this might be a site where fibronectin positive fibres are assembled. Since most of the matrix is deposited between the fibroblast and the culture dish during the first day in culture, cells were lysed in mild detergent (0·4 % Triton X-100 in PBS for 5 min) to expose the underlying ECM. As seen in Fig. 3, after treatment with anti-fibronectin IgG, microspheres decorate matrix fibres. Although a few beads bind to the culture dish where there are no obvious fibres, the majority of microspheres are deposited at regular intervals along strands of ECM. Comparable fibroblast cultures treated with pre-immune rabbit serum were beadless.

Fig. 3. SEM of the fibronectin-positive matrix secreted onto the culture dish by chick embryo fibroblasts. Cells were lysed from the culture dish with 0·4 % Triton X-100 to reveal matrix fibrils that were decorated regularly with microspheres (225 nm intervals). ×18000.

Fig. 4. SEM of a stage-4 chick embryo from which the surface ectoderm has been removed at the cranial end. Most of the exposed mesoderm is contained in the upper piece that is separated by a crack across the blastoderm cranial to Hensen's node (Hn). Higher magnifications of regions 'a' and 'b' appear in Figs 5 and 6. ×28.

Fig. 5. SEM of the mesoblast of a stage-4 chick embryo in region 'a' of Fig. 4, treated with the probe to fibronectin. Mesodermal cells are fibronectin positive, as indicated by the localization of microspheres. ×8420.
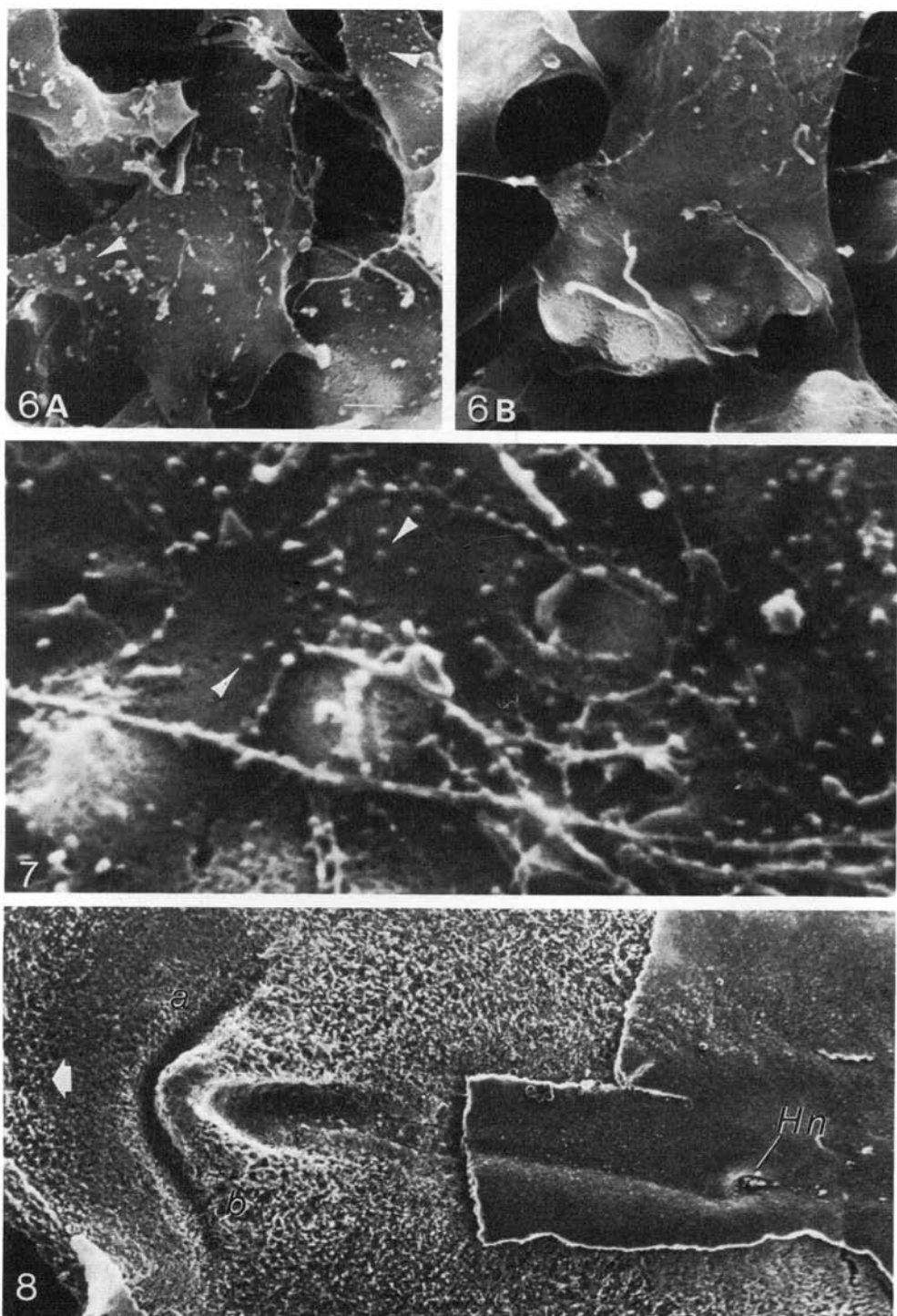
Figs. 6–8

Critchley *et al*. (1979), have shown by immunofluorescence that the cranial-most end of a stage-5 chick embryo is rich in fibronectin. At this time, most of the staining is confined to a crescent-shaped band cranial to the head process. Therefore, as a beginning point for this survey of the cranial distribution of cell-associated fibronectin, stage-4 to -5 chick embryos were fixed and surgically stripped of surface ectoderm (Fig. 4). Specimens were then treated with testicular hyaluronidase and thoroughly rinsed to remove as much ECM as possible. This treatment enhances decoration, presumably by making cell surfaces more visible and exposing fibronectin sites which might otherwise be masked by interaction with glycosaminoglycans. Enzyme-treated specimens were exposed to fibronectin probe or to pre-immune serum and observed with the SEM. Fibronectin was localized on mesoderm cells cranial to the head process (Fig. 5). Beads were individually localized on cell somas and most were distributed rather randomly. However, the somas of some mesodermal cells exhibited alignment of microspheres into short rows. Longer strands of beads were usually associated with fine filopodial extensions and matrix fibres. When mesodermal cells of the

Fig. 6. SEM of a stage-4 chick embryo mesoblast from region labelled 'b' in Fig. 4. (A) Mesodermal cells of cranial somitomeres are fibronectin positive, with more fibronectin localized on cell processes (white arrowheads) than on broad regions of the cell soma. (B) Control embryos treated with preimmune rabbit serum were essentially beadless. (A) and (B) ×5735.

Fig. 7. SEM of the basal surface of ectoderm removed from the cranial region of a stage-4 chick embryo similar to that removed from the specimen in Fig. 4. Treatment with our fibronectin probe showed fibronectin to be localized directly on the basal lamina (white arrowheads) as well as on small fibrils adherent to the basal lamina. ×12500.

Fig. 8. SEM of a stage-6 chick embryo from which ectoderm has been removed from much of the cranial end (white arrowhead points cranially). Mesoderm from regions 'a' and 'b' is shown in Figs 9 and 10. Hn = Hensen's node. ×78.

Fig. 9. SEM of mesoderm from region labelled 'a' in Fig. 8 treated with fibronectin probe. Microspheres are plentiful on cell somas and filopodia. ×8400.

Fig. 10. SEM of mesoderm from region labelled 'b' in Fig. 8 treated with fibronectin probe. Fibronectin is localized mostly on cell extensions and edges rather than cell somas (white arrowheads). ×4865.

Fig. 11. SEM of mesoderm from region labelled 'b' in Fig. 9, treated with pre-immune rabbit serum. Cell surfaces are beadless. ×6250.

Fig. 12. SEM of the cranial end of a stage-12 chick embryo from which the surface ectoderm has been removed from the right side (black arrowhead points cranially). Neural crest cells have emigrated extensively, forming prosencephalic (pro), mesencephalic (mes), and rhombencephalic (rhom) populations. A gap in crest emigration (white circle) occurs just cranial to the otic placode. ×105.

Fig. 13. SEM of prosencephalic crest, similar to that seen in Fig. 14, treated with fibronectin probe. Fibronectin is localized on crest cell somas and processes, and occasionally aligned in rows (arrowheads). ×8760.

Fig. 14. SEM of mesencephalic crest, similar to that seen in Fig. 14, treated with fibronectin probe. Fibronectin sites cover most of the neural crest cell surface and appear clumped in some regions where matrix is retained (arrows). ×12360.
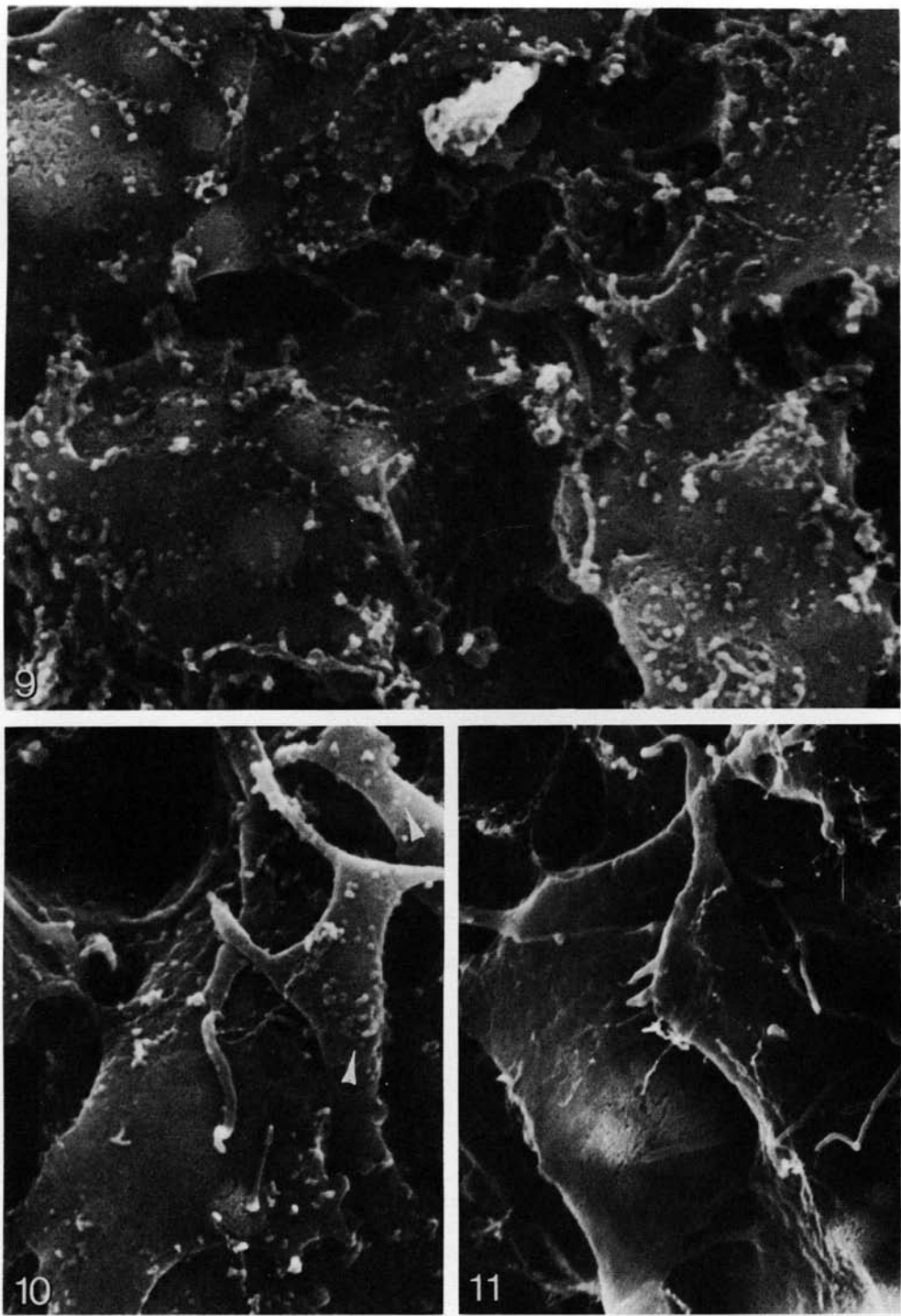
Figs. 9–11. For legend see p. 32-9.
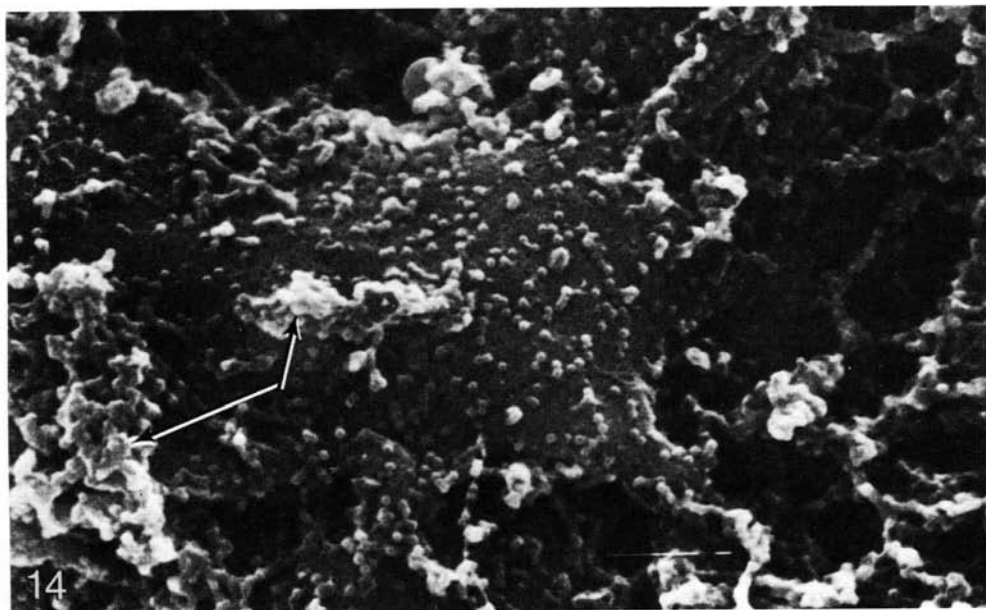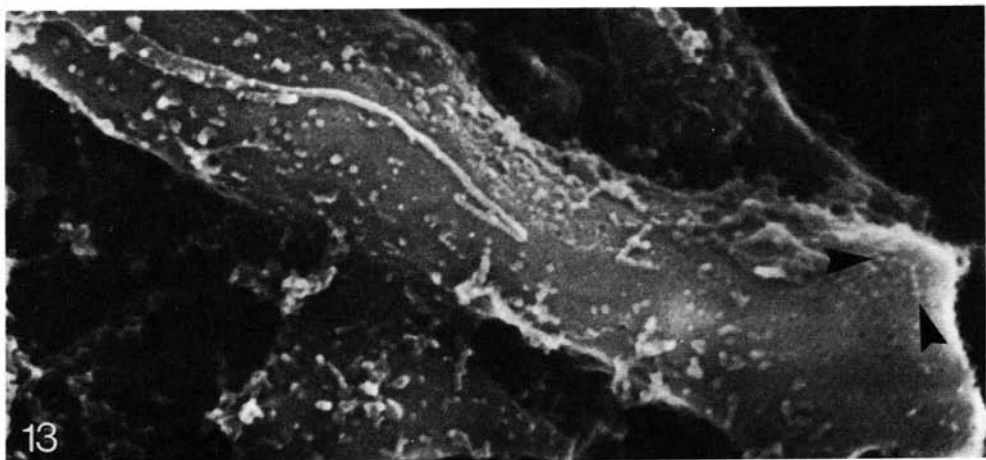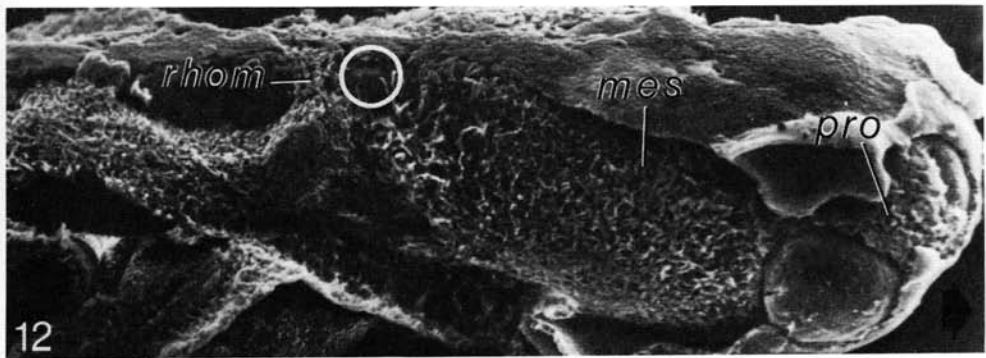
Figs. 12–14. For legend see p. 32-9.

first somitomeres were examined, they were also found to be fibronectin positive, but to a noticeably lesser extent than their more cranial neighbours (Fig. 6A). Microspheres decorated various portions of the cell somas, and even though some lamellipodia were moderately decorated, similar extensions sprouting from the same cell were not decorated. Examination of the mesoderm of control embryos treated with pre-immune rabbit IgG showed that the cells bound few microspheres, if any (Fig. 6B). In general, for control specimens, all mesodermal cell somas and processes were free of microspheres and less ECM was retained.

Examination of the ectoderm removed from stage-4 embryos treated with the fibronectin probe revealed that microspheres were bound to the basal surface (Fig. 7). The basal lamina is largely incomplete and there are holes through which epithelial processes protrude. Some of the microspheres are bound individually to bare regions of the basal lamina where it is complete. However, many of the spheres are aligned tandemly, along fibres and along the edges of epithelial cell processes. Control ectoderm treated with pre-immune serum was beadless (not shown).

By stage 6, most of the cranial mesoderm has formed and Hensen's node has regressed. By removing the surface ectoderm and applying our microsphere probe, we were able to localize fibronectin in all regions of the cranial mesoderm (Fig. 8). When an area of mesoderm cranial to the head process was examined with the SEM, it was found to be well decorated with microspheres (Fig. 9, approximately 1050 spheres/100 μm^2). Fibronectin sites were localized over most of the cell soma, although there was often an increased density on lamellipodia and filopodia. Fibronectin was also localized in rows along ECM fibres. In general, there are more microspheres bound at this time than at earlier stages (see Fig. 5, approximately 750 spheres/100 μm^2). The same was true for the mesodermal cells of the first somitomeres, in that there was more cell surface associated fibronectin at stage 6 than at stage 4 (Fig. 10, compare Fig. 6A). However, by stage 6, fibronectin seemed to be preferentially localized on filopodia and cellular edges rather than on the broader surfaces of the cell soma. When the most recently formed somitomere of a stage-6 embryo was examined near Hensen's node, its number and pattern of microsphere binding was most similar to the first somitomere seen earlier at stage 4. Likewise, there was no obvious gradient of fibronectin from the centre to the periphery of somitomeres.

Fig. 15. SEM of rhombencephalic crest, similar to that seen in Fig. 14, treated with fibronectin probe. Fibronectin sites are localized to the neural crest cell surface as well as the surrounding matrix. ×13 160.

Fig. 16. SEM of the basal lamina of surface ectoderm that covers the mesencephalic crest of a chick embryo (similar to that removed in Fig. 12), treated with our probe to fibronectin. Fibronectin is located on the surface of the lamina and on fibrous strands of ECM that course by (arrows). ×13 420.
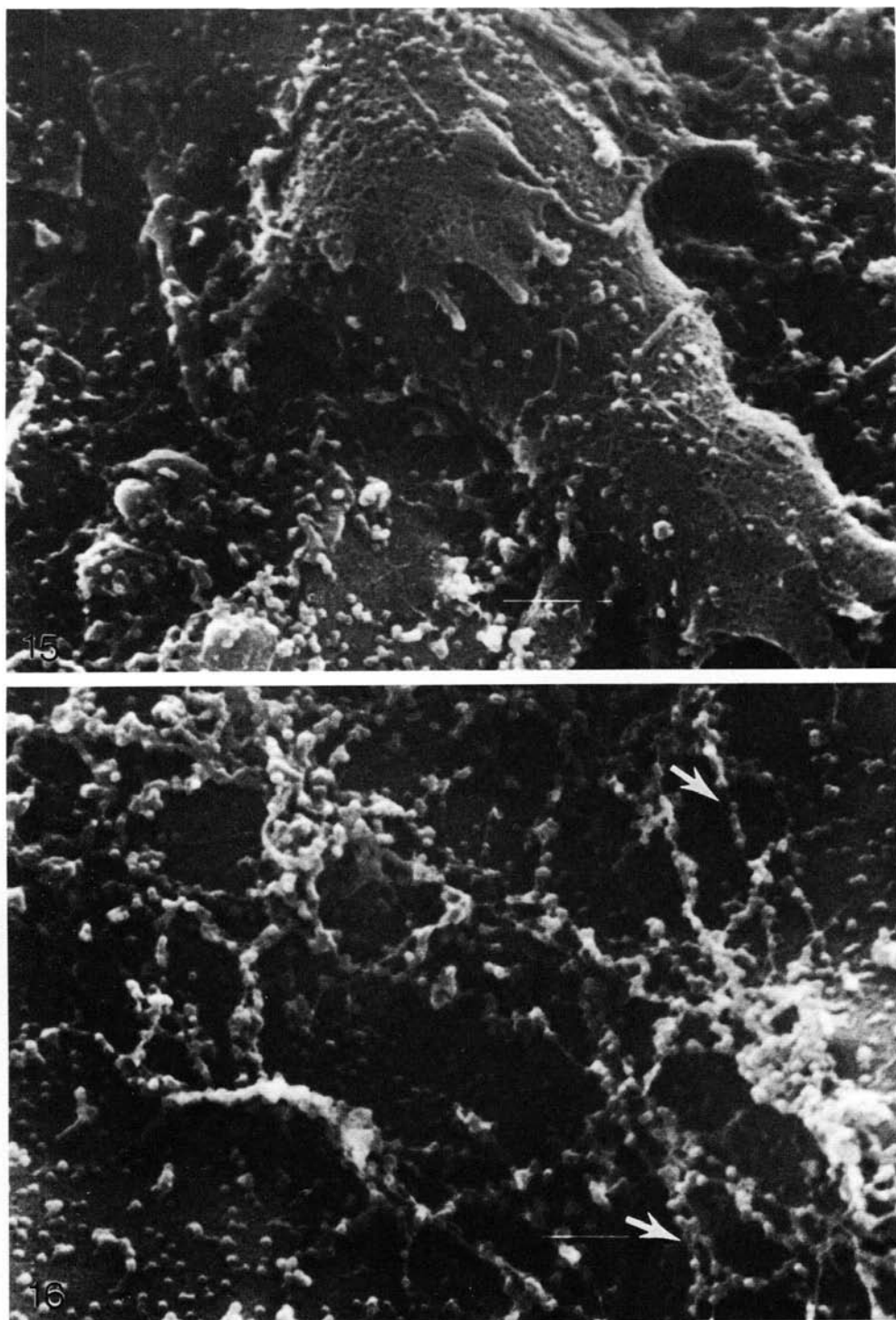
Figs. 15–16
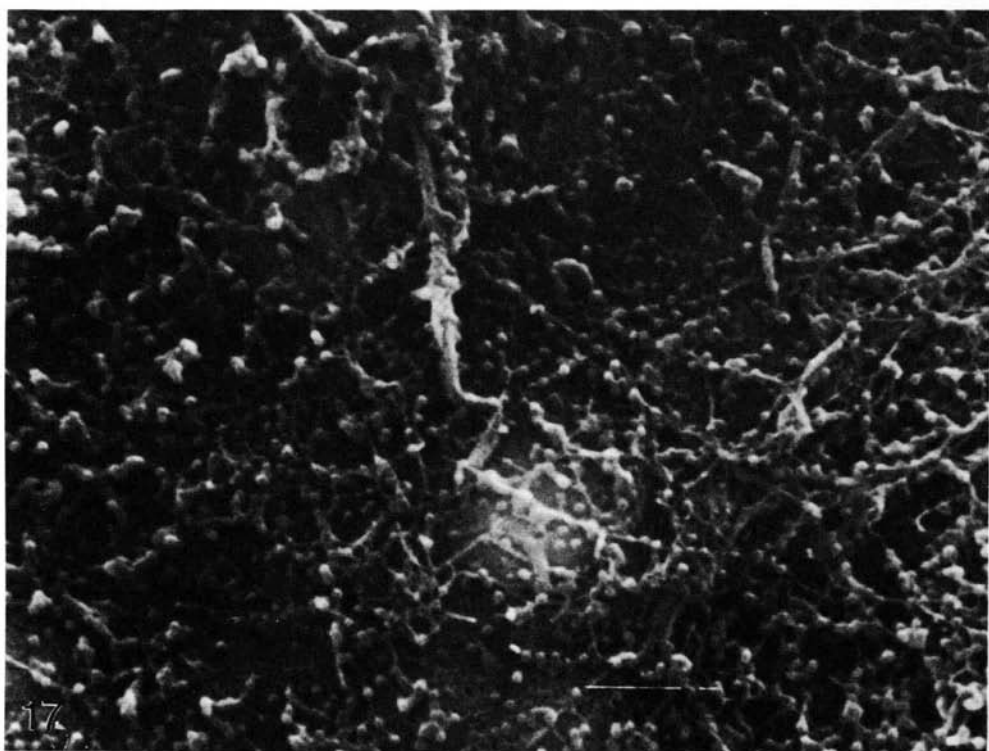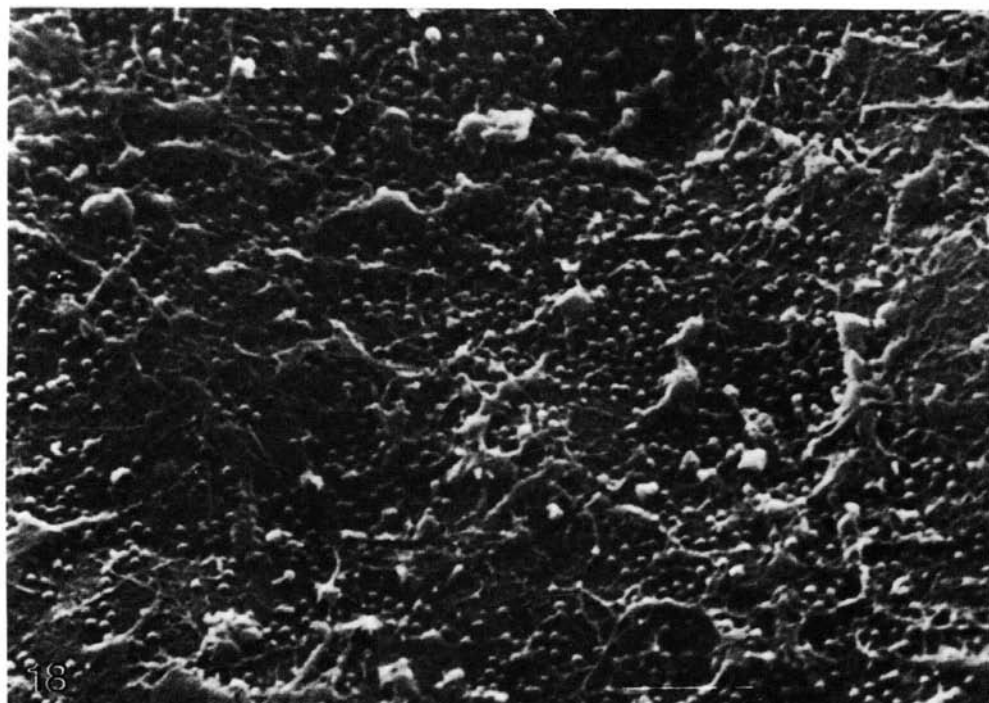
Figs. 17–18

Instead, there was mainly a segment-by-segment, cranial-to-caudal decrease in cell-surface-associated fibronectin. Stage-6 mesoderm treated with pre-immune rabbit IgG was beadless (Fig. 11) and much less ECM was retained.

A general increase in fibronectin labelling of the mesoderm was correlated with segmental age and position along the axis. By the time the cranial neural crest cells emerge, all the paraxial somitomeres are fibronectin positive. Although specific populations of crest were examined in detail, a single stage of neural crest dispersion will be used here as an example. At stage 11–12, (Fig. 12) most of the cranial neural crest is broadly distributed in the head. The prosencephalic crest has migrated rostrally over the optic vesicle, whereas the mesencephalic crest has spread as a dorsal shelf over the first four somitomeres. The rhombencephalic crests are represented by the otic populations, which have encircled the periphery of the otic placode in the surface ectoderm. Representative crest cells from each of the major brain regions were exposed to the fibronectin probe and examined with the SEM. As seen in Figs 13–15, all of the cranial neural crest cells are fibronectin-positive. However, the cranial-to-caudal gradient of fibronectin does not hold for the cranial crest. Even though the prosencephalic crest were fairly well labelled with microspheres, the mesencephalic crest were even more heavily labelled. Numerous microspheres were bound to almost every portion of the cell surface, and much fibronectin-positive ECM was retained as well. In contrast, the otic neural crest cells were about as fibronectin positive as the prosencephalic population, but were still not as heavily labelled as the mesencephalic crest. In general, all cranial neural crest cells in these dorsal locations were fibronectin positive from the time of their initial appearance at the midline. Crest cells tend to accumulate fibronectin as they spread from the midline and they soon become more fibronectin positive than the subjacent somitomeres.

When the surface ectoderm covering the mesencephalic crest was exposed to fibronectin probe and examined with the SEM, the basal lamina was quite well decorated with microspheres (Fig. 16). The basal lamina is complete in most places and the microspheres are individually bound to it in random fashion. Most of the fibronectin is bound to the scant ECM that is closely applied to the basal lamina. Microspheres are clustered in places where matrix fibres intersect, but are generally aligned in tandem along fibrils. In general, most of the surface ectoderm was moderately labelled, but other basal laminae were even more

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Fig. 17. SEM of the basal surface of the otic placode removed from a stage-12 chick embryo and treated with our probe to fibronectin. Although positive for fibronectin, little of the basal lamina is visible due to the retention of an extensive fibrillar network that also is laden with fibronectin. ×13 420.

Fig. 18. SEM of the basal lamina of the rhombencephalon of a stage-12 chick embryo from a region not traversed by cranial neural crest cells (white circle, Fig. 12), treated with fibronectin probe. The basal lamina is nearly uniformly fibronectin positive and relatively free of associated fibrillar ECM. ×12 875.

fibronectin positive. For instance, the undersurface of the otic placode (Fig. 17) is even richer in fibronectin. It is bound to the basal lamina and to the rich fibrillar network associated with the epithelial undersurface. Fibronectin occurs regularly along the thin fibrils being spaced about 225 nm apart. However, some basal laminae retain little fibrillar matrix. The basal surface of the neural ectoderm of rhombomere Rh_5, for example, is rather free of ECM fibres, but its basal lamina is covered with fibronectin (Fig. 18). Microspheres are bound individually and nearly blanket the basal lamina in some areas.

DISCUSSION

Efficacy of microsphere labelling

The use of the immunolatex spheres to localize specific molecular sites is an adaptation for the SEM of the immunofluorescence technique that has been so successful at the light microscopic level. The principal advantage of the immunolatex spheres is, of course, that one can more precisely define the location of antigenic sites and resolve them with the precision of the SEM. Extracellular molecules associated with the outer leaflet of the plasmalemma and glycocalyx are particularly suitable because these surfaces are most easily accessible to the SEM. Another more practical advantage is that fluorescent labelling diminishes with time, whereas the specimens prepared for SEM are stable nearly indefinitely and can be examined, stored, and re-examined at later times.

Apart from increased resolution, another major advantage of microsphere labelling is that it is a much more sensitive means of detection than fluorescence. The immunofluorescent technique involves treatment of specimens with chemicals that often react mildly and generate an autofluorescence or 'background' reaction. Even when this is held to a minimum, the positive fluorescence is almost always superimposed upon a faintly luminous background. Utilizing microspheres, the 'background' is almost always zero. In spite of the fact that we routinely used five times as much pre-immune serum as anti-fibronectin antibody, our control specimens were essentially beadless. This means that there is very little (if any) rabbit IgG binding non-specifically to the specimens. In addition, the sheep anti-rabbit IgG coated microspheres themselves are negatively charged and do not bind non-specifically because they actually repel cell surfaces as well as one another. Therefore, the microspheres must be physically bound to the specimen by virtue of their interaction with rabbit antibody, presumably localized on the site of the antigen. Since the microspheres will adhere to even a single bound rabbit IgG molecule, positive decoration is highly significant and almost certainly due to the treatment with the specific rabbit-made antibody.

Positive labelling with microspheres depends mostly on the quality of the rabbit-made antibody applied. In our case, we initiated antibody production in rabbits by injection with fibronectin isolated from chick plasma, so the source of

the antigen was from the same species that was used to test the probe. Purified rabbit IgG gave a single line of precipitation in ouchterloney gels when tested against chick plasma and other known sources of fibronectin. Furthermore, when a Western gel was run against total homogenates of stage-4 chick embryos, autoradiograms revealed that over 80 % of the proteins recognized by our antibodies corresponded to the fibronectin standard. It is even possible that the minor components might be breakdown products of fibronectin, which is highly protease sensitive. Furthermore, pretreatment with guinea pig anti-chicken fibronectin IgG greatly diminishes the subsequent binding of our rabbit anti-fibronectin IgG. This suggests that the guinea pig antibody binds to fibronectin sites and makes them unavailable for recognition by rabbit anti-fibronectin IgG. Finally, rabbit-made antibodies to fibronectin supplied by experts gave virtually identical results to our own. Therefore, we feel that an overwhelming number of antigen sites localized by the microspheres are in fact fibronectin.

Localization of fibronectin

Utilizing the immunolatex microspheres, we were able to localize fibronectin to the basal lamina of most epithelial cells. At stages 3–4 of chick development, the basal lamina is incomplete under the cranial surface ectoderm and epithelial cell processes actually protrude through holes in it. Fibronectin is relatively sparse on the basal lamina at this time and there is nearly as much fibronectin on associated matrix fibres as on the lamina itself. This is in agreement with the studies of Critchley *et al.* (1979) and Duband & Thiery (1982*a*), who showed the basement membrane at these stages was weakly fibronectin positive. However, by stage 12, the basal lamina underlying cranial surface ectoderm is complete and only the vague suggestion of epithelial cells are visible through it. Fibronectin is now more plentiful on the lamina and rather evenly distributed along it. The combination of fibronectin-positive basal lamina and associated fibrous matrix undoubtedly contributes heavily to the fibronectin-positive basement membrane recognized in this region by Duband & Thiery (1982*b*) with the light microscope. However, the basal lamina of nearby cranial structures are even more enriched in fibronectin than the surface ectoderm. The basal lamina of the rhomben-cephalon is literally blanketed with fibronectin and the highly decorated basal lamina of the otic placode is associated with an even denser mat of fibronectin-positive fibres. Therefore, in the cranial region, fibronectin is always found where a basal lamina is present, although it varies in concentration depending on the age and location of the particular lamina.

Fibronectin was also localized to the surface of newly gastrulated mesen-chymal cells at early primitive streak stages. Initially, cells at the cranial-most end of the axis are the most fibronectin positive, and those cells located in more caudal somitomeres (to either side of Hensen's node, in the 'somite forming centers' of Spratt (1955)), were less labelled. The failure of others (Critchley *et al.* 1979; Duband & Thiery, 1982*a*) to detect significant fibronectin in the cranial

mesoderm of these young embryos is probably a reflection of the insensitivity of the immunofluorescence technique. When we examined an individual cranial somitomere for fibronectin, there was no obvious gradient of labelling from centre to periphery of the unit. However, with time, cranial somitomeres do accumulate fibronectin and those segments established first become more heavily labelled than somitomeres most recently added to the axis near Hensen's node. Cheney, Seitz & Lash (1981) have shown a similar cranial to caudal gradient of fibronectin deposition in the segmental plates of older embryos. It has recently been shown (Meier, 1979; Solursh et al. 1979; Packard & Meier, 1983) that the segmental plates of birds also contain a tandem sequence of somitomeres undergoing morphogenesis that culminates in somite formation. Therefore, the general cranial to caudal gradient of fibronectin labelling of the mesodermal layer is likely to be a reflection of the progressive maturation and differentiation of segments that takes place during embryonic development.

   Cranial neural crest cells are fibronectin-positive at the time of their emergence from the midline and during their early migration between the surface ectoderm and paraxial mesoderm. The prosencephalic crest emigrates from the brain onto the paraxial mesoderm, and then returns to the rostral surface of the optic vesicles to participate in the development of numerous ocular tissues (see Noden, 1983). The lead crest cells, as well as those located more medially, show moderate amounts of fibronectin localized to the cell surface. The lead cells did show preferential localization of fibronectin to filopodia, but there were no obvious differences in fibronectin concentration among cells in this population. Mesencephalic crest are the first cranial crest released from the brain and migrate onto the paraxial mesoderm to cover the dorsolateral surface of the second, third, and fourth somitomeres (Anderson & Meier, 1981). Mesencephalic crest cell surfaces are enriched in fibronectin, approaching concentrations found for some basal laminae. This in spite of the fact that fibronectin is reportedly very sparse in the local extracellular matrix in this region (Duband & Thiery, 1982b). Newgreen & Thiery (1980) have shown that cultured mesencephalic crest cells synthesize fibronectin and it is likely that some of the fibronectin we visualize here is made by the mesencephalic crest cells themselves. However, localization of fibronectin to a particular cell surface does not prove that it was secreted by that cell. The rhombencephalic crest, as represented by the rostral otic crest, migrate into the intersegmental groove between the fifth and sixth somitomeres, around the rostral edge of the otic placode (Anderson & Meier, 1981). These cells remain confined in a sparsely fibronectin-positive groove in the mesoderm. This is in spite of the immediate proximity of the fibronectin-enriched basal lamina of more cranial rhombencephalon (devoid of crest cells) and the highly fibronectin-positive basement membrane of the adjacent otic placode. Fibronectin is fairly concentrated on the surface of the otic crest cells themselves, but gradually diminishes at more caudal (cervical) levels. Mayer et al. (1981) have shown fibronectin to be associated with the cell surface of trunk neural crest and

we have decorated trunk crest to a modest extent with our fibronectin probe as well (Meier & Drake, unpublished observations).

It seems likely that in the chick embryo, fibronectin becomes a ubiquitous component of the extracellular matrix shortly after gastrulation is initiated. Although there are regional differences in its concentration, fibronectin accumulates in the embryo in the extracellular matrix and in virtually every cranial basal lamina. Primary mesoderm cells and neural crest cells interact with fibronectin presumably by possessing fibronectin receptors, which are thought to be partially hydrophobic, intramembranous glycoproteins (Ruoslahti *et al.* 1981; Yamada, Akiyama & Hayashi, 1981). Therefore, increased localization of fibronectin to these cell populations probably represents an increase in the number of fibronectin receptor molecules in their cell membranes. If one presumes that the function of fibronectin in embryonic matrix is to serve as an adhesive molecule for interaction with cell surfaces, then it is logical to expect that neural crest cells, actively spreading into matrix compartments, would have much more cell surface associated fibronectin than more stationary mesodermal cells radially arranged in a stable somitomeric pattern, as is the case here. Since fibronectin is abundant, intrusions into specific cranial regions is therefore likely to depend on the appearance of crest cell fibronectin receptors. Although fibronectin localized along fibrous strands of matrix and in basal laminae may provide the appropriate conditions for cell movement, many fibronectin rich areas are not utilized as preferred substrata for crest migration and it is difficult to explain the migratory routes of cranial crest considering only the location of fibronectin. Topographical restraints resulting from cranial morphogenesis may also serve to further direct crest relocations by limiting or enhancing cell accessibility to fibronectin. In the final analysis the behaviour of crest cells toward fibronectin i.e., cell migration (Greenberg, Seppä, Seppä & Hewit, 1981) or the extension of neurites (Turner *et al.* 1983), may depend on what intracellular events are coupled to fibronectin receptors.

REFERENCES

ANDERSON, C. D. & MEIER, S. (1981). The influence of the metameric pattern in the mesoderm on migration of cranial neural crest cells in the chick embryo. *Devl Biol.* **85**, 385–402.

BURNETT, W. N. (1981). "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**, 195–203.

CHENEY, C. M., SEITZ, A. W. & LASH, J. W. (1981). Fibronectin, cell adhesion, and somite formation. *J. Cell Biol.* **87**, 94a.

CRITCHLEY, D. R., ENGLAND, M. A., WAKELY, J. & HYNES, R. O. (1979). Distribution of fibronectin in the ectoderm of gastrulating chick embryos. *Nature* **280**, 498–500.

DUBAND, J. L. & THIERY, J. P. (1982a). Appearance and distribution of fibronectin during chick embryo gastrulation and neurulation. *Devl Biol.* **94**, 337–350.

DUBAND, J. L. & THIERY, J. P. (1982b). Distribution of fibronectin in the early phase of avian cephalic neural crest cell migration. *Devl Biol.* **93**, 308–323.

ENGVALL, E. & RUOSLAHTI, E. (1977). Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer* **20**, 1–5.

GOODRICH, E. S. (1958). Morphology of the head region. In: *Studies on the Structure of Vertebrates*, Vol. 1, pp. 213–229. New York: Dover.

GREENBERG, J. H., SEPPÄ, S., SEPPÄ, H. & HEWIT, A. T. (1981). Role of collagen and fibronectin in neural crest cell adhesion and migration. *Devl Biol.* **87**, 259–266.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

KARNOVSKY, M. J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**, 137a.

KATOW, H., YAMADA, K. M. & SOLURSH, M. (1982). Occurrence of fibronectin on the primary mesenchyme cell surface during migration in the sea urchin embryo. *Differentiation* **22**, 120–124.

KLEBE, B. J. (1974). Isolation of a collagen-dependent cell attachment factor. *Nature* **250**, 248–251.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **277**, 680–685.

MAYER, B. W., HAY, E. D. & HYNES, R. O. (1981). Immunocytochemical localization of fibronectin in embryonic chick trunk and area vasculosa. *Devl Biol.* **82**, 267–286.

MEIER, S. (1979). Development of the chick embryo mesoblast. Formation of the embryonic axis and establishment of the metameric pattern. *Devl Biol.* **73**, 24–45.

MEIER, S. (1981). Development of the chick embryo mesoblast: morphogenesis of the prechordal plate and cranial segments. *Devl Biol.* **83**, 49–61.

MEIER, S. & DRAKE, C. J. (1982). Development of a latex-conjugated immunocytological marker for SEM analysis of quail-chick chimeras. *J. exp. Zool.* **224**, 25–37.

MEIER, S. & JACOBSON, A. G. (1982). Experimental studies of the origin and expression of metameric pattern in the chick embryo. *J. exp. Zool.* **219**, 217–232.

MEIER, S. & PACKARD, D. S., JR. (1983). Morphogenesis of the cranial segments and distribution of neural crest in the embryos of the snapping turtle, *Chelydra serpentina*. *Devl Biol.* (in press).

MITRANI, E. & FARBEROV, A. (1982). Fibronectin expression during the process leading to axis formation in the chick embryo. *Devl Biol.* **91**, 197–201.

MOLDAY, R. S., DREYER, W. J., REMBAUM, A. & YEN, S. P. S. (1975). New immunolatex spheres: visual markers of antigens on lymphocytes for SEM. *J. Cell Biol.* **64**, 75–88.

NEWGREEN, D. & THIERY, J. P. (1980). Fibronectin in early avian embryos: synthesis and distribution along the migration pathways of neural crest. *Cell Tissue Res.* **211**, 269–291.

NODEN, D. M. (1983). The role of neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Devl Biol.* **96**, 144–165.

OTTO, N., TAKAMIYA, N. & VOGT, R. (1973). A two-stage method for cross-linking antibody to ferritin by glutaraldehyde. Comparison between one-stage and two-stage method. *J. Immunol. Methods* **3**, 137–146.

PACKARD, D. S., JR. & MEIER, S. (1983). An experimental study of the somitomeric organization of the avian segmental plate. *Devl Biol.* **97**, 191–202.

ROGERS, S. L., LETOURNEAU, P. C., PALM, S. L., McCARTHY, J. & FURCHT, L. T. (1983). Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. *Devl Biol.* **98**, 212–220.

ROMANI, M., VIDAL, G., TAHOURDIN, C. S. M. & BUSTIN, M. (1980). Solid phase radioimmunoassay for chromosomal components. *J. biol. Chem.* **225**, 468–474.

RUOSLAHTI, E., ENGVALL, E. & HAYMAN, E. G. (1981). Fibronectin: current concepts of its structure and function. *Collagen Res.* **1**, 95–128.

RUOSLAHTI, E., PIERSCHBACHER, M., HAYMAN, E. G. & ENGVALL, E. (1982). Fibronectin: a molecule with remarkable structural and functional diversity. *Trends in Biochem. Sci.* **7**, 188–190.

RUOSLAHTI, E., VAHERI, A., KUUSELA, P. & LINDER, E. (1973). Fibroblast surface antigen: a new serum protein. *Biochem. Biophys. Acta* **322**, 352–358.

SIEBER-BLUM, M. & YAMADA, K. M. (1981). Cellular fibronectin promotes adrenergic differentiation of quail neural crest cells *in vitro*. *Expl Cell Res.* **133**, 205–295.

SOLURSH, M., FISHER, M., MEIER, S. & SINGLEY, C. T. (1979). The role of extracellular matrix in the formation of the sclerotome. *J. Embryol. exp. Morph.* **54**, 75–98.

SPRATT, N. T., JR. (1955). Analysis of the organizer center in the early chick embryo. I. Localization of prospective notochord and somite cells. *J. exp. Zool.* **128**, 121–163.

SUGRUE, S. P. & HAY, E. D. (1982). Interaction of corneal epithelium with exogenous collagen, laminin, and fibronectin: role of endogenous protein synthesis. *Devl Biol.* **92**, 97–106.

THIERY, J. P., DUBAND, J. L. & DELOURÉE, A. (1982). Pathways and mechanisms of avian trunk neural crest cell migration and localization. *Devl Biol.* **93**, 324–343.

TURNER, D. C., LAWTON, J., DOLLENMEIER, P., EHRISMANN, R. & CHIQUET, M. (1983). Guidance of myogenic cell migration by oriented deposits of fibronectin. *Devl Biol.* **95**, 497–504.

YAMADA, K. M. (1978). Immunological characterization of a major transformation-sensitive fibroblast cell surface glycoprotein. Localization, redistribution, and role in cell shape. *J. Cell Biol.* **78**, 520–541.

YAMADA, K. M., AKIYAMA, S. K. & HAYASHI, M. (1981). Fibronectin structure and function, and its interactions with glycosammoglycans. *Biochem. Soc. Trans.* **9**, 506–508.