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Original Article

Collagen I, laminin, and tenascin: ultrastructure and correlation with avian neural crest formation

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ABSTRACT We have investigated the distribution of type I collagen, tenascin, and laminin in younger chick embryos than have previously been studied in detail. The initial appearance of type I collagen, but not tenascin and laminin, is exactly correlated with the beginning of neural crest migration, suggesting a role for collagen I in the migration. Light microscopy of whole mounts of 2-day-old chick embryos reveals that type I collagen is expressed in a rostral to caudal gradient; it localizes to the notochord sheath before accumulating around the neural tube and somites. Collagen I and tenascin also associate with central somite cells. Surprisingly, no extracellular matrix can be detected among the early sclerotomal cells, which suggests that little or no cell migration is involved in this epithelial-mesenchymal transformation. Electron microscopy using peroxidase antiperoxidase reveals that tenascin is present in nonstriated, 10 nm wide fibrils and in interstitial bodies, both of which have previously been reported to contain fibronectin. However, collagen I only occurs in the 10 nm fibrils and larger striated fibrils. This is the first ultrastructural study to assign tenascin to fibrils and interstitial bodies and to describe its appearance and disappearance from embryonic basement membranes. The discussion emphasizes the possible importance of type I collagen in neural crest cell migration and compares the ultrastructural associations of the ECM molecules present at this early embryonic stage.

KEY WORDS: *extracellular matrix, embryogenesis, neural crest, sclerotome, epithelial somite, immunoelectron microscopy, whole mount immunohistochemistry*

Introduction

Two important epithelial-mesenchymal transformations occur simultaneously in the trunk of the 2-day-old chick embryo: the formation of neural crest from neural tube and formation of sclerotome mesenchyme from ventromedial somite wall. The first formed crest cells migrate as individuals through a dorsal pathway between somite and ectoderm and into a ventral pathway alongside the neural tube in the 16 somite embryo. The dorsal pathway develops later than the ventral pathway in the midtrunk and earlier in the rostral trunk (Newgreen *et al.*, 1986). Later, presumptive neurons and Schwann cells in the ventral pathway migrate through the rostral half of the newly formed sclerotome (Rickmann *et al.*, 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987; Tan *et al.*, 1987; Newgreen *et al.*, 1990). The abundant extracellular matrix (ECM) around the neural tube and somite (Cohen and Hay, 1971; Bancroft and Bellairs, 1976; Tosney, 1978) has been considered to play a role in the formation of these mesenchymal cells (Newgreen and Gibbons, 1982; Tosney, 1982).

Fibronectin has for some time been the ECM molecule thought to be the most important in promoting both emigration and migration of neural crest (Newgreen and Thiery, 1980; Rovasio *et al.*, 1983; Boucaut *et al.*, 1984; Thiery *et al.*, 1985). Recently, a new ECM molecule has been described that seems to be more specifically found in neural crest pathways than fibronectin; the latter is ubiquitous in the embryo. The new molecule, tenascin (Chiquet-Ehrismann *et al.*, 1986), also called cytotactin (Grumet *et al.*, 1985; Hoffman *et al.*, 1988), hexabrachion (Erickson and Inglesias, 1984), or J1-220 glycoprotein (Faissner *et al.*, 1988), often occurs as a hexamer with 6 arms, each of which is about 250 kD and is attached to a central knob. Smaller associations of subunits can occur (Erickson and Bourdon, 1989). In addition to reports of correlated localization *in vivo* of crest and tenascin (Crossin *et al.*, 1986; Tan

Abbreviations used in this paper: DAB, diaminobenzidine; D, dimensional; ECM, extracellular matrix; PAP, peroxidase antiperoxidase; TBS, Tris buffered saline pH 7.4; TEM, transmission electron microscopy.

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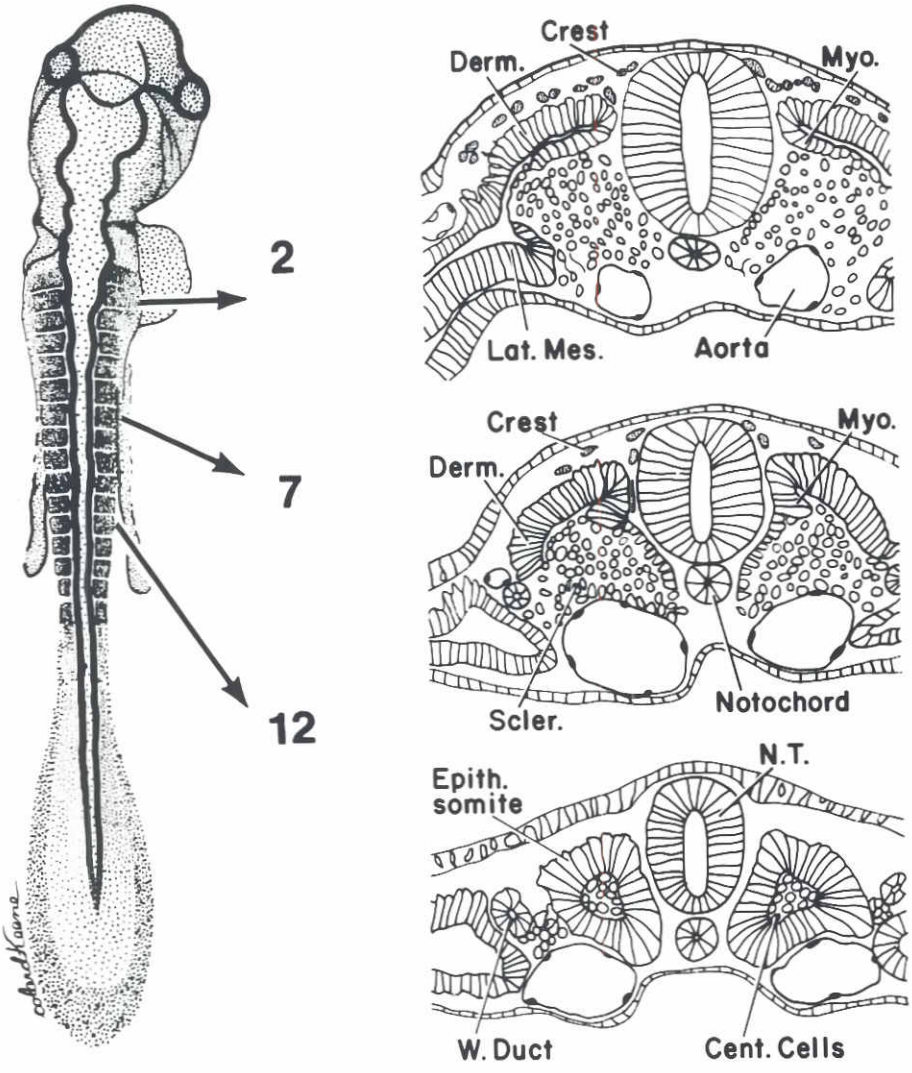


Fig. 1. Camera lucida drawing of sections of the 16 somite (stage 12) chick embryo at 2 days. *A whole mount is shown on the left. The levels studied in detail in this report show the epithelial (Epith.) somite (level of somite 12, lower right), beginning of neural crest (Crest) and sclerotome (Scler.) formation (level of somite 7, middle right), and a more advanced stage of mesenchyme formation (level of somite 2, upper right). Derm., dermatome; Myo, myotome; Lat. Mes., lateral mesoderm; N.T., neural tube; W., Wolffian; Cent., central.*

et al., 1987; Mackie *et al.*, 1988b), *in vitro* studies suggest that crest can migrate on tenascin (Halfter *et al.*, 1989). An RGD cell binding site is present in chicken and human tenascin (Friedlander *et al.*, 1988), but not in mouse tenascin (Weller *et al.*, 1991). A RGD-sensitive receptor (integrin) for human tenascin has been isolated (Bourdon and Ruoslahti, 1989). However, there are also *in vitro* data showing tenascin is a poor substratum for crest attachment (see discussion).

The immunohistochemical studies of early avian embryos have neglected or even dismissed type I collagen as a possible substratum for neural crest emigration and migration. Duband and Thiery (1987) localized type I collagen to crest pathways in 2-3-day-old avian embryos, but concluded that fibronectin was the important attachment molecule, because studies *in vitro* suggested that attachment of crest to collagen is via fibronectin (Greenberg *et al.*, 1981). However, these and related (see Thiery *et al.*, 1985) studies used cells deposited on top of 2D collagenous substrata. Within 3D collagen gels, neural crest cells migrate on collagen without fibronectin (Tucker and Erickson, 1984; Bilozur and Hay, 1988). The survival of Mov13 mutant mice to 13-15 days of gestation (Jaenisch *et al.*, 1983) seems to suggest that type I collagen is not necessary for early events such as crest migration. This mutant has a retroviral insert in the collagen α1(I) gene that is said to inactivate the gene. However, the α1(I) gene is expressed in odontoblasts and osteoblasts of Mov13 mice (Schwarz *et al.*, 1990). Therefore, it cannot be concluded that this collagen gene is turned off in all tissues of the Mov13 mouse embryo.

In this study, we examine for the first time the distribution of type I collagen in the trunk of the 2-day-old chick embryo (Hamburger and Hamilton stage 12). At this 16 somite stage, crest and sclerotome are forming in the midtrunk and rostral trunk, but not in the caudal trunk (last 6 somites). We modify a whole mount, light microscopic technique (Dent and Klymkowsky, 1987) to show that the gradient of type I collagen expression correlates more specifically with the presence of migrating crest than does the distribution of either tenascin or laminin.

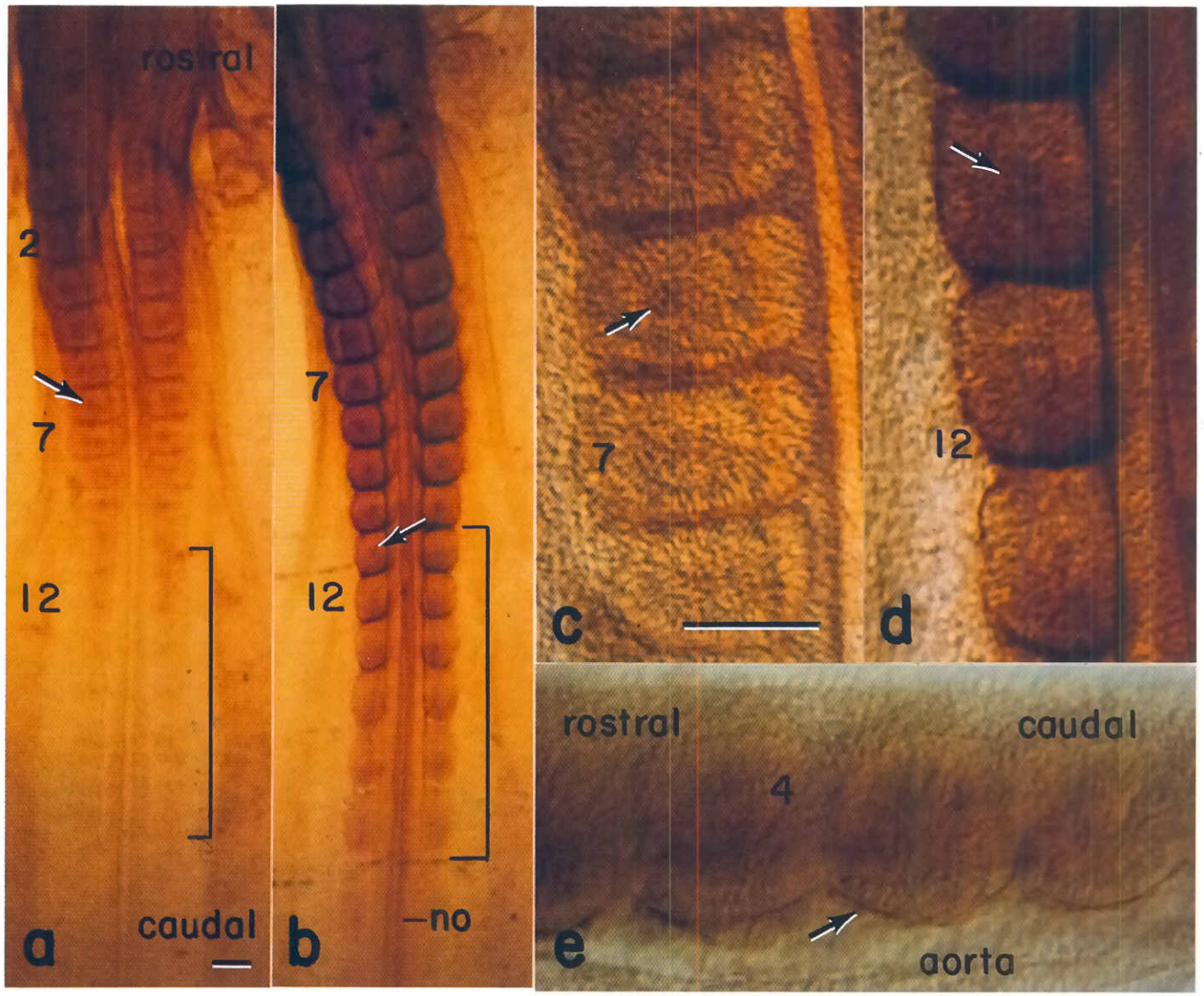


Fig. 2. PAP immunostaining of type I collagen (a,c,e) and tenascin (b,d) in whole mounts of stage 12 embryos. *Peroxidase staining for type I collagen is weak or absent in the caudal 6 somites (bracket, a). Staining appears in the center of each somite (arrow, a,c), associated with the central cells (lower right, Fig. 1). Tenascin is clearly present on the caudal 6 somites (bracket, b), around and in the center (arrow, b,d) of each. The notochord is also stained (no, b). A lateral view of a whole mount of a stage 10 embryo (e) shows that type I collagen first accumulates on the ventral surfaces of the somite (arrow) in association with the dorsal aorta. c-d are Nomarski photographs. Bars 100 nm.*

Type I collagen, tenascin, and laminin have not previously been localized at the ultrastructural level in young embryos, although they are known, respectively, to distribute in older tissues in striated fibrils (Hendrix *et al.*, 1982), between striated fibrils (Lightner *et al.*, 1989), and/or in basement membranes (Courtoy *et al.*, 1983). We show that tenascin occurs in the earliest basement membranes, but disappears rostrally from basement membranes in 2-day-old embryos. Tenascin also occurs in 10 nm wide fibrils and in interstitial bodies. The distribution of tenascin in so many submicroscopic structures was not suspected from studies of its more limited ultrastructural distribution in older tissues.

Results

Whole mount immunohistochemistry

The distributions of type I collagen, laminin, and tenascin were investigated in permeabilized whole mounts of 16 somite embryos using the PAP technique. Since the rostral part of the embryo develops earlier than the caudal part, a rostral to caudal gradient of development is visible in the whole mounts and in sections taken at different levels along the length of the embryo (Figs. 1, 2). Examination of serial sections of paraffin embedded embryos reveals that neural crest cells are migrating from neural tube (and

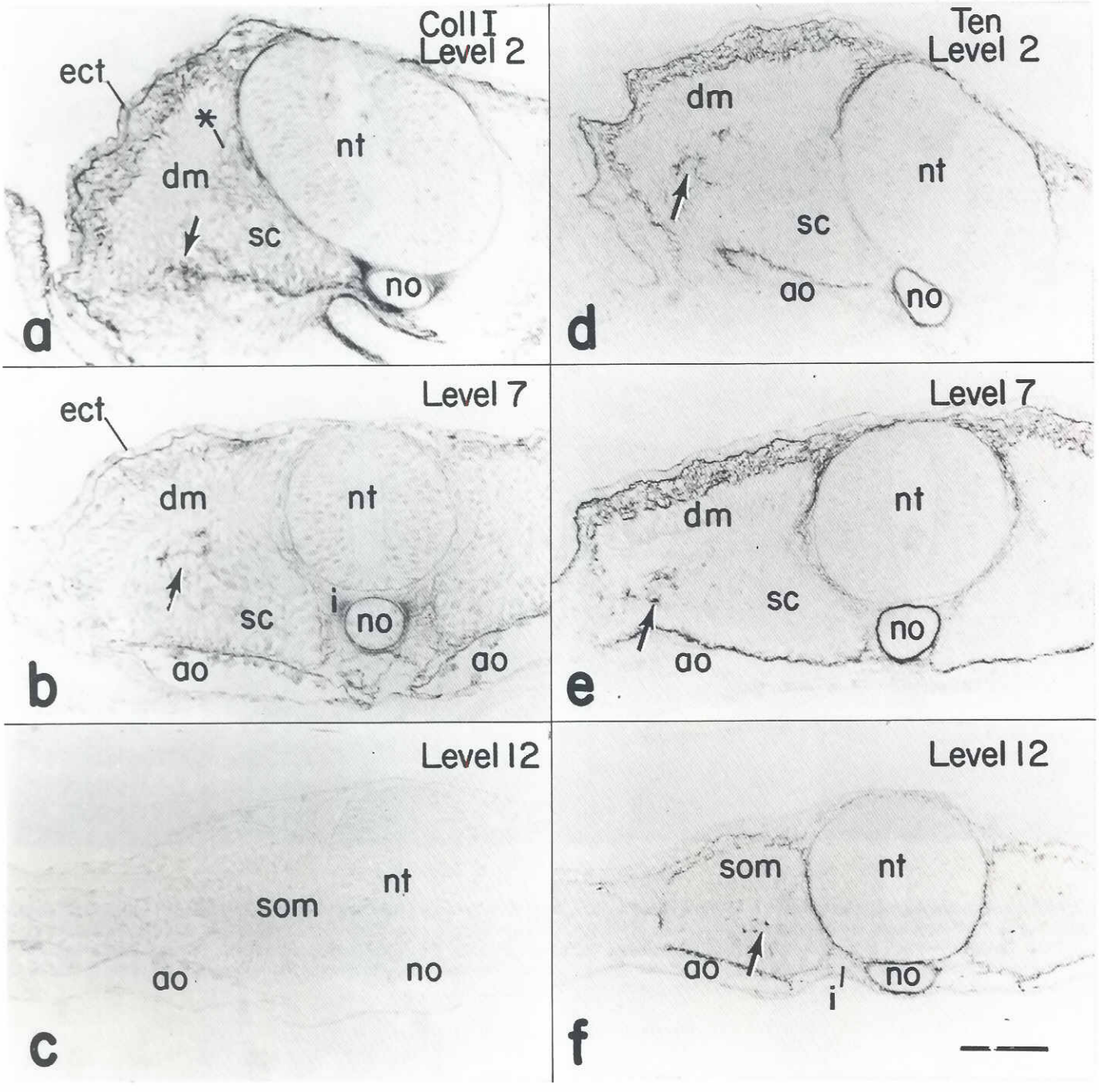
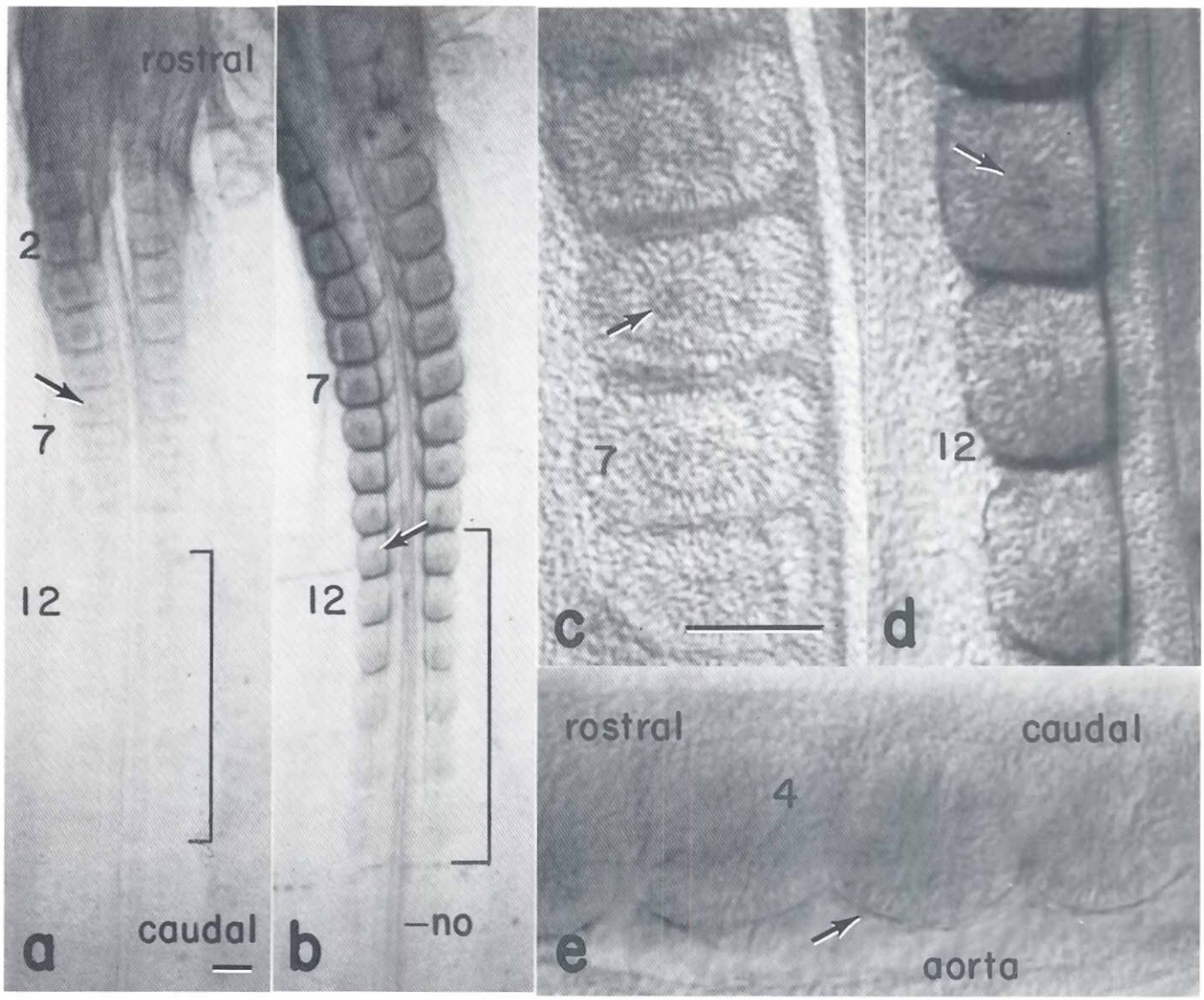


Fig. 3. Transverse sections of whole mounts through somite 2 (a,d), somite 7 (b,e) and somite 12 (c,f) of stage 12 embryos, viewed with the light microscope after PAP immunolocalization. (a) *Type I collagen staining is extensive in the rostral portion of the embryo. It is present under the ectoderm (ect), over the dorsal surface of the dermamyotome (dm), around the notochord (no), and in an area (arrow) under the sclerotome (sc) believed to represent developing vasculature. Collagen staining is also detected on the dorsolateral surface of the neural tube (nt) and extends into the sclerotome (asterisk).* **(b)** *In the midtrunk, type I collagen is detected above the dermomyotome, on the central cells of the somite (arrow), along the aorta (ao), and in the notochord sheath and adjacent interstitium (i).* **(c)** *Type I collagen is absent or reduced in the caudal portion of the embryo where the somites (som) are epithelial and no crest cells have formed.* **(d)** *In the rostral trunk, tenascin is widely distributed. Staining (arrow, d) between the dermomyotome and the sclerotome probably derived from the central cells.* **(e)** *In the midtrunk, the same pattern of staining for tenascin is seen.* **(f)** *Tenascin is present caudally around epithelial somite 12, the ventral neural tube, notochord, and aorta. The central cells in the somite (arrow) are stained. Bar 100 nm.*



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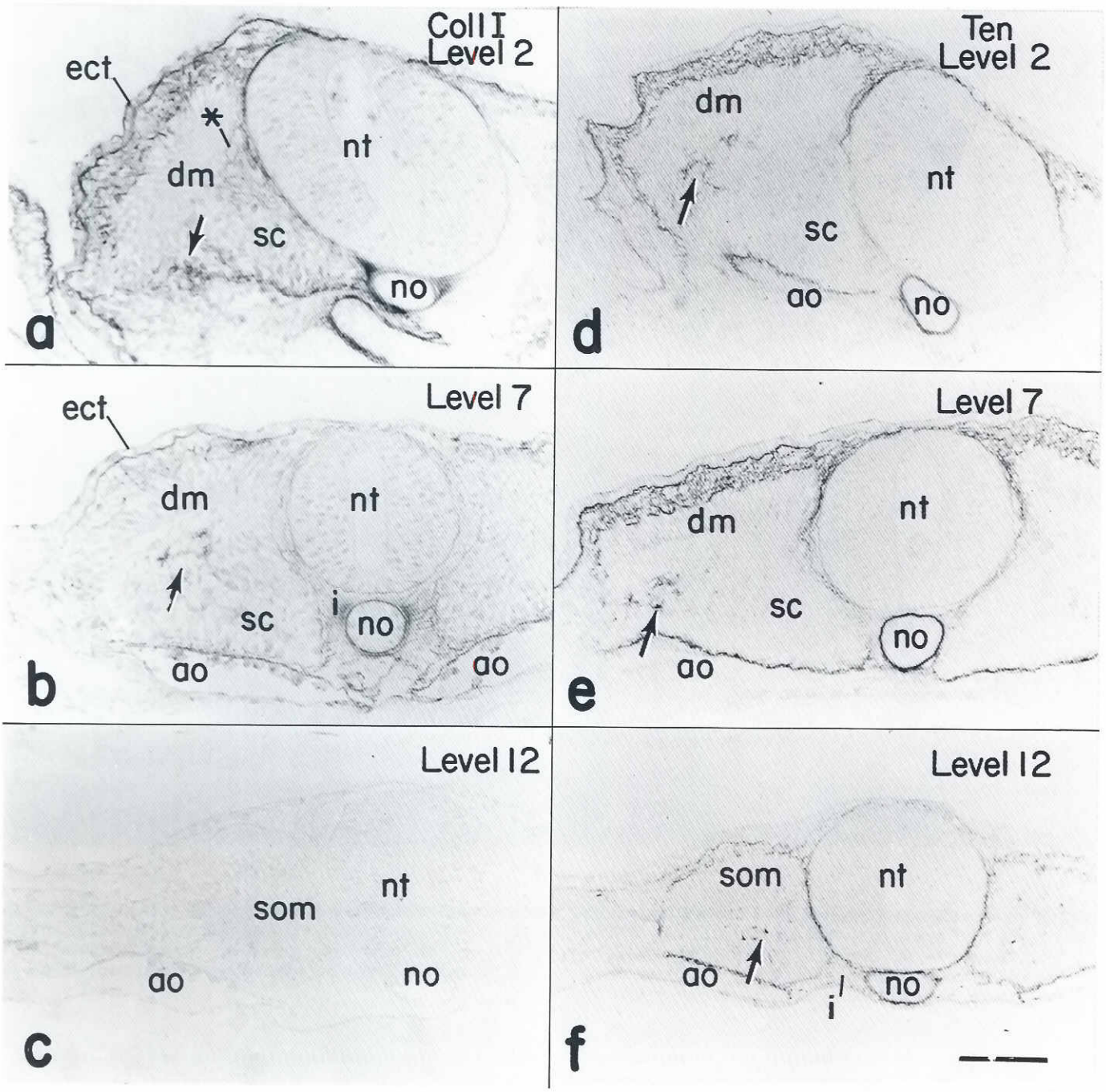

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sclerotome has dispersed) at the level of somites 2 and 7, but not at somite level 12 (Fig. 1).

Fig. 2a shows the distribution of type I collagen, as revealed by the PAP method, in a whole mount of an embryo at the stage shown in Fig. 1. Little or no staining for type I collagen is seen in the mesenchyme of the primitive streak or segmental plate (not shown) or over the 6 caudal epithelial somites (bracket, Fig. 2a). Type I collagen is first clearly detected at the level of somite 7, where crest is forming (Fig. 1), as an accumulation of peroxidase product between and in the center of the somites (Fig. 2a,c).

The accumulation of the type I collagen defines both a rostral to caudal and a ventral to dorsal gradient on somite surfaces in the developing embryo. Fig. 2e demonstrates the distribution of type I collagen in the midtrunk region of a 10 somite embryo viewed from the side. Type I collagen is detected initially on the ventral surface of the somites in association with the developing aortae (arrow, Fig. 2e) and later accumulates on the dorsal surface.

Tenascin is expressed more extensively than type I collagen and is detected along the length of the embryo strongly through somite 13 and weakly through somite 16 (Fig. 2b). As soon as somites form from the segmental plate, tenascin begins to accumulate at their peripheries. Staining is stronger over the caudal than over the rostral halves of the epithelial somites (somite 12, Fig. 2b,d). Staining for tenascin begins to increase in the center of the somites at level 11-12 (arrow, Fig. 2b,d). More rostrally, tenascin accumulations are extensive. The notochord (no, Fig. 2b) is the first structure to stain for tenascin. The primitive streak does not contain detectable tenascin (not shown).

Laminin does not exhibit a gradient of expression as observed in the whole mounts (not shown). It is distributed uniformly down the length of the embryo, including the segmental plate and the primitive streak levels. Label is primarily associated with the neural tube, somite surfaces and overlying ectoderm.

Light microscopic sections

Transverse sections of the embryos (Figs. 1, 3) allow the distribution of the PAP label to be correlated with the morphology of the developing tissues. Plastic sections of blocks stained for ECM are illustrated in Fig. 3 and paraffin sections in Fig. 1. In order that the orange DAB product show in these black and white photographs (Fig. 3), the blue counterstain over the cells has been filtered out during photography, but in the original sections, it is clear that migrating crest cells are present at levels 2 and 7, but not at level 12, as shown in Fig. 1. Since it shows cellular detail at the levels (2,7,12) illustrated in Figs. 3-11, Fig. 1 should be frequently consulted for orientation.

There are numerous crest cells in the dorsal pathway at somite level 2 (Fig. 1) and this pathway is rich in collagen and tenascin (Fig. 3a,d). At level 7, crest cells are present in the dorsal pathway and a few can be ventrally near the myotome (Fig. 1). Collagen I is just making its appearance (Fig. 3b).

Between level 7 and 10, crest cells are restricted to the ventral pathway. Caudal to somite 10, we did not detect migratory crest cells in serial sections (lower right, Fig. 1). Sections taken through the epithelial somite 12 (Fig. 3c) confirm Fig. 2a, showing that little or no type I collagen is present in the caudal portion of the embryo, where somites are epithelial and crest cells have not yet emigrated. In contrast to type I collagen at this level, tenascin does occur on the surface and in the center of the epithelial somite, the adjacent neural tube, within the notochordal sheath, over the aortae, and between the epidermis and the somite (Fig. 3f).

Type I collagen surrounds the notochord at midtrunk and rostral levels of the embryo (no, Fig. 3a,b) and occurs over the surface of the dorsal aorta (ao, Fig. 3b). Tenascin is present in these areas (Fig. 3d,e), as well as caudally (Fig. 3f). Collagen (i, Fig. 3b) but no tenascin is present in the notochord interstitium, through which sclerotome will later migrate. Tenascin staining is present in the notochordal sheath (no, Fig. 3e). Both collagen and tenascin are abundant over the dorsal wall of the somite and the neural tube at level 7, as well as over the central somite cells (arrows, Fig. 3b,e). Rostrally, tenascin is disappearing from the ventral neural tube surface (Fig. 3d).

Residues of labeled matrix (arrow, Fig. 3b,e,d) next to the dermomyotome probably derived from somite central cells and likely will become part of the ventral crest pathway (Rickmann *et al.*, 1985) between the rostral sclerotome and dermomyotome. The origin of this pathway and the relative roles of tenascin (Tan *et al.*, 1987) and collagen in its formation will be the subject of further study using older embryos.

Little or no staining for either type I collagen or tenascin is seen in the sclerotome *per se* (sc, Fig. 3). The ventromedial wall of the somite appears to be attached to the neural tube by ECM and its initial expansion may be due in part to the growth of the embryo rather than to cell migration into ECM (see discussion).

Electron microscopic observations

Whole mount immunolabeling demonstrates that both collagen and tenascin appear along the pathways of neural crest cell migration. The expression of type I collagen is, however, more directly correlated temporally and spatially with the migration of the crest cells. We investigated the ultrastructural organization of collagen, tenascin, and laminin along these pathways, using TEM immunolocalization en bloc by the PAP procedure (Mayer *et al.*, 1981).

Type I collagen is present in the midtrunk region of the stage 12 embryo in the areas where neural crest cells are starting to migrate. PAP staining reveals small collagen fibrils 10 nm in diameter in the neural crest pathway between the sclerotome and the neural tube at level 7 (fibrils, Fig. 4a). They also occupy the intercellular space between the neural tube and ectoderm and the somite and ectoderm. In some areas, the fibrils lie in close association with the basement membrane of the neural tube. We did not detect in sections, the oriented fibrils on the somite face described by Newgreen (1989). The band of collagen-rich 10 nm fibrils surrounding the notochord forms a dense sheath connected to more diffusely distributed 10 nm fibrils in the surrounding area. That this more diffuse area (Fig. 4b) is not tightly associated with neural tube is suggested by the occasional appearance of a space (presumably artefactual) between it and the neural tube after fixation.

Many of the type I containing fibrils are oriented with their long axis parallel to the surface of the notochord and neural tube as reported by Tosney (1978) and Ebendal (1977). The DAB product is detected in a 60 nm periodic pattern (Fig. 4c), although the 10 nm fibrils do not show striations. Where the fibrils are closely apposed to the plasma membrane, PAP staining can be seen associated with the cell surface (large arrows, Figs. 4c, 5a) as electron dense patches in the plane of the plasma membrane. This staining is an artifact due to the diffusion of the DAB product into the poorly fixed membrane (Courtoy *et al.*, 1983). The aldehyde-fixed blocks of tissue were taken through antibodies, PAP, and DAB before osmium fixation and therefore lipid that OsO_4 would preserve is lost from

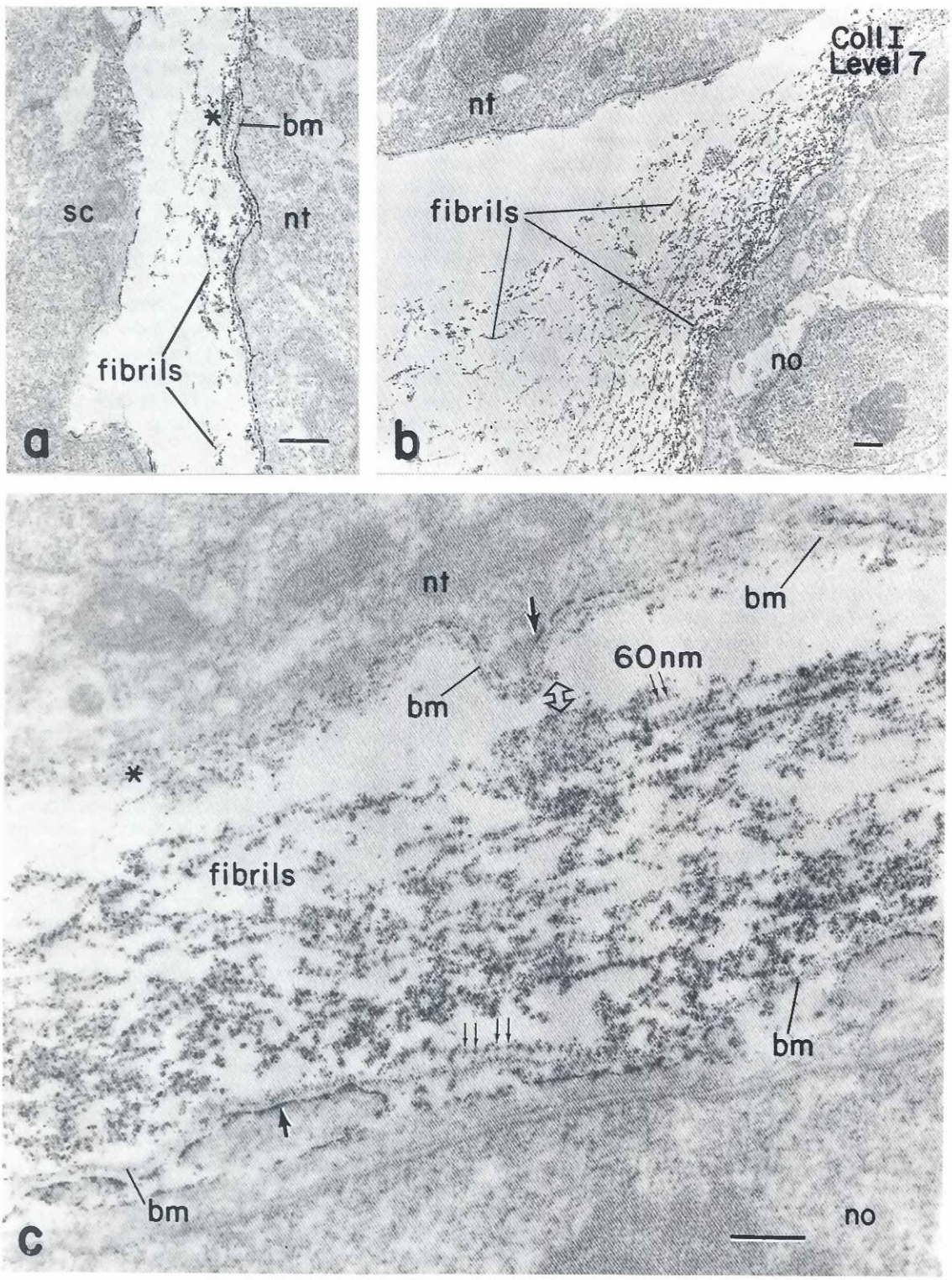

Fig. 4. PAP ultrastructural immunolocalization of type I collagen along the neural tube (nt) and notochord (no) at the level of somite 7 of a 16 somite chick embryo. *PAP labeling can be detected along 10 nm wide fibrils which lie between the sclerotome (sc, a) and neural tube, and the neural tube and notochord (b,c). The fibrils may form small aggregates (*, a). The basement membrane (bm) of the neural tube is not stained (c), but may have associated with it an amorphous material (double-headed arrow, c), which gives the impression in tangential section that the bm is stained (*, c). The 10 nm wide fibrils show a 60 nm periodicity (small arrows,c) in their PAP staining pattern. Cell surface staining (solid arrows, c) is an artifact of the PAP procedure. Bars 1 nm (a,b) and 0.5 nm (c).*

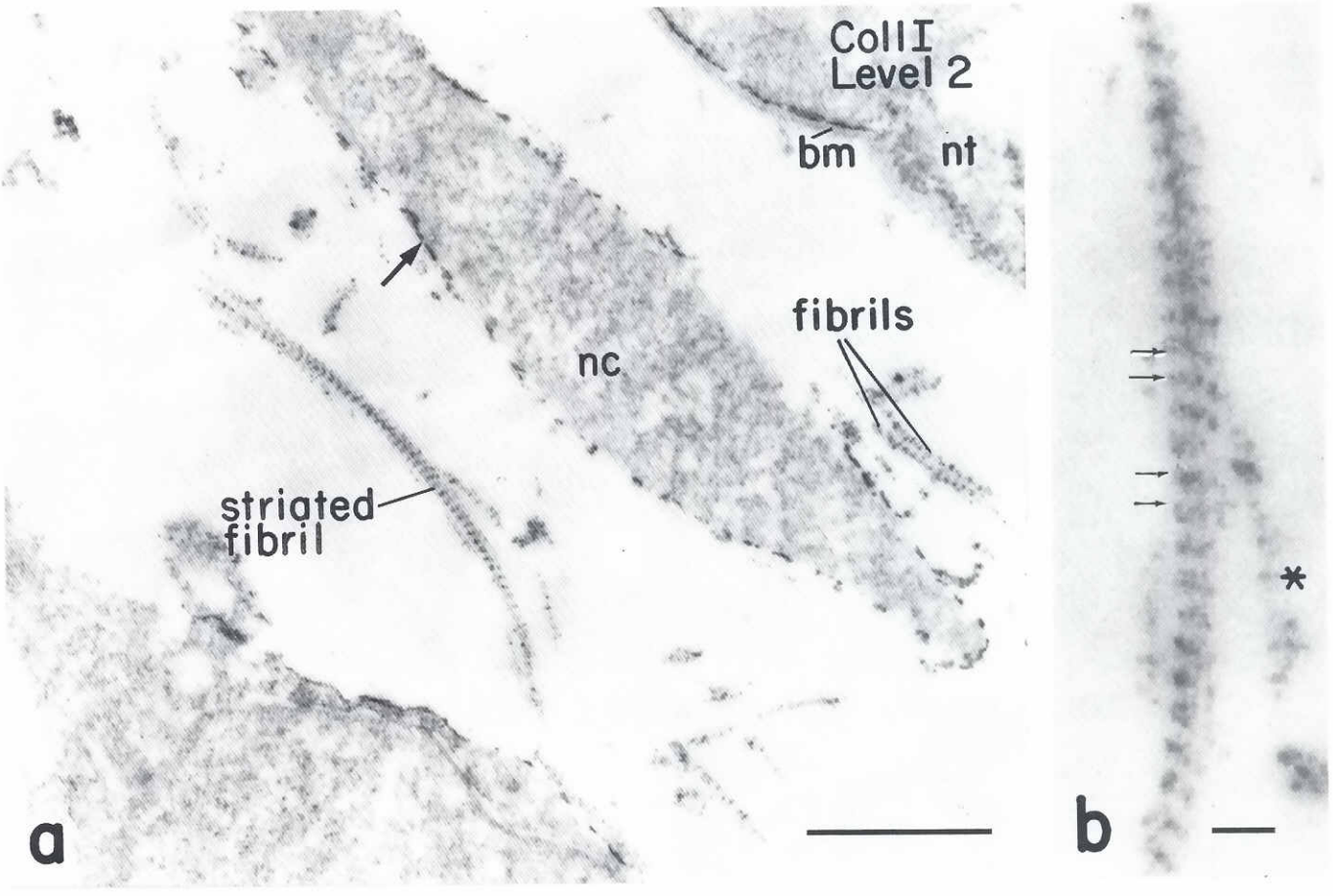

Fig. 5. PAP ultrastructural immunolocalization of striated type I collagen fibrils at a rostral level near the neural tube (nt) and neural crest (nc). *Fibrils of 10 nm in diameter can still be seen, but also thicker fibrils 20-50 nm in diameter are observed with visible striations. These fibrils show a 60 nm periodicity of PAP staining (small arrows, b) and are associated with 10 nm wide fibrils (*, b). The staining on the surface (arrow, a) of the neural crest cell is a PAP artifact. The basement membrane (bm) of the neural tube is unlabeled (a). Bars 1 nm (a) and 120 nm (b).*

membranes permeabilizing them (Mayer *et al.*, 1981).

The basement membrane (bm, Fig. 4c) or so-called basal lamina (Hay, 1991) is not positive for type I collagen. The nearby fibrils are labeled by DAB-PAP-anticollagen complexes and these precipitates do not diffuse from one layer of ECM to another as suggested by Lightner *et al.* (1989).

The relationship of neural crest to type I collagen is best appreciated in rostral areas where crest cells are numerous. The migrating crest cell (nc, Fig. 5a) is associated with 10 nm wide collagen fibrils. Larger fibrils with a definite striated pattern are also present (Fig. 5a,b). Smaller fibrils lie adjacent to striated fibrils, as if they were being incorporated into the larger fibrils (asterisk, Fig. 5b). Thus, the collagenous matrix encountered by the neural crest is primarily fibrillar.

Type I collagen cannot be detected in the caudal portion of the embryo (level 12, Fig. 6a). The negative reaction for type I collagen at these levels, as judged here by immunohistochemistry on sections, as well as whole mounts, serves as an excellent control for our immunological approach. Duband and Thiery (1987) used immunofluorescence to conclude that type I collagen is present in "basement membranes" as viewed by light microscopy. The authors did not illustrate a negative control, nor did they investigate the localization of type I collagen by electron microscopy. We also illustrate unstained basement membranes by electron microscopy in sections that contain labeled type I collagen fibrils (Figs. 4c, 5a). Other controls that attest to the specificity of the ECM staining include a lack of staining when the ECM antibody is omitted or absorbed against its specific antigen.

No neural crest has emigrated at level 12, but tenascin is expressed abundantly (Fig. 6b). PAP labeling demonstrates that tenascin is associated with the basement membranes and 10 nm wide fibrils within the extracellular spaces in this relatively young region of the embryo (Fig. 6b,c). Heavy labeling is detected along the ventral surface of the neural tube and notochord surface (Fig. 6b,c). Many of these unstriated fibrils show a 60 nm periodicity of deposition of DAB product (Fig. 6c).

In the midtrunk region, tenascin (Figs. 7, 8) is distributed with type I collagen throughout the extracellular embryonic spaces. Around the dorsal neural tube at level 7, tenascin-rich fibrils are associated with neural crest cells (nc, Fig. 7). These 10 nm wide fibrils may form small aggregates (inset, Fig. 7). Since all the 10 nm fibrils at level 7 are labeled by anticollagen (Fig. 4), it is likely that

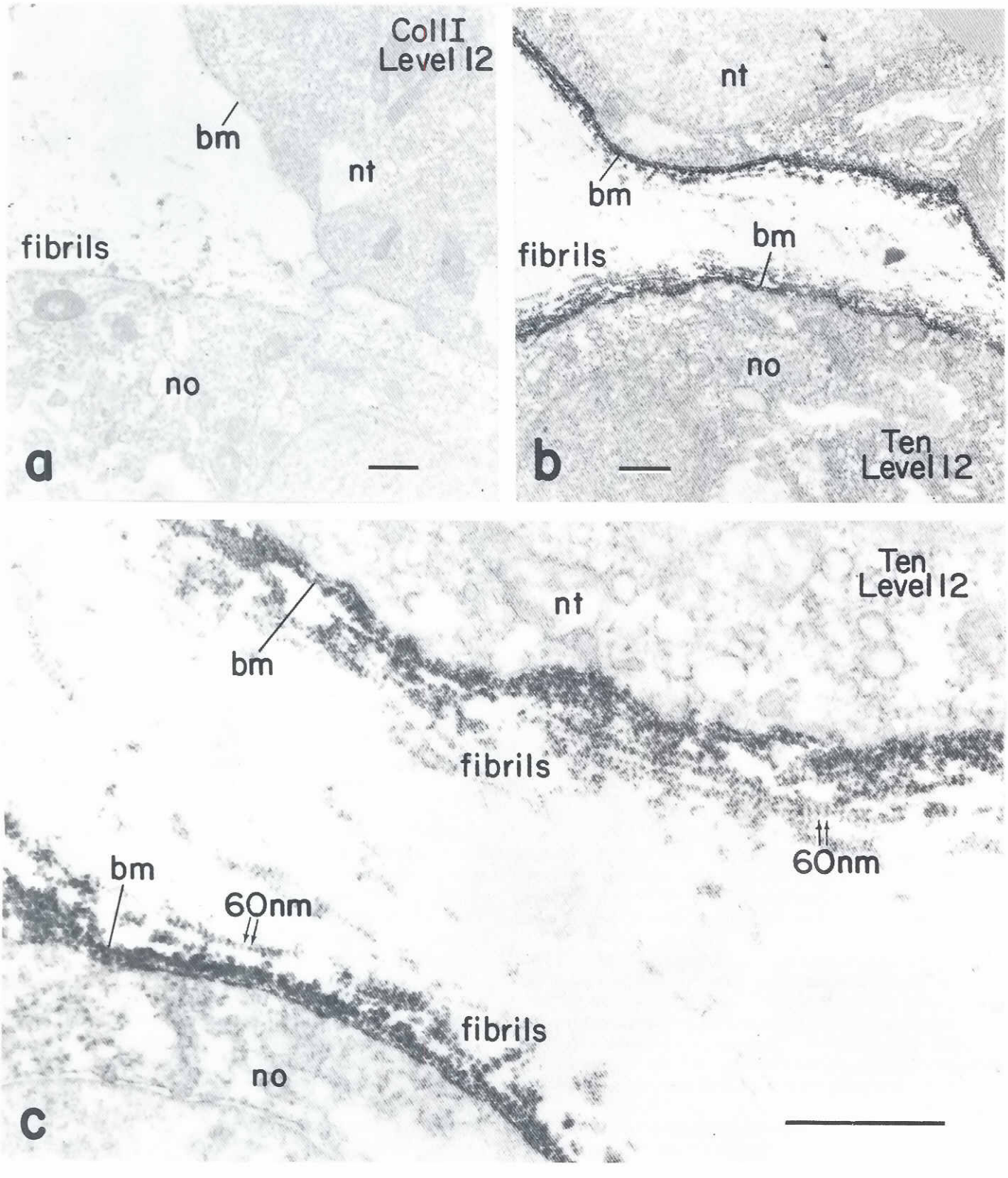

Fig. 6. PAP ultrastructural immunolocalization of type I collagen (a) and tenascin (b, c) between the notochord (no) and neural tube (nt) in the caudal part of the embryo. *No type I collagen appears to be associated with fibrils or basement membranes (bm) in this area (a). Heavy tenascin staining can be seen on the basement membrane (bm) of the neural tube and notochord and the adjacent 10 nm wide fibrils (b,c). Only the fibrils in the notochordal sheath stain; the label does not extend very far into the adjacent matrix. Bars 1 nm.*

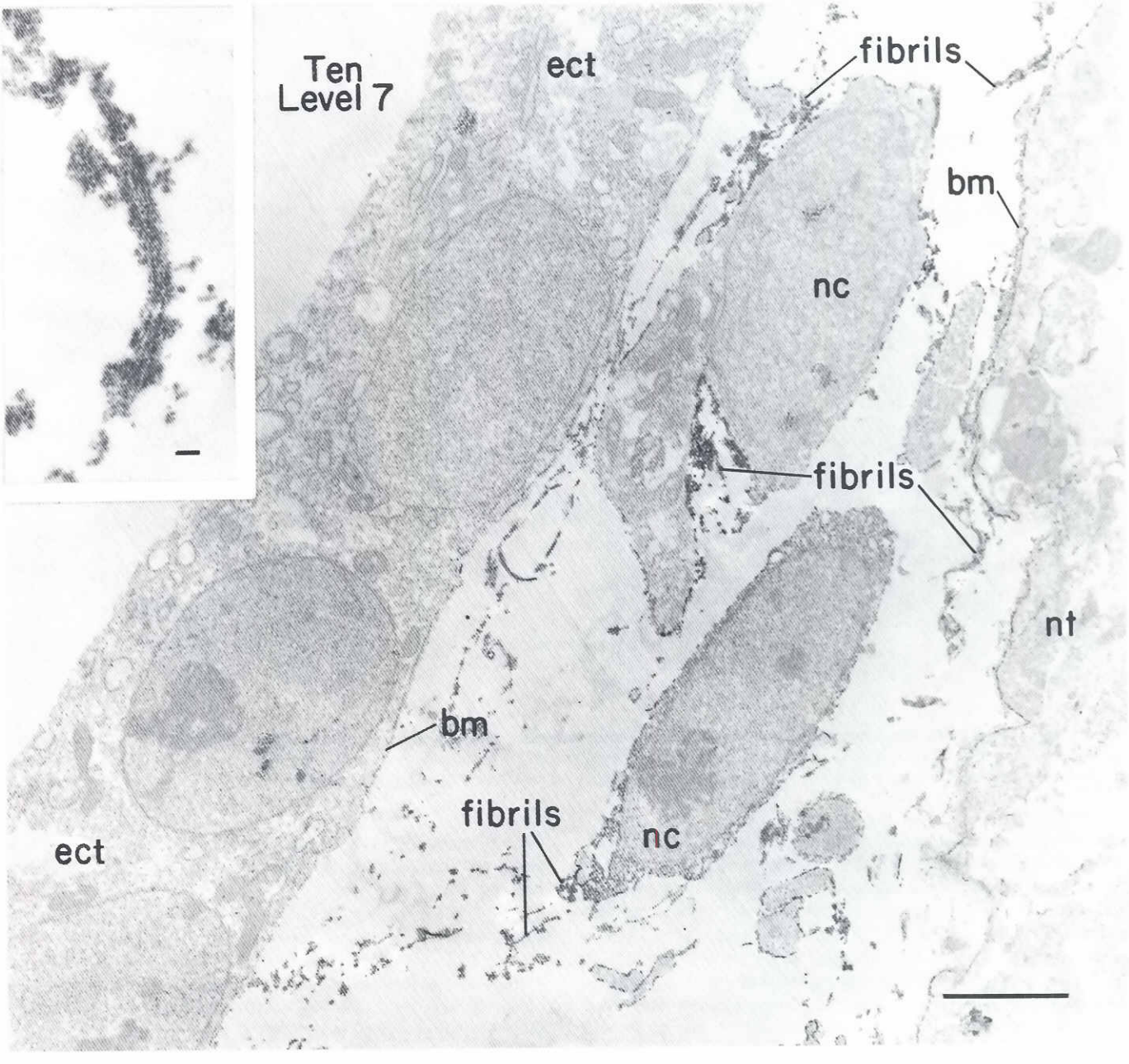

Fig. 7. PAP ultrastructural immunolocalization of tenascin dorsolateral to the neural tube at level 7. *Neural crest cells (nc) migrating between the dorsal neural tube (nt) and the ectoderm (ect) contact many 10 nm wide fibrils. At higher magnification, tenascin labeling is seen as DAB aggregates associated with fibrillar material (inset). There is little or no tenascin labeling of the basement membrane (bm) of the neural tube or ectoderm in the midtrunk. Bars 1 nm (a) and 120 nm (b).*

they contain type I collagen as well as tenascin. Tenascin is reduced in amount around the ventral neural tube in the midtrunk region (Figs. 3e, 8a), but is still abundant in the notochordal sheath as 10 nm fibrils (Fig. 8b). The numerous collagen fibrils between notochordal sheath and neural tube (fibrils, Fig. 8a) are not labeled for tenascin. The basement membrane of the notochord, which was labeled caudally, shows little or no label in the midtrunk (bm, Fig. 8a,b), nor does that of the epidermis or neural tube (bm, Fig. 7).

The ultrastructure of tenascin-containing structures in the rostral somites (level 2, Fig. 9) is similar to that of the midtrunk level. The labeled 10 nm wide fibrils may be aggregated into larger structures (fibril, Fig. 9). The basement membrane (bm) of the dermamyotome (myo, Fig. 9), is labeled for tenascin. The label in this region also appears to be associated with the interstitial bodies (isb, Fig. 9) of Low (1970). We will return to this point (Fig. 11).

Laminin is a major component of all basement membranes, or

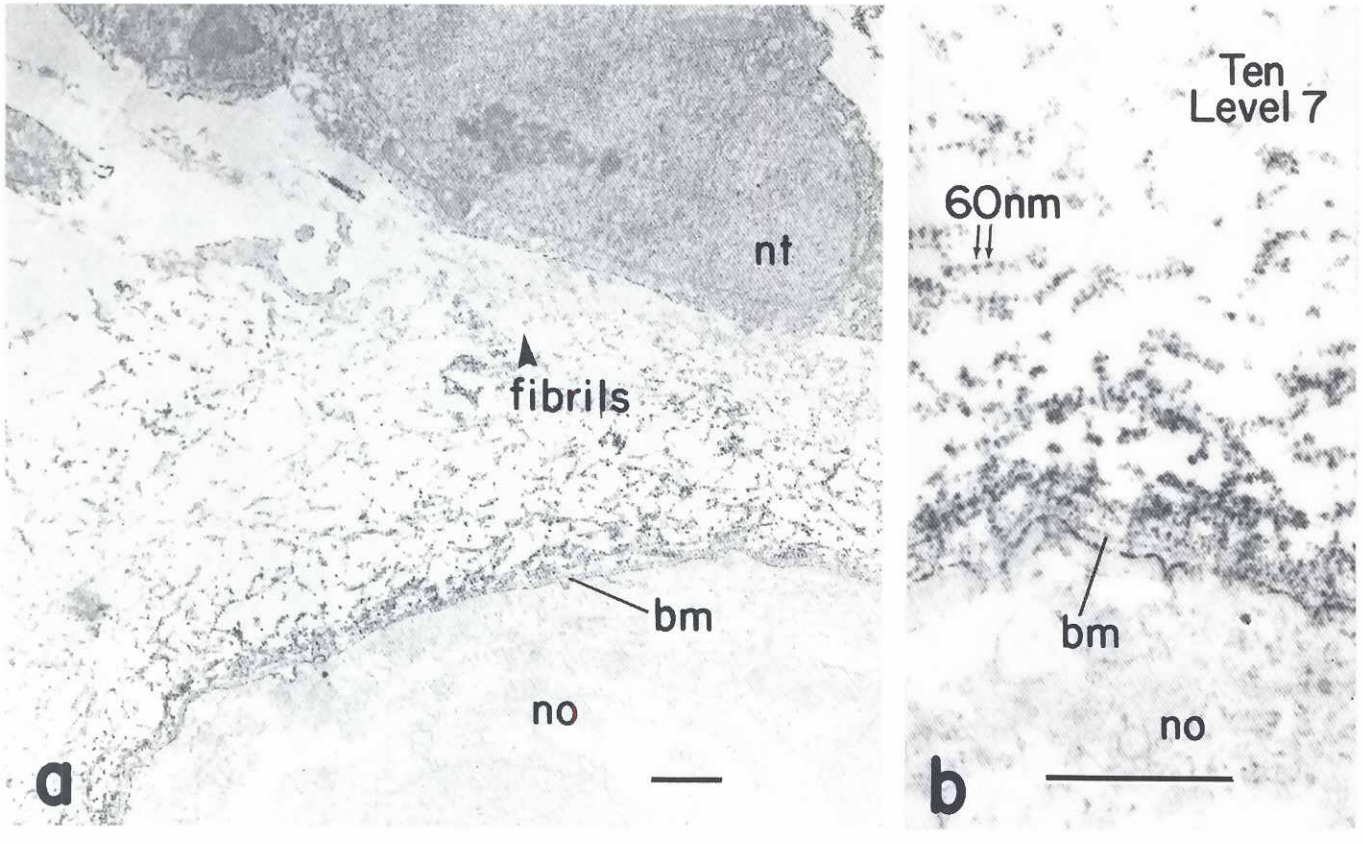

Fig. 8. PAP ultrastructural immunolocalization of tenascin in the midtrunk notochordal sheath. *Tenascin is distributed primarily in 10 nm wide fibrils around the notochord (no), where they run parallel to the notochordal basement membrane (bm), which is not stained at this level. The basement membrane and extracellular matrix next to the ventral neural tube (nt) do not react for tenascin in the midtrunk. Bars 1 nm.*

so-called basal laminae, studied to date by electron microscopy. We stained laminin by the PAP immunolocalization method and found DAB product in basement membranes of all the embryonic tissues of the early embryo (bm, Fig. 10). Laminin is seen in the basement membranes of the somite (Fig 10b), neural tube (Fig. 10a), the notochord (Fig. 10c) and the ectoderm and dermamyotome (Fig. 10d). Little or no label is found among cell processes adjacent to the neural tube (cp, Fig. 10a). Antilaminin labels 10 nm wide fibrils with 60 nm periodicity immediately adjacent to the basement membrane of the neural tube (Fig. 10a, inset) and notochord (Fig. 10c). The label appears to be specific as it was not present on fibrils at a distance from the basement membrane (*, Fig. 10c).

To be certain that interstitial bodies do not contain significant amounts of laminin, as suggested by Martins-Green and Erickson (1987), we examined the area vasculosa (Fig. 11). Here, the basement membrane is hypertrophied into large folds and essentially nothing but interstitial bodies are present in adjacent matrix. Immunolabeling of the area vasculosa with anticollagen/PAP reveals that type I collagen is absent from the interstitial bodies and the convoluted basement membrane (Fig. 11a). Some type I collagen staining was detected as fibrillar material around migrating mesenchymal cells in the area (not shown). Antibodies to tenascin label the interstitial bodies and basement membrane (Fig. 11b,d). Thus, the distribution of tenascin in this area is similar to that of fibronectin (Mayer *et al.*, 1981). Additionally, we found that laminin staining is confined to the highly convoluted basement membrane (bm, Fig. 11c) and is absent from the underlying interstitial bodies (isb, Fig. 11c,e).

Discussion

Recently, several investigators assigned to tenascin, a primary role in initiating and directing neural crest migration, because its distribution in the early avian embryo is more "restricted" to crest pathways than that of fibronectin or laminin (Crossin *et al.*, 1986; Tan *et al.*, 1987; Mackie *et al.*, 1988b). We show in this paper that the distribution of type I collagen is even better correlated than that of tenascin with crest migration. By staining whole mounts of permeabilized two-day-old embryos with antibodies to ECM molecules, we were able to demonstrate for the first time, a rostral-caudal gradient of type I collagen deposition along the trunk. No neural crest cells are present in caudal areas (level of somites 11-16) lacking type I collagen. Tenascin labeling, however, is present strongly through somite 13 and is detectable at the level of somite 16. Laminin and fibronectin (Mayer *et al.*, 1981) are even more widely distributed. Thus, type I collagen is the matrix molecule most specifically associated with the beginning of neural crest migration in the embryo.

It is tempting to think that contact with collagen type I fibrils is necessary for the extension of filopodia and pseudopodia into

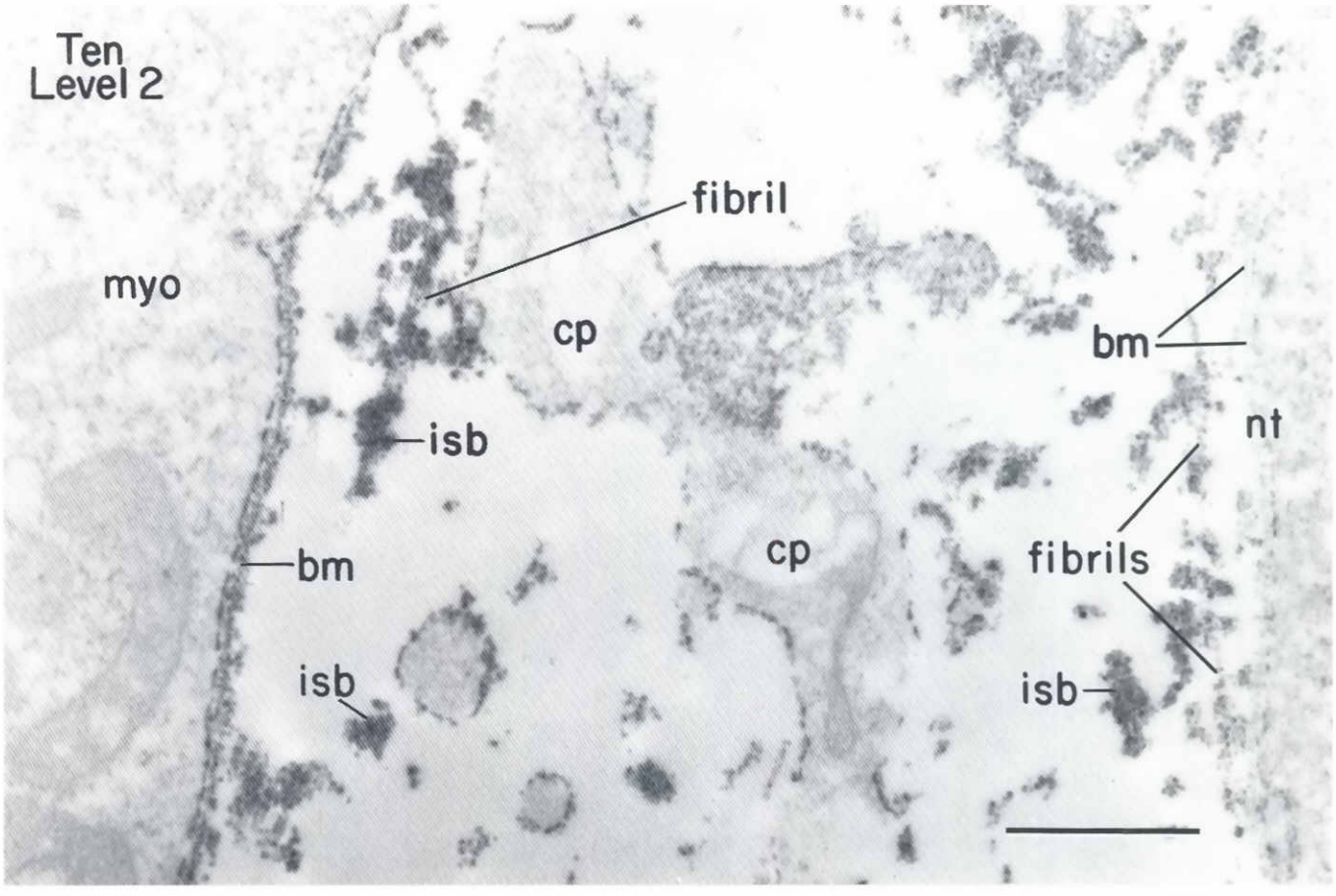

Fig. 9. PAP ultrastructural localization of tenascin between a rostral myotome (myo) and neural tube (nt) shows dense aggregates of PAP product that probably correspond to interstitial bodies (isb). *The 10 nm wide tenascin-positive fibrils occur individually or in aggregates. The basement membrane (bm) of the newly formed myotome is labeled at this rostral level, but that of the neural tube is not. The cell processes (cp) probably belong to neural crest cells. Bar 1 nm.*

adjacent ECM by the crest cells. The ability of neural crest to move out of the 2-day-old neural tube into a type I collagen gel containing no fibronectin has been demonstrated *in vitro* by Tucker and Erickson (1984) and Bilozur and Hay (1988). Contact with collagen fibrils in 3D array stimulates a variety of epithelia to transform into mesenchymal cells (Greenburg and Hay, 1986, 1988). However, neural crest can migrate from neural tube *in vitro* into 3D basement membrane gel (Bilozur and Hay, 1988) and onto fibronectin- or laminin-coated planar substrata (Thiery *et al.*, 1985). In judging the roles of ECM molecules in initiating and supporting neural crest migration in the embryo, therefore, it becomes important to assess their actual distribution *in vivo* at the time of crest formation.

Crossin *et al.* (1986) described, by immunofluorescence of sections, a gradient of "cytotactin" in the embryonic avian trunk in the sense that the last 2-3 somites were negative or weakly stained. Mackie *et al.* (1988b) reported that the most caudal somites are negative for tenascin. We show a tenascin gradient that extends to the most caudal somite. Thus, the tenascin pattern does not correlate as exactly as that of collagen I with initial crest migration. Tenascin has been identified in the rostral half of the sclerotome, a very specific ventral crest pathway, but descriptions correlating the relation to crest migration are not in agreement (Tan *et al.*, 1987; Bronner-Fraser, 1988; Mackie *et al.*, 1988b; Stern *et al.*, 1989; Newgreen *et al.*, 1990) and the role of collagen and fibronectin are unknown.

Experimental approaches to the role of tenascin in crest formation and migration are also not in agreement. Bronner-Fraser (1988) injected anti-tenascin into embryos in the cranial region and observed abnormal crest, but the antibodies had no effect in the trunk. When the crest cells are grown on tenascin, they become round in shape as if weakly adherent (Mackie *et al.*, 1988b; Halfter *et al.*, 1989). Tan *et al.* (1987) reported that crest prefers attachment to fibronectin over tenascin. Others have reported that neither cells nor fibronectin attach well to tenascin (Erickson and Bourdon, 1989; Lightner *et al.*, 1989; Lightner and Erickson, 1990) and that tenascin inhibits spreading of amphibian dorsal lip cells on fibronectin (Riou *et al.*, 1990). However, Halfter *et al.* (1989) observed the rate of quail crest migration to be greater on tenascin than on fibronectin or basement membrane.

Laminin is widely distributed in the 2-day-old avian embryo and there is no rostral-caudal gradient of staining as viewed in the whole mounts. Duband and Thiery (1987) reported that laminin and type

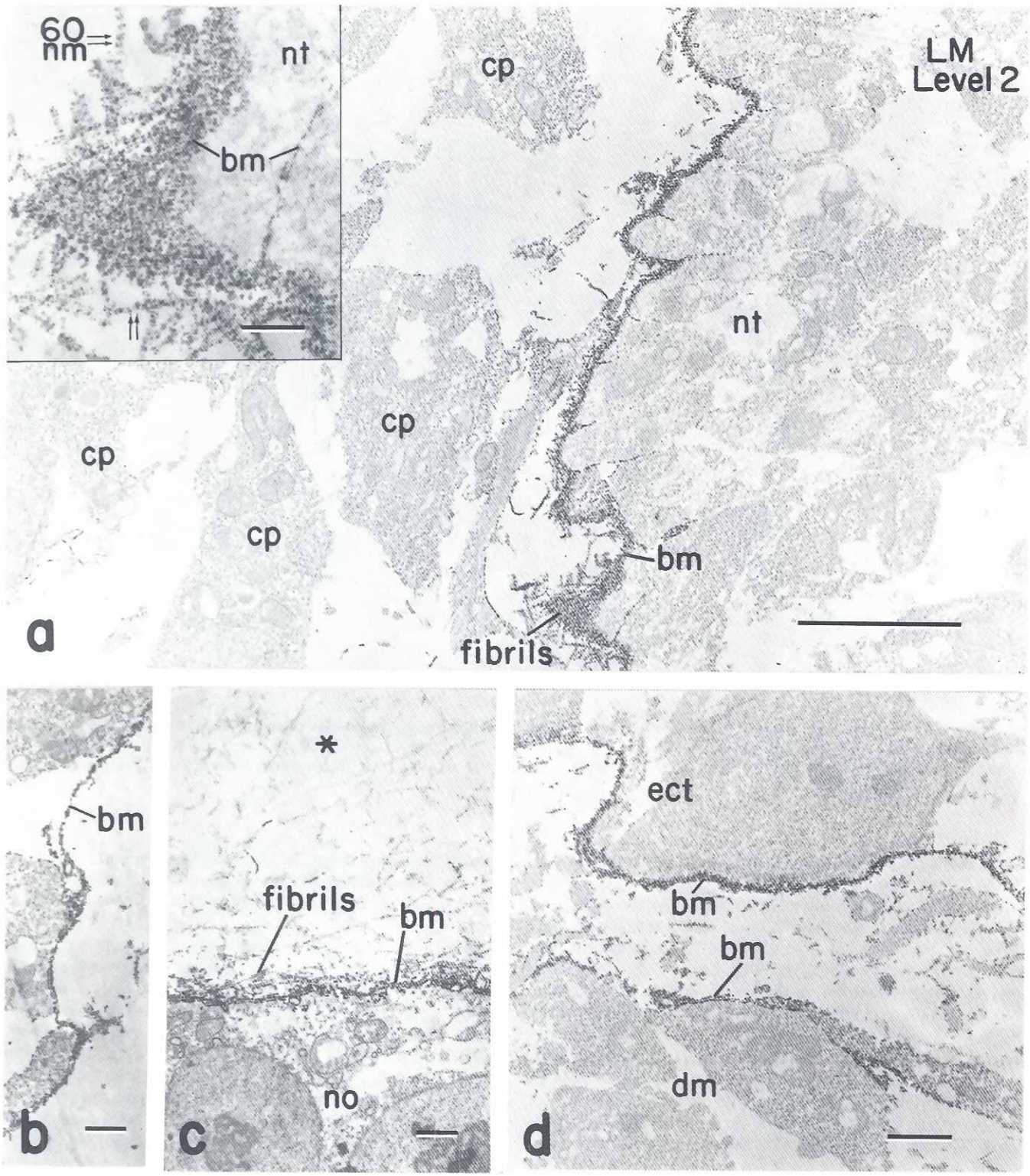

Fig. 10. PAP ultrastructural immunolocalization of laminin in basement membranes (bm) of the stage 12 chick neural tube (nt, a), somite (b), notochord (no, c), ectoderm (ect, d) and dermomyotome (dm, d). *No staining is seen among cell processes (cp) adjacent to the neural tube, but the fibrils in close association with the neural tube basement membrane do stain for laminin with a 60 nm PAP periodicity (inset, a). Fibrils near the notochord (c) are also labeled, and here again the label does not extend very far into the adjacent ECM (*, c). Bars 5 nm (a), 0.5 nm (inset), and 1 nm (b,c,d).*

IV collagen disappear from the dorsal neural tube prior to crest formation, but it seems more likely that a complete basement membrane does not form in this area until after the crest emigrates (Martins-Green and Erickson, 1987; Martins-Green, 1988). It is generally assumed that absence of a dorsal neural tube basement membrane assists the movement of crest into the interstitial ECM. Quail neural crest cells migrate on top of basement membrane *in vitro* without penetrating it (Erickson, 1987) and they can be seen to contact but not to penetrate neural tube and epidermal basement membrane *in vivo* (Hay, 1978). Contacts with laminin in the embryo presumably do add to the overall efficiency of crest migration, as do contacts with other ECM molecules (Rovasio *et al.*, 1983; Bilozur and Hay, 1988).

We were surprised to find that there is little or no type I collagen, tenascin, or laminin in the first-formed sclerotome. However, it has been reported that the ventromedial wall of the somite becomes mesenchymal by increasing intercellular hyaluronate and swelling up in situ (Solursh *et al.*, 1979). Little or no ECM was visualized in the sclerotome, and the large intercellular spaces could be demolished by hyaluronidase (Solursh *et al.*, 1979; Newgreen *et al.*, 1986). The ventromedial epithelial cells transforming to mesenchyme may migrate little if at all at this early stage, when differential growth and folding of the embryo as well as hyaluronate accumulation could serve to displace them (Gasser, 1979; Solursh *et al.*, 1979). Later, they will move around the notochord and neural tube in pathways rich in collagen and other ECM. Further study of sclerotome might allow one to separate acquisition of mesenchymal shape from the process of cell migration itself.

The central cells in the lumen of the epithelial somite stain for tenascin and type I collagen (this report) and for laminin and perhaps fibronectin (Duband *et al.*, 1987). When the sclerotome disperses, we show that this central cell ECM remains attached to the dermatome in the ventral pathway that crest seems to follow when it invades the somite (Loring and Erickson, 1987; Keynes and Stern, 1988; Newgreen *et al.*, 1990). The central cells may be the source of the tenascin staining attributed to the sclerotome (Tan *et al.*, 1987; Mackie *et al.*,1988b). The function of the central somite cells and their ECM has received little attention in the past and is another topic deserving of future study.

At the ultrastructural level, we have localized type I collagen and tenascin in 10 nm wide fibrils, previously reported to contain fibronectin (Mayer *et al.*, 1981), that are loosely scattered throughout the dorsal and ventral neural crest pathways. The fibrils are tightly packed and more numerous immediately next to the notochord where crest cells do not migrate. Lightner *et al.* (1989), using colloidal gold immunohistochemistry, localized tenascin in amorphous patches of material between, but never on, striated collagen fibrils in adult human dermis. The collagen fibrils in the rostral trunk and midtrunk of the early chick embryo that co-stain for tenascin are immature, in the sense that they are small and are not striated. It is possible that larger, more mature collagen fibrils, like many basement membranes, lose their affinity for tenascin in older animals. In support of this idea, tenascin (J1-220) has been shown to be associated with collagen fibrils in denervated muscle but disappears after reinnervation (Sanes *et al.*, 1986). Tenascin has also been reported to be associated with collagen fibrils in developing bone and wounded skin (Mackie *et al.*, 1987, 1988a).

Although the early embryonic 10 nm wide fibrils are not striated as viewed by TEM after routine lead staining (Mayer *et al.*, 1981), the PAP reaction for both collagen and tenascin reveals a periodicity of DAB precipitate at 60 nm intervals along the fibrils. It is likely that the periodicity of the DAB precipitate on the 10 nm fibrils is due to epitopes, recognized by our antibodies, which are repeated at 60 nm intervals. The period may not necessarily be related to collagen as the 10 nm wide tenascin-rich fibrils in the caudal trunk of the embryo do not contain type I collagen, at least not as recognized by our polyclonal antibodies. Is it possible that another type of collagen is present caudally? The fiber-forming type III collagen clearly is expressed later than type I (Duband and Thiery, 1987) and is not a candidate. Kosher and Solursh (1989) reported that type II collagen, while abundant in the stage 15 avian embryo, is absent caudally. We have confirmed that type II collagen is also absent caudally in the stage 12 embryo (Vanderburg and Hay, unpublished). These are the fibrous collagens most likely to occur in the early embryo and the only ones studied to date.

The interstitial matrix of the early embryo characteristically contains, in addition to proteoglycan aggregates, hyaluronic acid, and 10 nm fibrils, the interstitial bodies described by Low (1970). Interstitial bodies are 50-200 nm in diameter, irregular shaped patches of amorphous "fibrillogranular" material similar in ultrastructure to basement membrane, they occur at both caudal and rostral levels in the 16 somite chick embryo, and they are thought to play a role in cell migration. They contain proteoglycan and fibronectin (Mayer *et al.*, 1981). We found in the present study that they do not contain type I collagen, as one might have predicted from their amorphous structure. We thought they would contain laminin because of the lamina-like structure, but this did not prove to be the case. Martins-Green and Erickson (1987) also reported undetectable levels of laminin in interstitial bodies, and they were able to detect type IV collagen in the bodies. We found for the first time that interstitial bodies do contain tenascin. We demonstrate this very clearly by examining the area vasculosa, which is particularly rich in interstitial bodies (Mayer *et al.*, 1981). The function of this abundant embryonic matrix structure remains an enigma.

The basement membrane (basal lamina) in all regions of the 2-day-old chick embryo is rich in fibronectin (Mayer *et al.*, 1981) and, as shown in the present study, in laminin. Laminin is also deposited on the 10 nm wide fibrils in the immediate vicinity of several embryonic basal laminae, probably a nonspecific association reflecting high levels of laminin production by the adjacent embryonic epithelia. However, we failed to confirm the report that laminin is dispersed throughout the embryonic ECM (Krotoski *et al.*, 1986). Early on, the basement membranes are rich in tenascin, but tenascin disappears from the neural tube and notochord basement membranes rostrally. Variability in association of tenascin with basement membranes in the adult has also been noted (Crossin *et al.*, 1986; Mackie *et al.*, 1988a; Lightner *et al.*, 1989). Rather than having any structural role in basement membranes, tenascin may associate with specific basement membranes to perform other functions conducive to epithelial development.

The staining patterns we report here do not support the idea that tenascin performs a "spacer" function (Lightner *et al.*, 1989) within the embryonic ECM. Tenascin-rich amorphous patches the diameter of hexabrachions (Lightner *et al.*, 1989) were not observed in the present study. Further ultrastructural studies of a variety of tissues of different ages are needed to substantiate the idea that tenascin contributes to the organization of mature collagen fibrils.

In conclusion, we demonstrate that the first type I collagen accumulation colocalizes with the onset of neural crest migration in the trunk of the very early avian embryo. Tenascin, fibronectin, and

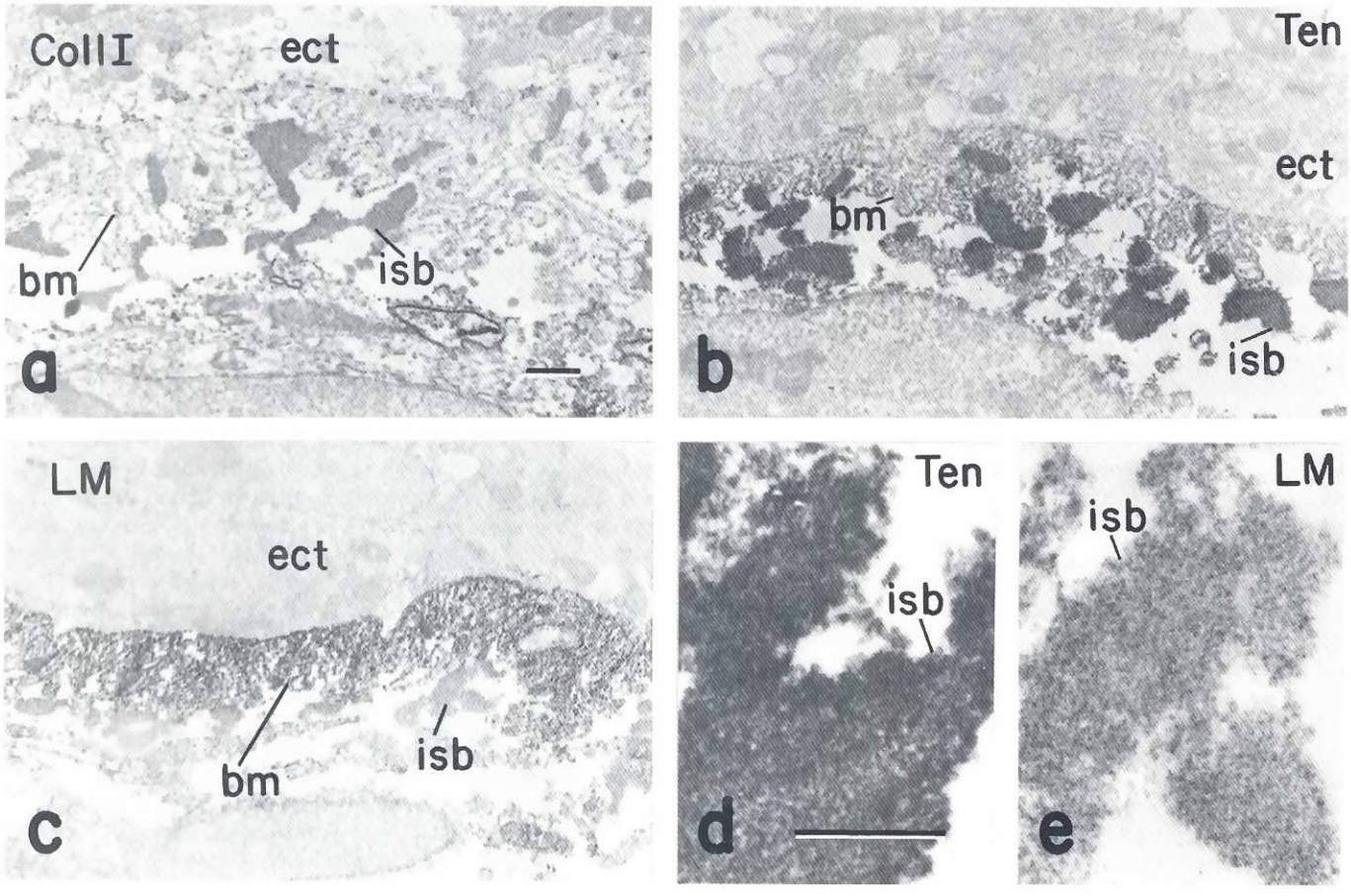

Fig. 11. PAP ultrastructural immunolocalization in interstitial bodies of the area vasculosa. *The basement membrane (bm) of the ectoderm (ect) is thrown into highly convoluted folds and is surrounded by large interstitial bodies (isb). Type I collagen is absent from the basement membrane and the interstitial bodies (a), but tenascin is found within the interstitial bodies (b and d) and basement membrane (b). Laminin is confined to the basement membrane (c) and is absent from the interstitial bodies (c and e). Bars 1 nm (a,b,c) and 0.5 nm (d,e).*

laminin are not as closely correlated with the emergence of neural crest. Taken together with our studies of crest migration in 3D collagen matrices *in vitro*, these results indicate that type I collagen is likely to be an important ECM molecule promoting crest motility in the embryo. We show that collagen and tenascin are not present in the sclerotome at the time it disperses into mesenchyme, and we suggest a role for central somite cells in ECM formation. At the ultrastructural level, we found that tenascin is widely distributed in the fibrils, interstitial bodies, and basement membranes of the very early embryo, but is not located as a spacer between fibrils as has been proposed for adult skin. Collagen I occurs in many of these same fibrils, but not in basement membranes or interstitial bodies. We suggest that while neural crest can attach to laminin, fibronectin, and tenascin *in vitro*, type I collagen is in the best position to be an essential component for the initial migration of crest cells in the embryo.

Materials and Methods

Antibodies

Mouse monoclonal antibodies M1-B4 (anti-tenascin, Chiquet and Fambrough, 1984) and 31-2 (anti-laminin, Bayne *et al.*, 1984) were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Biology, Johns Hopkins University School of Medicine and the Department of Biology, University of Iowa, under NIH contract N01-HD-6-2915. Anti-type I chicken collagen IgG prepared in guinea pig and adsorbed against type III collagen was a gift of Dr. Charles Little (University of Virginia). Mouse antiperoxidase coupled to peroxidase (PAP, Sigma Chemical Co., St. Louis, MO) was used to localize mouse monoclonal antibodies via a linker antibody. Guinea pig antiperoxidase was used in the PAP reaction to detect guinea pig polyclonal antibody. The linker was goat or rabbit antibody against mouse or guinea pig, respectively (Sigma Chemical Co.).

Whole mounts

Whole mounts were prepared by a modification of the procedure of Dent and Klymkowsky (1987) developed for amphibian embryos. Two-day-old chick embryos (16 somite, Hamburger/Hamilton stage 12) were removed from the surface of the egg, rinsed in phosphate buffered saline and placed in periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974) for 1 hr. The embryos were washed 3 times in 0.15 M NaCl, 50 nM Tris buffer pH 7.4 (TBS), then permeabilized in 20% DMSO in methanol for 16 hr at 4°C. They were stored in methanol at -20°C until use. For immunostaining, the embryos were washed three times in TBS to quench any remaining aldehyde. Embryos were placed into individual microfuge tubes and blocked with 3% goat serum (when using mouse monoclonal antibodies) or 3% rabbit serum

(with polyclonal anti-type I collagen) in TBS for 30 min at room temperature. The serum was removed, replaced with 100 ul of hybridoma supernatant, control supernatant, or anti-type I collagen IgG at 10 ug/ml in TBS containing 1% goat or rabbit serum, and incubated overnight at 4°C. The embryos were then washed three times in TBS for at least 6 hr and placed in linker antibody (goat anti-mouse IgG or rabbit anti-guinea pig IgG) diluted 1:20 in TBS overnight at 4°C. After washing in TBS, the embryos were incubated in peroxidase antiperoxidase (PAP) antibody complex diluted 1:100 in TBS containing 1% goat or rabbit serum overnight at 4°C. To localize the PAP complexes, the embryos were washed three times and reacted with 500 ul of 0.05% diaminobenzidine (DAB), 0.01% hydrogen peroxide in 0.1 M Tris buffer pH 7.4 for 5 min at room temperature. In order to stabilize the DAB product, the formaldehyde prefixed embryos were immediately washed twice in TBS, dehydrated in a graded series of alcohols and embedded in epon between two glass cover slips. The whole embryos were photographed using a Zeiss Axiophot microscope equipped with Nomarski optics and a blue filter.

To obtain transverse sections of the whole mounts, the cover slips were removed by placing them in hydrofluoric acid for five to ten minutes until the coverglass dissolved, then washed extensively in 1.0 M TBS pH 7.4. The thin slice of epon was then reembedded and thick sectioned. Sections were photographed as above except that a BG12 filter was used to visualize the peroxidase product.

Electron microscopic immunolocalization

Immunolocalization at the electron microscopic level can be achieved directly using the above procedure if the embryos are postfixed in 1% OsO_4 before the dehydration step. However, to optimize membrane and cellular detail, some embryos were not treated with DMSO, but were cut into fragments. We chose the peroxidase antiperoxidase (PAP) immunohistochemical method (Sternberger, 1979), not only because it is readily applicable to both light and electron microscopic studies, but also because we have previously shown it penetrates ECM in unembedded tissue slices better than particles like ferritin (Mayer *et al.*, 1981).

The fragments were prepared for electron microscopy by the method of Mayer *et al.* (1981), except that embryos were fixed in periodate-lysine-paraformaldehyde prior to exposure to antibodies. They were postosmicated after exposure to antibodies and embedded in epon. The antibody incubation procedure was essentially as above and access to ECM was via the cut edges of the tissue fragments. Thin sections were viewed in a JEOL 100CX electron microscope at 60kV. They were not stained with uranyl acetate or lead citrate.

Controls

Immunohistochemical controls were routinely run to check the specificity of staining. These included the omission of the linker antibody, PAP complex, or primary antibody. In the case of the polyclonal anti-type I collagen, the IgG was used after absorption to chicken type I collagen immobilized on nitrocellulose. Control experiments gave no staining in either the whole mount procedure or in immunolocalization with sections.

Acknowledgments

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