

## The transient expression of type II collagen at tissue interfaces during mammalian craniofacial development

ANDREW WOOD<sup>1</sup>, DOREEN E. ASHHURST<sup>2</sup>, ANDREW CORBETT<sup>3</sup> and PETER THOROGOOD<sup>4,\*</sup>

<sup>1</sup>The Institute of Neuroscience, 219 Heustis Hall, Eugene, Oregon 97403, USA

<sup>2</sup>Anatomy Department, St George's Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE, UK

<sup>3</sup>Department of Computing Services, University of Southampton, Highfield, Southampton SO9 5NH, UK

<sup>4</sup>Department of Oral Biology, Institute of Dental Surgery, Eastman Dental Hospital, 256 Gray's Inn Rd., London WC1X 8LD, UK

\* Author for correspondence

### Summary

Using immunocytochemical techniques, the spatiotemporal distribution of the major collagen isoform of cartilage, type II collagen, has been investigated during early craniofacial development in the mouse embryo. Early and transient expression was associated with the otic and optic vesicles, the ventrolateral surfaces of the developing brain, olfactory conchi, endocardial and mesocardial tissues, the lateral and basal surfaces of the pharyngeal endoderm and beneath the ectoderm of the branchial arches. A number of these locations are sites of epithelial–mesenchymal tissue interaction believed to generate the component parts of the chondrocranium; here, type II collagen appears transiently in advance of overt chondrogenesis in the mesenchyme. At such sites, immunofluorescence is typically localised along the basal surface of the epithelial partner, with the strongest reaction detected between the basal aspects of the otic and rhombencephalic epithelia. Immunoelectron microscopy, using pre-embedding immunostaining and a protein G-gold technique, reveals that the type II collagen is adjacent to, but not integral with, the basal

laminae. Gold particles are clearly associated with 10–15 nm fibrils of the extracellular matrix in the reticulate lamina region.

The pattern of type II collagen expression in the mouse closely correlates with that demonstrated previously in the quail, indicating a high degree of phylogenetic conservation between these two vertebrate species. These findings are consistent with the hypothesis that the pattern of epithelial secretion of type II collagen, or a co-expressed matrix molecule, constitutes a morphogenetic signal, realised as a matrix-mediated tissue interaction, and specifying the form of the vertebrate chondrocranium. Three-dimensional reconstruction of early type II collagen distribution, and of the subsequent chondrocranial cartilages, reveals that chondrocranial form can be derived from a 'pre-pattern' of epithelially derived type II collagen expressed at epithelial–mesenchymal tissue interfaces.

Key words: type II collagen, craniofacial morphogenesis, chondrogenesis, neural crest, placodes.

### Introduction

One of the fundamental questions surrounding vertebrate morphogenesis is precisely how specific and consistent patterns are generated and co-ordinated. This question is especially pertinent to observers of the neural crest – a 'dynamic tissue' – arising at the apices of the neural folds and dispersing widely throughout the embryo during ontogeny. Such cell translocation creates unprecedented opportunities for interaction with other tissues and elements of the extracellular milieu encountered *en route* and this, together with the intrinsic heterogeneity displayed by the crest population itself, results in an extensive catalogue of derivatives (reviewed Weston, 1982; Le Douarin, 1982). One such derivative is cartilage in the embryonic head.

During development, cephalic neural crest cells make a substantial contribution to the chondrocranium which, together with the developing brain, functions as a structural template for subsequent formation of the bony skull (De Beer, 1937; Horstadius, 1950).

Recently, it has been proposed that early and transient expression of extracellular type II collagen contributes fundamentally to chondrocranial morphogenesis by specifying a chondrogenic fate to responsive mesenchyme cells (Thorogood, 1987, 1988). In the avian embryo, an early and transient distribution of type II collagen, unlike types I and IV, laminin and fibronectin, maps precisely to epithelial–mesenchymal interfaces where matrix-mediated interactions are known to be causally involved in generating the constituent parts of the cartilaginous neurocranium

(Thorogood *et al.* 1986). These sites include the basolateral aspects of the diencephalon, mesencephalon and rhombencephalon, and the basal surfaces of the optic, otic and olfactory epithelia (Thorogood *et al.* 1986; Croucher and Tickle, 1989; Fitch *et al.* 1989). Significantly, not only is the distribution of type II collagen at such interfaces coincident with the future chondrocranial pattern, but also the timing of its appearance correlates precisely with the duration of the tissue interactions concerned. Evidence is now emerging that such early and transient type II collagen, in the avian system at least, is derived from adjacent epithelia in which mRNA for this collagen can be detected long before its appearance within the adjacent mesenchyme cells at a stage when they differentiate into chondrocytes (Hofmann and von der Mark, unpublished data; M. Hayashi, personal communication; and see Discussion).

The precise role of this collagen at this developmental stage is unclear. It could serve a signalling role in the matrix-mediated interactions which are known to occur (Smith and Thorogood, 1983). Alternatively, or maybe additionally, it may serve a role in arresting neural crest cell migration at such sites, as proposed elsewhere (Thorogood, 1988). Our preliminary results indicate that a subpopulation of cephalic neural crest cells express at the cell surface a  $34 \times 10^3 M_r$  protein, Anchorin CII. This is a member of the lipocortin/calpactin family, a group of structurally related, membrane-associated proteins characterised by an ability to bind to phospholipids in calcium-dependent manner and thought to mediate in certain signal transduction events (Haigler *et al.* 1989; Moss and Crumpton, 1990). Anchorin was originally discovered at the surface of avian chondrocytes where its high binding affinity to type II collagen is believed to mediate attachment of chondrocytes to the pericellular matrix (von der Mark *et al.* 1986). The role of the molecule at the surface of certain neural crest cells is not established but the known properties of this molecule, and of the family of proteins to which it belongs, is compatible with a potential role in either signal transduction and/or trapping by a selective adhesion.

Chondrocranial pattern has been highly conserved during vertebrate evolution. Therefore, irrespective of precise function, any morphogenetic model or hypothesis that incorporates this developmentally regulated expression of type II collagen should be applicable outside of the system upon which the initial observations were made. Thus, early and transient localization of type II collagen at tissue interfaces should occur in the embryos of other vertebrate taxa. This paper specifically addresses this issue by investigating, immunocytochemically, the pattern of early expression of type II collagen in the mammalian embryo using the mouse as a model system. Furthermore, by building three-dimensional reconstructions of both early collagen distribution and subsequent chondrocranial primordia, it has been possible to demonstrate how chondrocranial pattern might be assembled, based

upon a prepattern of type II collagen secreted by the epithelium.

## Materials and methods

A colony of adult Porton mice was maintained on a 12 h light cycle. E7.5 through to E16 embryos were obtained by counting time zero as midnight of the day preceding disclosure of a vaginal plug and the stage subsequently verified using morphological criteria at the time of fixation. Females were killed by cervical dislocation and the decidua removed to phosphate-buffered saline (PBS) at pH 7.2 (Dulbecco 'A', Oxoid Ltd, UK). After dissection to remove fetal membranes, embryos for light microscopical immunocytochemistry were fixed intact in pre-cooled 95% ethanol according to the technique of Sainte-Marie (1962). For light microscopy, embryos were further dehydrated in an ascending ethanol series, cleared, embedded in 56°C m.p. paraffin wax and 5 µm serial sections cut and dried down on glass slides. Sections for immunofluorescence were stored for a maximum of one week at 4°C before staining. Embryos to be processed for immunoelectron microscopy were partially dissected after 30 min fixation in 4% paraformaldehyde (4°C) in PBS at pH 7.2, using sharpened tungsten needles. Slices approximately 100 µm thick were removed to fresh paraformaldehyde containing 0.25% glutaraldehyde for a further 60 min and subsequently processed according to the protocol described below.

### Antibodies

Affinity-purified, polyclonal goat anti-rabbit type II collagen antibodies were used for most of the work and their preparation and characterization has been described elsewhere (Page *et al.* 1986); specificity of affinity-purified antibody was checked by pre-adsorption with purified type II collagen, which eliminated any immunoreactivity. Affinity-purified, polyclonal rabbit anti-chicken type II collagen antibodies, kindly provided by Dr Klaus von der Mark (von der Mark *et al.* 1976; Sasse *et al.* 1981) were also used. Identical results were obtained with both antibodies.

### Light microscope immunocytochemistry

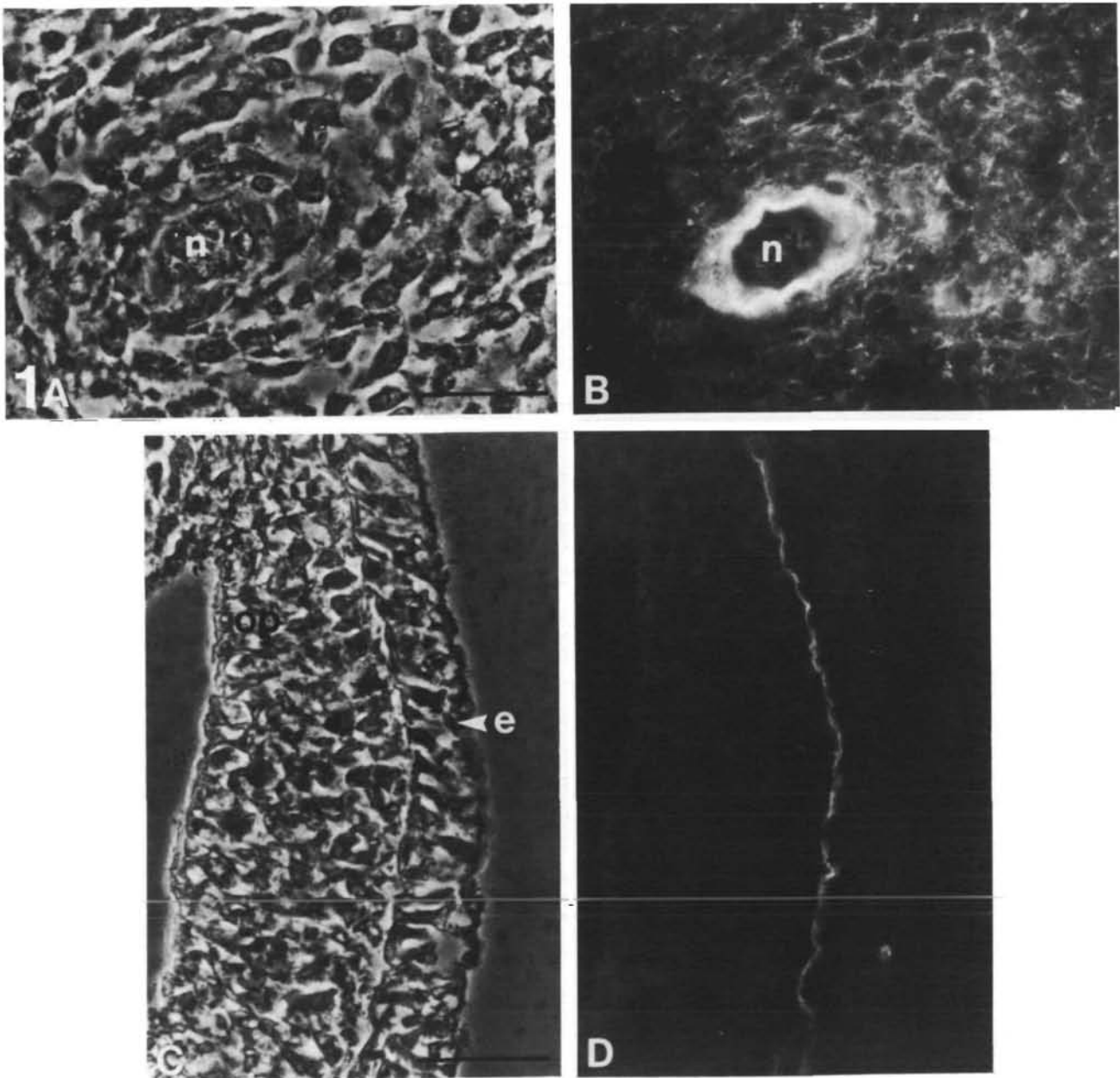
Sections were dewaxed, rehydrated through a series of ethanol dilutions to PBS. After three washes for 3 min each, the sections were incubated in a humidified atmosphere at 37°C in 1 mg ml<sup>-1</sup> testicular hyaluronidase (Sigma, UK) in PBS, to eliminate possible masking of type antigenicity by hyaluronate. After three 5 min washes in PBS, sections were incubated for 15 min in 2% L-lysine in PBS at room temperature and then washed a further three times in PBS for 5 min each. Prior to the addition of the primary antibody, sections were also incubated in 4% bovine serum albumin (BSA) (Sigma, UK) containing 10% heat-inactivated normal goat or donkey serum in PBS for 15 min as a further measure to block non-specific binding of the second (conjugated) antibody. Following three 5 min washes in PBS, sections were incubated with either of the primary antibodies at a dilution of either 1:40 or 1:160, the antisera being appropriately diluted in PBS containing 1% BSA. After incubation for 30 min at room temperature, sections were washed three times in PBS (5 min each wash) and incubated for 30 min, under dark conditions, with a 1:40 dilution of fluorescein isothiocyanate-(FITC)-conjugated goat anti-rabbit IgG, or FITC-conjugated donkey anti-goat IgG (Amersham Ltd, UK), containing 1% heat-inactivated mouse serum (to quench autofluorescence). Sections were washed a further three times and mounted in

Citifluor mountant (Agar Aids Ltd, UK). Each slide included control sections for which either PBS or preimmune serum was substituted for the primary antibody, and others for which PBS was substituted for the secondary antibody; all controls were routinely negative. Sections were examined using an Olympus BH-2 photomicroscope set for phase and epifluorescence, using  $\times 10$  and  $\times 40$  objectives. Photographic records were made using Kodak Tri-X 35 mm film rated at 400ASA, using a standard 60 s exposure for all fluorescence photographs.

#### Immunoelectron microscopy

Following prefixation (see above), tissue was fixed for 1 h in

PBS at 4°C and then incubated in 1 mg ml<sup>-1</sup> testicular hyaluronidase (Sigma Ltd, UK) for 1 h in a humidified atmosphere. Tissue slices were then washed in PBS for a further hour and incubated in goat anti-rabbit type II collagen antibody at a 1:40 dilution for 8 h at 4°C on a rotator. Negative controls consisted of PBS or preimmune serum substitution for the primary antibody. Tissue was subsequently washed overnight in PBS at room temperature and incubated in protein G-gold (Janssen Life Sciences Products Division, UK) at a 1:3 dilution for 5 h at room temperature with rotation. Protein G was selected for its higher affinity to goat IgG than protein A; gold particle size was 5 nm. Following a further wash for 3 h in PBS, tissues were further



**Fig. 1.** Phase-contrast (A,C) and immunofluorescent (B,D) micrographs of E9.5 rhombencephalic notochord and optic vesicle in transverse section. The perinotochordal sheath (A,B) is strongly type II collagen-positive and staining extends into the adjacent mesenchyme dorsally. Positive staining can also be seen around the optic vesicle, the strongest immunolocalization being immediately under the surface ectoderm. (n, notochord; e, ectoderm; op, optic vesicle). Bars = 27  $\mu$ m (A) and 24  $\mu$ m (C).

fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate at pH 7.3 for 2 h and rinsed overnight in 0.13 M cacodylate, pH 7.3. Postfixation was carried out in 1% osmium tetroxide in 0.13 M veronal acetate buffer for 1 h at room temperature. Tissues were then given a cacodylate buffer rinse for 1 h, dehydrated in an ascending ethanol series and transferred to a TAAB/propylene oxide 1:1 mixture for infiltration before embedding and polymerisation in TAAB resin. Ultrathin sections were cut at 1  $\mu\text{m}$  on an LKB ultratome, mounted on copper grids and either stained using lead citrate and uranyl acetate, according to Reynolds (1963) or left unstained to facilitate detection of gold particles. Sections were viewed in a Jeol (JEN 1200 EX) transmission electron microscope, at an accelerating voltage of 80 kV.

### Three-dimensional reconstruction

E9.5 to E16 embryos were fixed in 10% paraformaldehyde, dehydrated, embedded in paraffin wax (see earlier) and serially sectioned, in a transverse plane, at 20  $\mu\text{m}$  intervals. Sections mounted on glass slides were stained with haematoxylin and eosin; later stages were counterstained with alcian blue (1% in 3% acetic acid pH 2.6, containing 0.25 M  $\text{MgCl}_2$ ) to stain cartilage. Sections were viewed using a high-resolution, charged couple device, JVC video camera (model TK-870) attached to an Olympus BH-2 photomicroscope fitted with  $\times 4$ ,  $\times 10$  and  $\times 25$  planapo-chromatic objectives. The resulting images were contrast-enhanced, digitized and measured using a VIDS IV digital image processor (Analytical Measuring Systems Ltd, Cambridge, UK). XY coordinate data from serial sections were compiled from on-screen tracings, using an interactive cursor tablet and transferred to an IBM 3090 model 150 VF main-frame computer. Cephalic aspects of embryos were reconstructed from this database using an IBM 5080 high resolution graphics workstation and the Winsom Solid Modeller programme (kindly provided by IBM Scientific Centre, Winchester, UK). The collection of XY coordinate data was confined to the outer aspects of the ectoderm, the basal and luminal surfaces of the neuroepithelium, the outer aspect of the notochord, the basal and luminal aspects of the olfactory, optic and otic primordia, and the outer surface of cartilaginous primordia in E10, E12 and E16 embryos.

## Results

### Light microscope immunocytochemistry

Description of the immunocytochemical localization of type II collagen will be treated in chronological order. Staining patterns were qualitatively identical for both antibodies used and any differences were restricted only to intensity of fluorescence and the degree of non-specific background fluorescence.

No staining was detected between E7.5 and E8.5 in any of the tissues observed, whether in the head or trunk. The first detectable appearance of type II collagen occurred at E9 as very faint fluorescence between the basal surfaces of the invaginating otic vesicle and neural epithelium, and as a faint halo surrounding the notochord (not illustrated). By E9.5 staining was much stronger around the notochord and extended a short distance into the adjacent mesenchyme (Fig. 1A,B). Strong staining was also detected at the interface between the basal surfaces of the lateral aspect of the optic vesicle and adjacent ectoderm but did not extend around the remainder of the vesicle (Fig. 1C,D). Between E9.5 and E10.5, staining around the otocyst, and on the ventrolateral aspect of the adjacent (rhombencephalic) neural tube increased in intensity. The strongest reaction was found between the two structures (Figs 2 and 3). At E10.5, type II immunoreactivity was detected surrounding the olfactory vesicles subjacent to the epithelium. A positive but patchy immunoreactivity was also found along the basal surface of the neural tube throughout the mesencephalic and rhombencephalic regions (Fig. 2 and see later, three-dimensional reconstruction). Other positive tissues during this period were the endocardial and mesocardial tissues adjacent to the dorsal aorta (Fig. 4), lateral and basal surfaces of the pharyngeal endoderm, and central and apically located limb mesenchyme (beneath the apical ectodermal ridge – not illustrated). At E11, fluorescence in the otic region was adjacent to

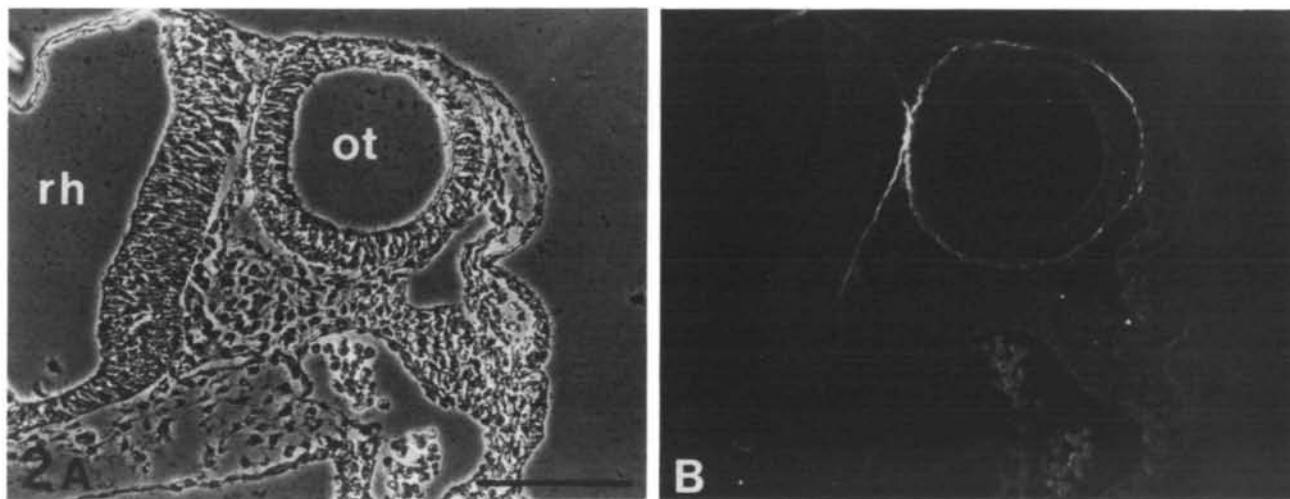
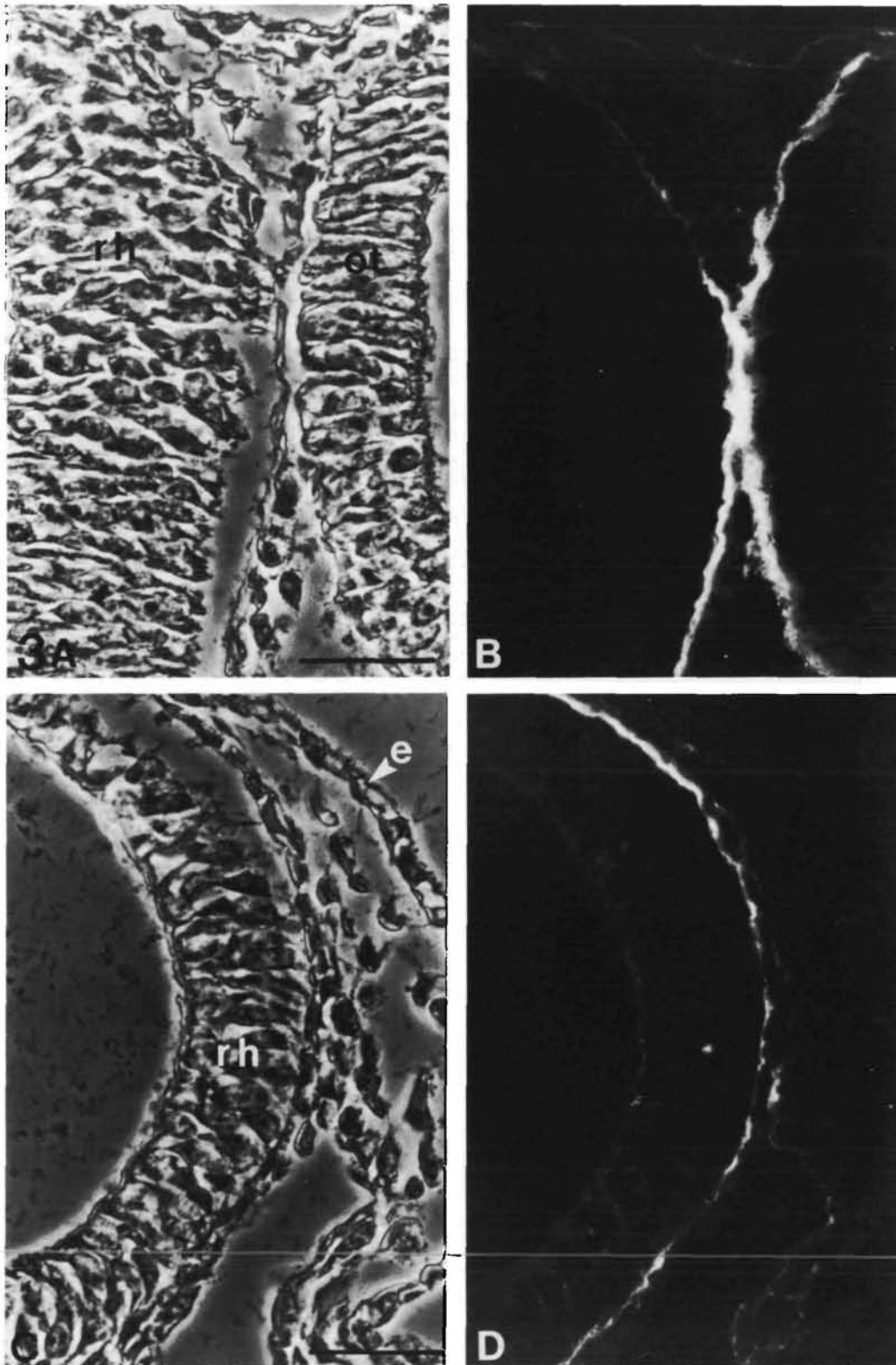
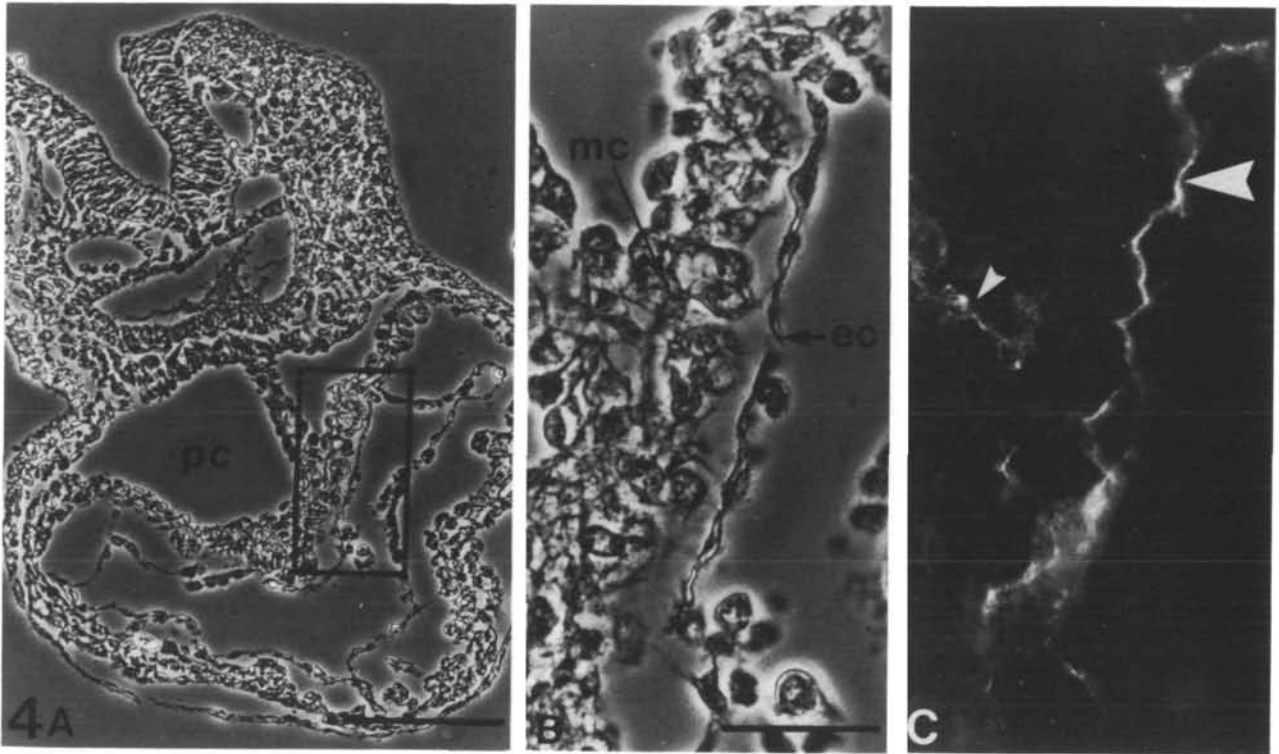


Fig. 2. Phase-contrast (A) and immunofluorescent (B) micrographs of E10 hindbrain and otic vesicle in transverse section. Type II collagen is immunolocalised around the entire otic vesicle (ot) and laterally on the rhombencephalon (rh), but is strongest where the basal surfaces of the two structures are apposed (see Fig. 3). Apparent positive reaction on erythrocytes lower right is autofluorescence. (ot, otic vesicle; rh, rhombencephalon). Bar=80  $\mu\text{m}$ .



**Fig. 3.** Higher magnification phase contrast (A,C) and immunofluorescent (B,D) micrographs of E10 hindbrain and otic vesicle shown in Fig. 2. Note strong immunolocalization of type II collagen at the point where the basal aspects of the rhombencephalic neuroepithelium and otic vesicle epithelium are juxtaposed (A,B). C and D illustrate the extent of staining around the remaining aspects of the otic vesicle and the absence of staining under the ectoderm. Very sparse staining is observed within the periotic mesenchyme and does not increase until E10 when the otic cartilages begin to form. (rh, wall of rhombencephalon; ot, otic epithelium; e, ectoderm). Bar=30  $\mu$ m.



**Fig. 4.** Phase-contrast (A,B) and immunofluorescent (C) micrographs of an E9 embryo in transverse section, showing the heart primordium. The boxed area in A is illustrated at higher magnification in B and C, revealing quite strong immunolocalization of type II collagen associated with the interface between endocardial and mesocardial layers of the heart wall (large arrow) and also deep within the mesocardium itself (small arrow). (pc, pericardial cavity; mc, myocardium; ec, endocardium). Bars=104  $\mu\text{m}$  (A) and 24  $\mu\text{m}$  (B).

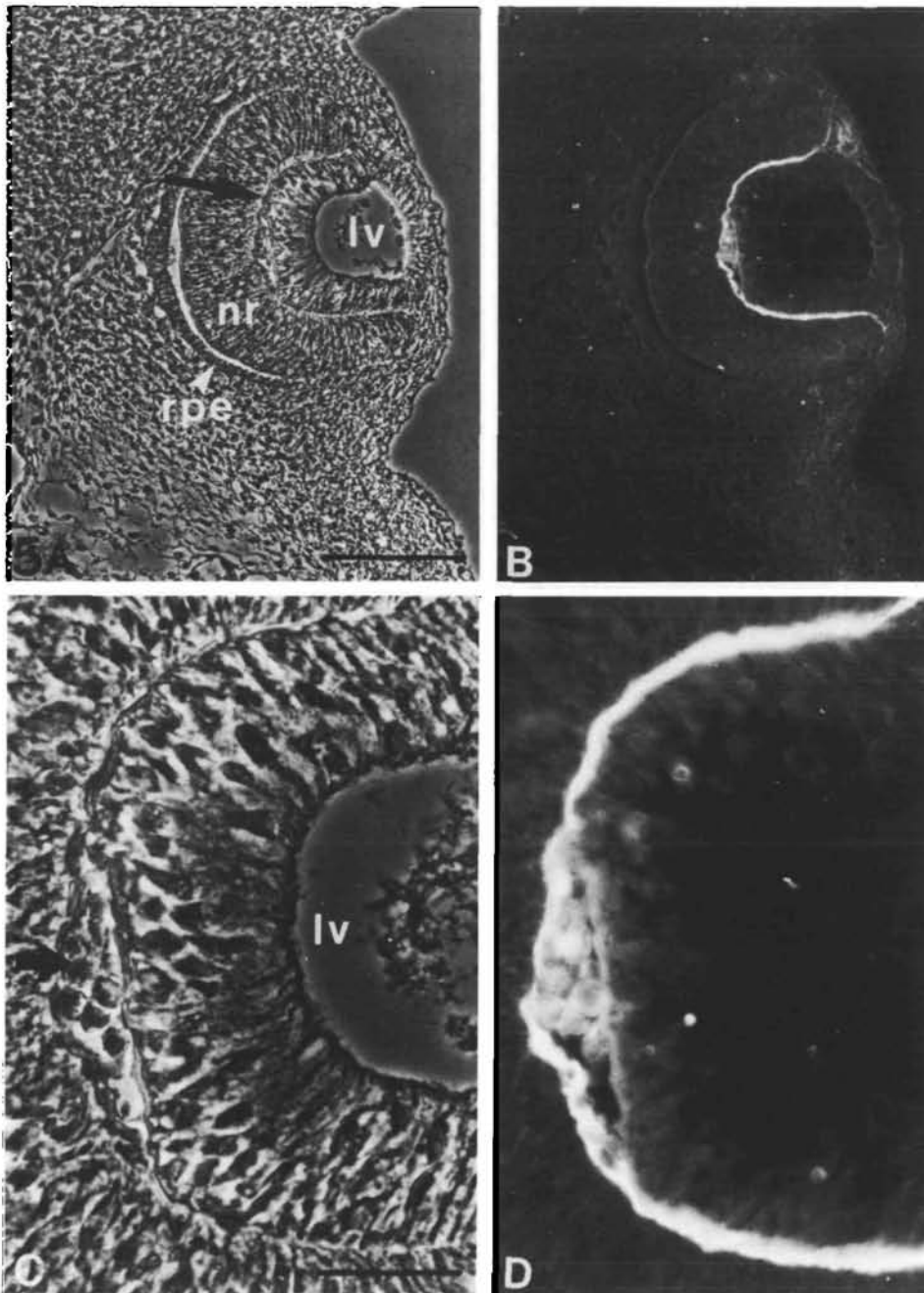
and between the basal surfaces of the vestibular and cochlear epithelia, but also extended into the periotic mesenchyme (not illustrated). By E11.5, strong staining was present in the forming posterior chamber of the eye between the medial surface of the lens and the neural retina; immunoreactivity appeared to be associated almost exclusively with the basal surface of the retina (Fig. 5). In addition, very strong staining was detected at the base of the infundibulum and the hypophyseal pouch, and this persisted through E12 (Fig. 6A,B). Between E11 and E13.5, type II immunoreactivity in the olfactory location became restricted to the precartilaginous mesenchyme of the nasal septum and nasal capsules, and also subjacent to the epithelium surrounding the forming nasal passages. By E12.5, staining around the notochord was intense and extended well into the perinotochordal mesenchyme. Faint staining was evident in the central region of the mandibular processes. Staining had now intensified around the otic epithelia, for instance basal and apical surfaces of the sacculus, utriculus, ampulus and cochlea were all strongly positive and by E13.5 heavy staining was present within the periotic mesenchyme, which was starting to display a morphology characteristic of mature cartilage (Figs 7 and 8). All cartilages of the inner ear, in particular the tympanic ring surrounding the cochlear and saccular epithelia, were strongly positive at this stage. By now type II immunofluor-

escence staining had entirely disappeared within the eye but had increased dramatically beneath the notochord as overt chondrogenesis progressed. By this stage, the facial, branchial arch, vertebral and limb cartilages all illustrated a positive fluorescent reaction for type II collagen (Fig. 8E, F).

#### *Immunoelectron microscopy*

The otocyst/rhombencephalic interface at E12 was selected for investigation of the ultrastructural distribution of type II collagen, since this interface gave the strongest positive reaction with both antibodies. No gold particles were observed in any of the sections from negative control blocks (Fig. 9A). In sections from blocks that had been subjected to pre-embedding immunogold staining, the perinotochordal sheath served as a positive reference tissue and was always observed to be strongly positive, as judged by the distribution of gold particles.

In sections not stained with lead and uranyl salts, 5 nm gold particles were clearly visible subjacent to the otic and neuroepithelial basal laminae but were not necessarily an integral part of either structure, typically being found either in the extracellular areas immediately adjacent to the basal lamina (the 'reticulate lamina' – Bernfield, 1984), or along the outer aspect of the lamina densa itself (Fig. 9B). In sections stained with lead and uranyl salts, the location of the gold



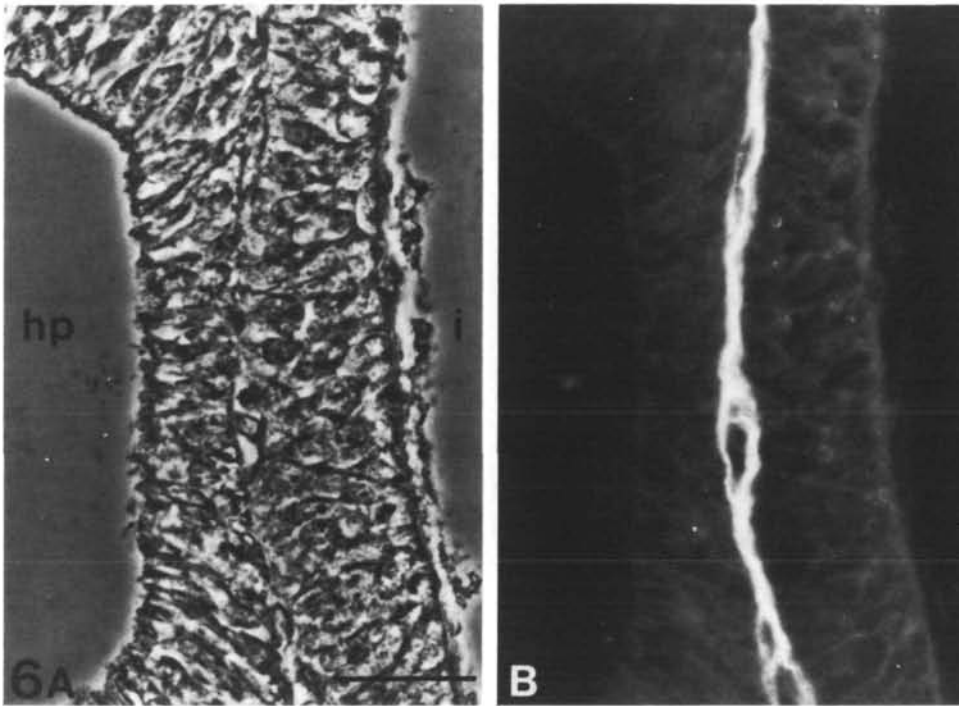
**Fig. 5.** Phase-contrast (A,B) and immunofluorescent (C,D) micrographs of E11.5 optic cup, illustrated at both low and high magnifications. At this stage, type II collagen is largely confined to the forming posterior chamber of the eye, between the basal (inner-facing) surface of the neural retina and the medial aspect of the lens vesicle (arrow, A); the pericocular mesenchyme is negative. At higher magnification, it appears that the immunolocalization may also be associated with the formation of the hyaloid plexus (arrow, C). (rpe, presumptive pigmented retina; nr, neural retina; lv, lens vesicle). Bars = 96  $\mu\text{m}$  (A) and 24.4  $\mu\text{m}$  (C).

particles was revealed as being spatially coincident with 10–15 nm fibrils in the reticulate lamina (Fig. 9C, D). The lamina densa of the adjacent neuroepithelium was always less distinct than that of the otic epithelium but still had associated gold particles, albeit fewer.

This localization of gold particles, within the reticulate lamina and on the outer surface of the lamina densa, extended around the entire otocyst and along the entire lateral and ventral surface of the rhombencephalon. Gold particles were exclusively located extracellularly and never found within the otic or neuroepithelial cells. At this stage and at this axial level, all other epithelia and mesenchymal cells were negative.

#### *Three-dimensional reconstruction*

Initially, graphic reconstruction of the ectoderm XY coordinate data was checked against published scanning electron microscopy accounts of external morphology of the embryonic mouse head (Sulik and Schoenwolf, 1985; Tamarin and Boyde, 1977) in order to assess the accuracy of the overall reconstruction. Subsequently, comparable assessment was made with respect to accuracy of reconstruction of the neural tube (e.g. Bush *et al.* 1990) and the chondrocranium (Gruneberg, 1953). Reconstructions were viewed from a range of perspectives – lateral, ventral, dorsal and three-quarter oblique. In all cases, the reconstructions compared well



**Fig. 6.** Phase-contrast (A) and immunofluorescent (B) micrographs showing a line of strong immunolocalization present between the basal surfaces of the infundibulum and the hypophyseal pouch (Rathke's pocket) in a transversely sectioned E11 embryo. The close apposition between these two structures probably precludes the role of any mesenchymal component in the deposition of type II collagen at this site. A positive immunolocalization continues through E12 but has ceased by E13. (in, infundibulum; hp, hypophyseal pouch). Bar = 30  $\mu$ m.

with published images and were considered to represent accurately the intact structure *in situ*. For reasons of clarity and space, only the outer aspects of structures or organs are depicted in the reconstructions illustrated here.

Fig. 10A and B illustrate the distribution of type II collagen at E10 seen from a lateral perspective. The most prominent locations are around the optic and otic primordia, notochord, and on the ventrolateral surface of the rhombencephalic neural tube (the patchy distribution over the mesencephalic region is not shown). By E12, this pattern has extended to include the olfactory primordia and the previous distribution associated with the basal surface of the optic vesicle has now become largely confined to the forming posterior chamber of the eye and hence is only glimpsed at the margin of the optic cup (Fig. 10C,D). Type II collagen is no longer detected in any appreciable amount around the ventrolateral aspects of the neural tube, except medially along the floor of the forebrain, seen only from a ventral perspective. The otocyst and the notochord retain their prominent distribution of type II collagen.

For reasons of clarity, adjacent cartilage elements of the E16 chondrocranium were grouped together and colour-coded (see Fig. 11 legend for details). Given differences of size and proportion, due to (allometric) growth, there is a striking spatial correlation between aspects of the earlier patterns at E10 and E12, and the form of the chondrocranium. Prominent cartilages (parachordals, otic capsules) develop at the ventrolateral aspects of the rhombencephalon, around the otocyst and medially around the anterior notochord (Fig. 11). Likewise, more rostrally, the other most prominent cartilages (trabeculae, olfactory capsule/nasal cartilages) form ventrally under the prosencepha-

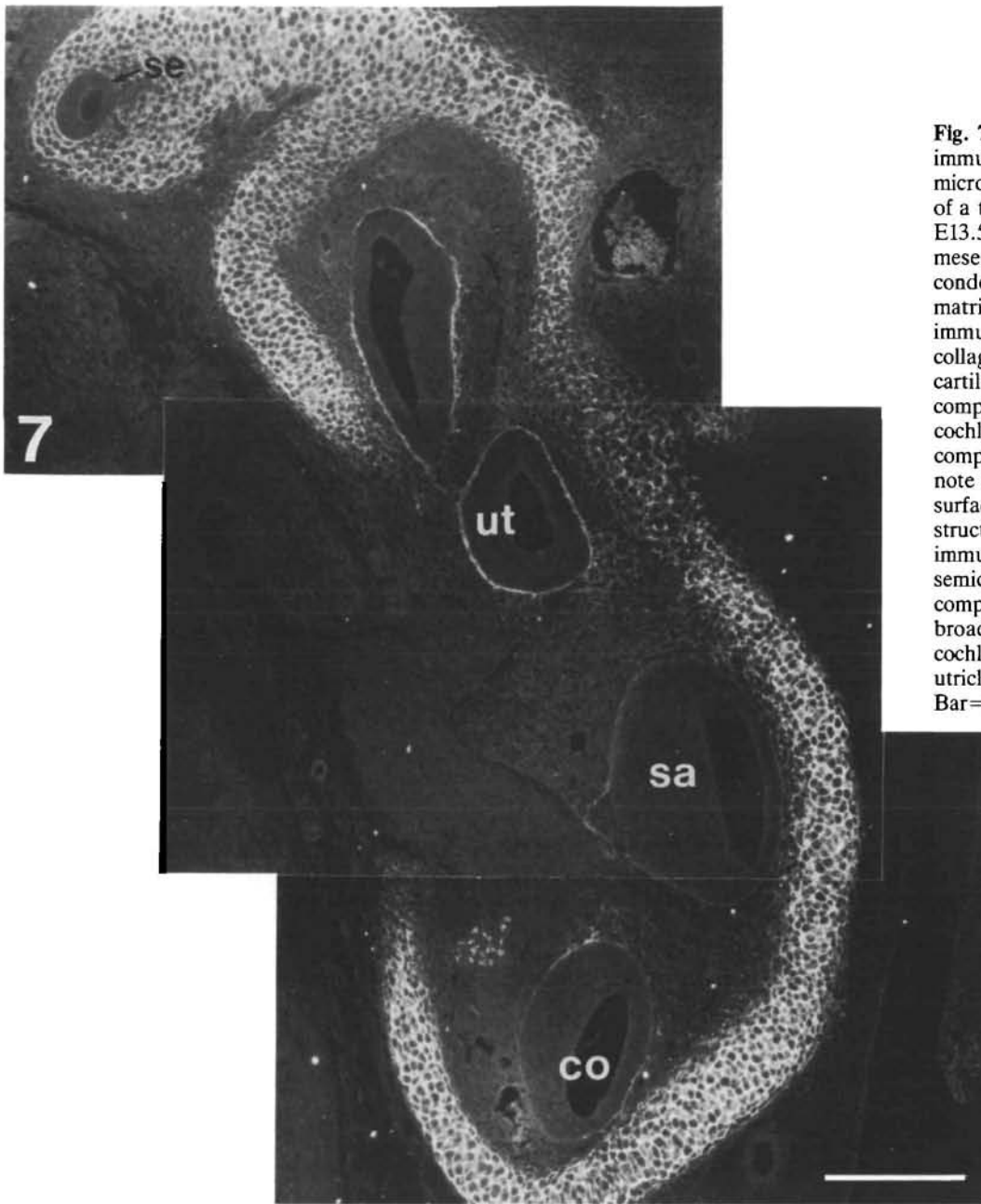
lon and around the olfactory conchii. In the mammalian chondrocranium, which lacks an optic/scleral cartilage, the region between these two axial levels ('prosencephalic' and 'rhombencephalic'), contains rather less cartilage *per se* (Fig. 11A) and this correlates with a lack of strongly positive immunolocalization of type II at 'mesencephalic' levels in the earlier stages.

We were unable to find an unambiguous correlation between Meckel's cartilage with any early and transient type II collagen distribution within the mandibular arch, apart from that found associated with the endoderm of the forming pharynx (see earlier). However, in a study of murine mandibular chondrogenesis, the presence of type II collagen in the 'sub-basement membrane area' of the mandibular processes was noted (Richman and Diewert, 1987), although the rate of skeletogenesis described is somewhat faster than that observed in the present study.

## Discussion

This study demonstrates the transient appearance of immunocytochemically detectable amounts of type II collagen in close proximity to the epithelial basal lamina at a number of craniofacial locations in the embryonic mouse – notably surrounding the otic vesicle and olfactory conchii, between the basal surface of the optic vesicle and lateral ectoderm, around the notochord, and on the basolateral surfaces of the developing brain. Predictably, chondrogenic primordia that appear later during development are strongly type II collagen-positive, since this molecule plays an important structural role within the cartilaginous stroma (Miller and Matukas, 1969). The early distribution parallels, both regionally and temporally, the ontogeny of type II



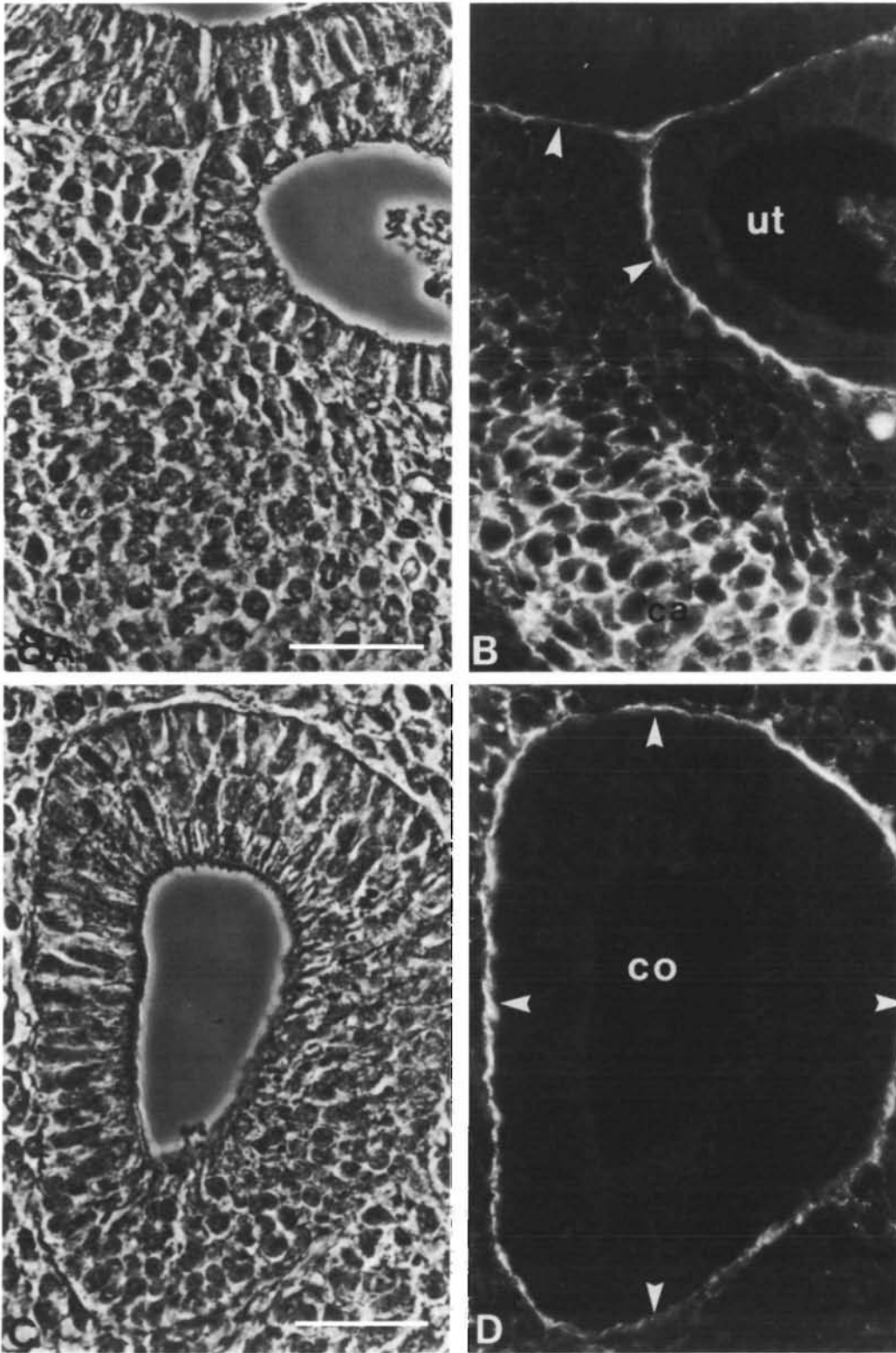


**Fig. 7.** Montage of immunofluorescent micrographs of the otic region of a transversely sectioned E13.5 embryo. The periotic mesenchyme is heavily condensed and the cartilage matrix is strongly immunopositive for type II collagen. The periotic capsular cartilage now almost completely surrounds the cochlear, saccular and utricular components of the inner ear; note also that the basal surfaces of these epithelial structures remain immunopositive. The semicircular canals are completely embedded in a broad capsular cartilage. (co, cochlea; sa, sacchule; ut, utricle; se, semicircular canal). Bar=130  $\mu$ m.

collagen expression at equivalent stages of development in the avian embryo, which suggests a high degree of phylogenetic conservation between these two vertebrate taxa. This correlation constitutes only circumstantial evidence that any proposed morphogenetic mechanisms arising from this pattern of expression are also conserved between taxa. However, the highly precise spatial correlations between sites of chondrogenesis-promoting tissue interactions and the discrete and transient secretion of type II collagen, demonstrated now in both avian and mammalian systems, is at least consistent with the hypothesis that epithelial production of type II collagen may be causally involved in specification of chondrocranial form (Thorogood, 1988).

All known or assumed sites of chondrogenesis-

promoting tissue interactions in the axial skeleton have been demonstrated to involve a transient or prolonged occurrence of type II collagen in the interface ECM. This observation, together with the fact that type II collagen itself is the principal protein synthesized by the cells whose subsequent differentiation is promoted by the interaction, suggests that the collagen has a signalling role in the matrix-mediated interaction. The 'direction' of signalling in this sense would mean epithelium to (responsive) mesenchyme. Given the enormous spatial relocation of mesenchymal cells that takes place during craniofacial morphogenesis (due either to active migration of individual cells or their passive displacement by growth of adjacent tissues), the various epithelia may play an important role as a framework of positional reference (Thorogood, 1988).



**Fig. 8.** Phase-contrast (A,C and E) and immunofluorescent (B,D and F) micrographs. Details are illustrated, from Fig. 7, of the interface between pre-chondrogenic and chondrogenic periotic mesenchyme (A,B) and the utricular (A,B) and cochlear epithelia (C,D). The basal surface of these organ rudiments are strongly type II collagen-positive (arrowheads) and this is clearly discrete from that present within the (by now metachromatic) matrix of the capsular cartilage itself. All rudiments of the inner ear appear to express type II collagen between E13 and E14.5, at which time immunoreactivity becomes entirely restricted to the capsular cartilages.

The evidence for an epithelial origin of this putative signal molecule is accumulating. The ultrastructural location of type II collagen in the reticulate lamina of the epithelia and *not* around mesenchyme cells, is highly suggestive of an epithelial origin (The absence of gold particles within the basal lamina itself may simply reflect the barrier to penetration during the pre-

embedding procedure by the lamina densa). At certain sites, the location of type II collagen, between closely apposed epithelia (Fig. 6), almost precludes a mesenchymal origin. More direct evidence for an epithelial origin comes from *in situ* hybridization. Using cDNA probes to avian type II collagen mRNA, message has been detected not only in corneal epithelium (Hayashi

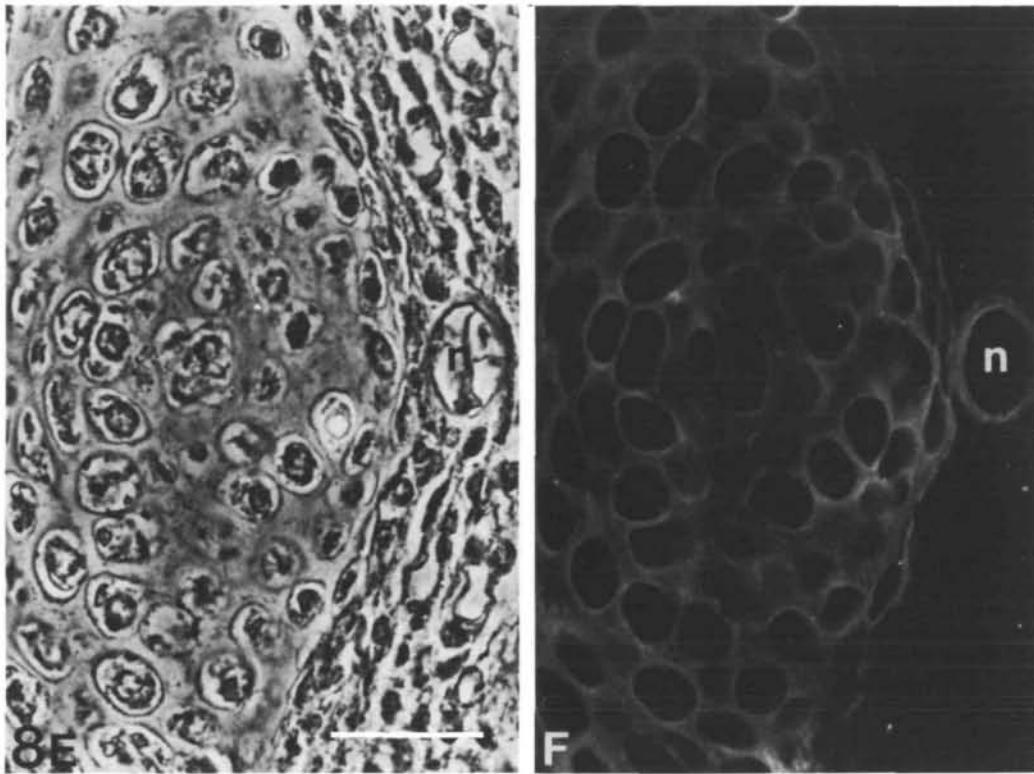


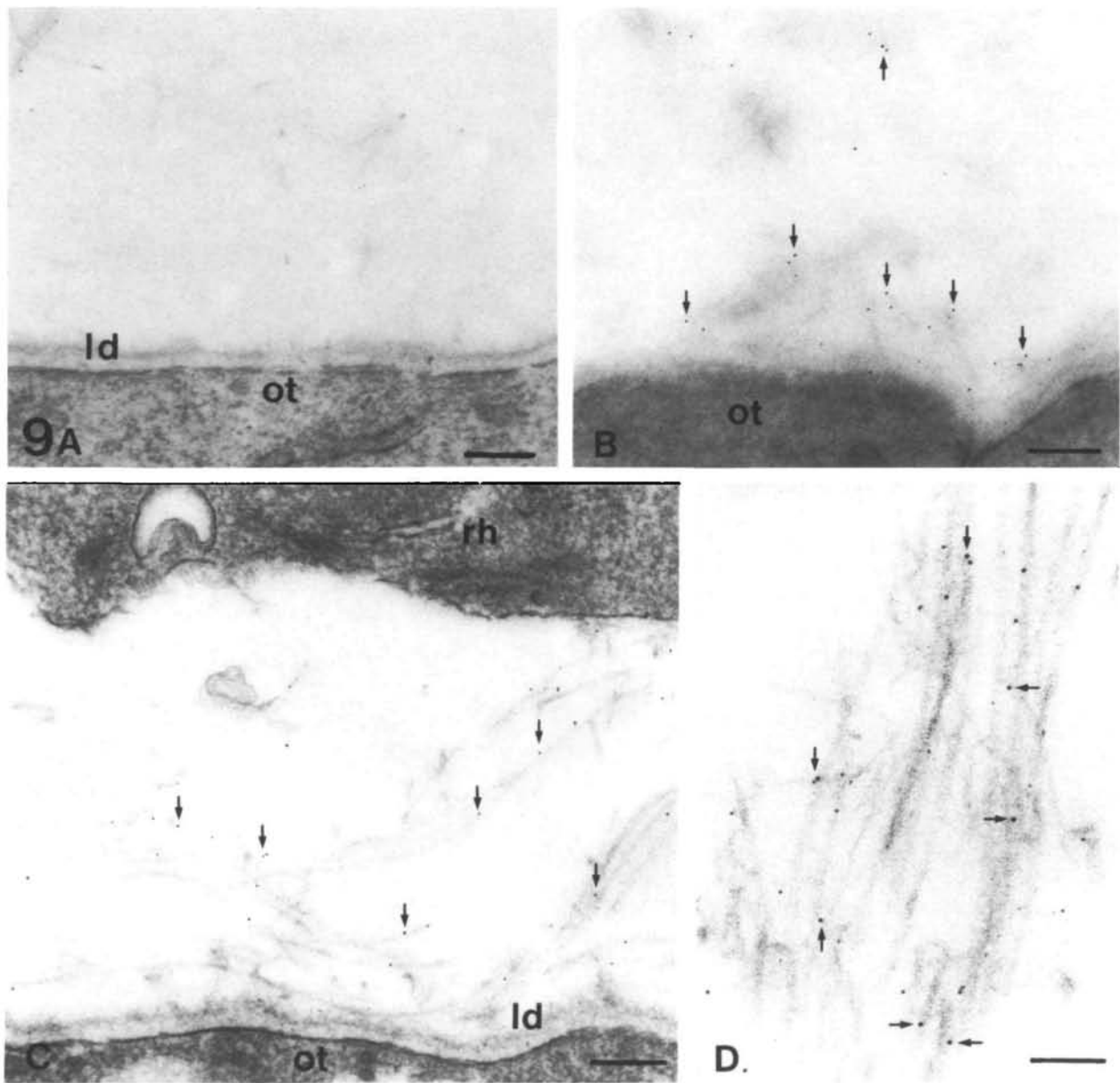
Fig. 8. (E,F) The notochord and basioccipital cartilage in a transversely sectioned E13.5 embryo. Immunolocalization in the perinotochordal sheath has now considerably diminished compared with earlier stages (see Fig. 1) and the cartilage matrix is strongly immunopositive for type II collagen. (ca, capsular cartilage; co, cochlear epithelium; ut, utricular epithelium; n, notochord). Bars=30  $\mu$ m (A) and 34  $\mu$ m (C,E).

*et al.* 1988) but, of special relevance here, in otic, optic and neural epithelia and in notochord cells (M. Hayashi, pers. comm.: Hofmann and von der Mark, unpublished data). All of these last four tissues are epithelial partners in chondrogenesis-promoting interactions and the mRNA is detectable well in advance of detectable type II message in the responding mesenchyme. Furthermore, transcription of the type II collagen gene in a comparable range of epithelial tissues has very recently been demonstrated in murine embryos (Cheah *et al.* 1991), providing a strong correlation with the distribution of the protein as described here.

Although evidence for a signalling role is accumulating, it remains at best circumstantial. To date there is no direct evidence of signal reception/transduction taking place at the surface of the responding mesenchymal cells. Furthermore, there is no functional evidence that the signal molecule is type II collagen itself and it remains possible that it is a matrix molecule coexpressed with type II collagen, perhaps another cartilage-specific matrix molecule. Obvious candidate molecules are a minor cartilage collagen or a cartilage-specific proteoglycan. However, present evidence indicates that the minor cartilage collagen, type IX, is not expressed at sites of chondrogenesis-promoting tissue interactions (Fitch *et al.* 1989) and therefore type IX appears to be a marker of overt differentiation in the avian system. A survey of the distribution of certain proteoglycans during avian craniofacial development

suggests that, while cartilage-specific proteoglycan may be coexpressed with type II collagen at some sites of interaction (e.g. around the otic vesicle and notochord), it is absent at others (e.g. around the optic vesicle, Heath and Thorogood, 1989).

In seeking a role for the early and transient type II collagen, it should be noted that this collagen is also transiently observed elsewhere at sites unassociated with chondrogenesis-promoting interactions. Thorogood *et al.* (1986) report that type II collagen is present underneath ectoderm around the head and Koshier and Solursh (1989) describe type II also being present within the heart primordium. Comparable observations have been made here for the mammalian system, in so far as positive staining was found in the endocardium and mesocardium (Fig. 4; and Cheah *et al.* 1991). It is remotely possible that type II collagen at such sites reflects some developmentally atavistic or phylogenetically ancient role (e.g. reflecting dermal skeletogenesis in an ancestral form) but it is more likely that it serves some direct, but as yet undefined, function in contemporary development. In this respect, it is of interest that molecular polymorphism of type II collagen produced by chondrocytes occurs due to the alternative mRNA splicing of an exon encoding for a cysteine-rich domain at the NH<sub>2</sub> terminal of type II procollagen (Ryan and Sandell, 1990). Whether different forms of type II collagen are produced at these non-chondrogenic embryonic sites remains to be established.

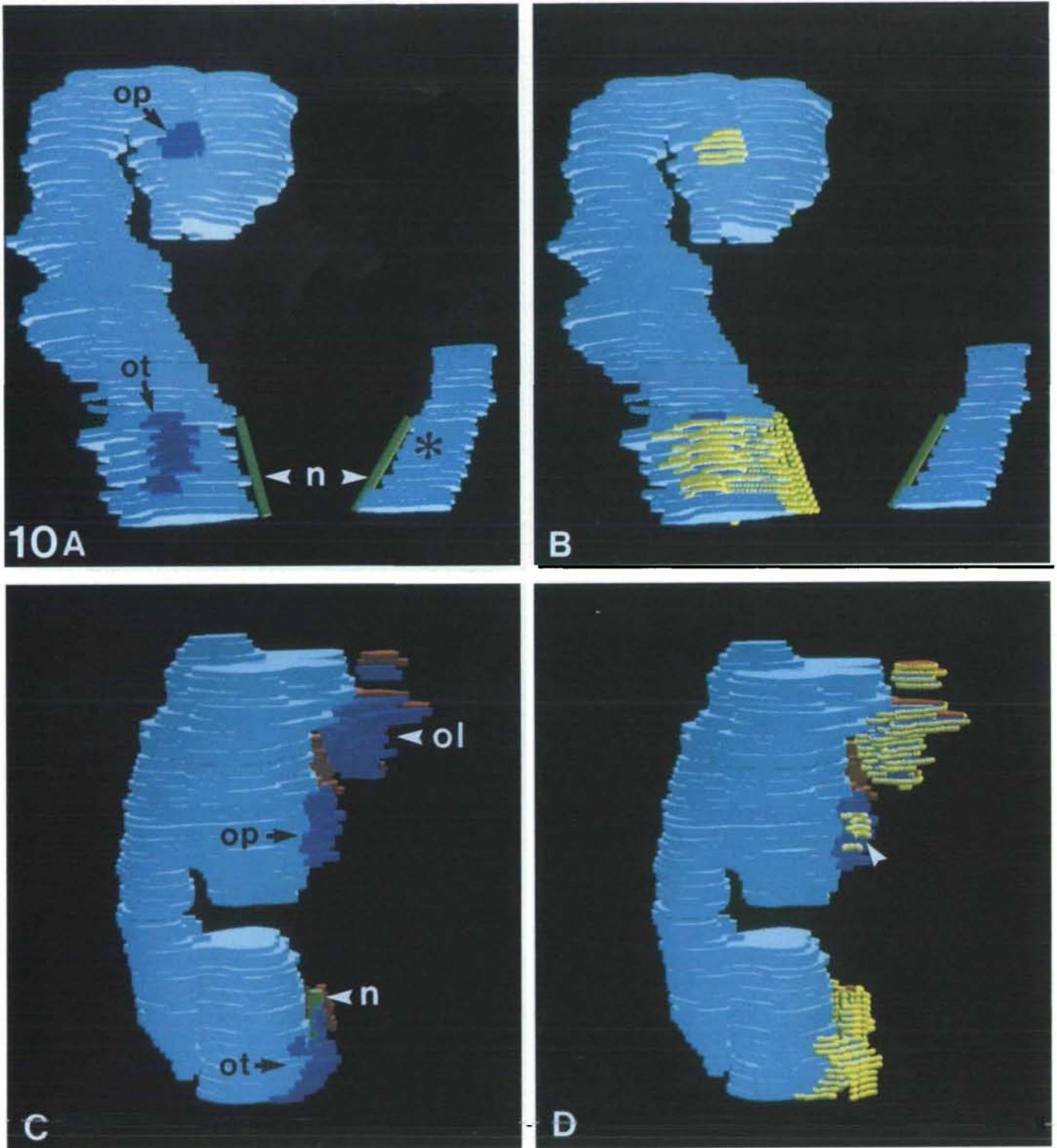


**Fig. 9.** Transmission electron micrographs of immunogold preparations of E12 otocysts (A,B unstained and C,D stained with uranyl and lead salts). (A) Negative control of basal surface of otic epithelium. (B) Immunopositive interface between basal surfaces of the rhombencephalon and the otocyst with gold particles (arrows) in the intervening reticulate lamina; note association of gold particles with amorphous clumps of extracellular material. (C) Conventional staining with uranyl and lead salts reveals the 'amorphous clumps' to be bundles of fibrils and shown to decorated with the gold particles (arrows). (D) Higher magnification of field similar to C reveals virtually exclusive association of gold particles (arrows) with extracellular fibrils. (ot, basal aspect of otic epithelium; ld, lamina densa; rh=basal aspect of rhombencephalic neuroepithelium). Bars=200 nm (A,B, and C) and 100 nm (D).

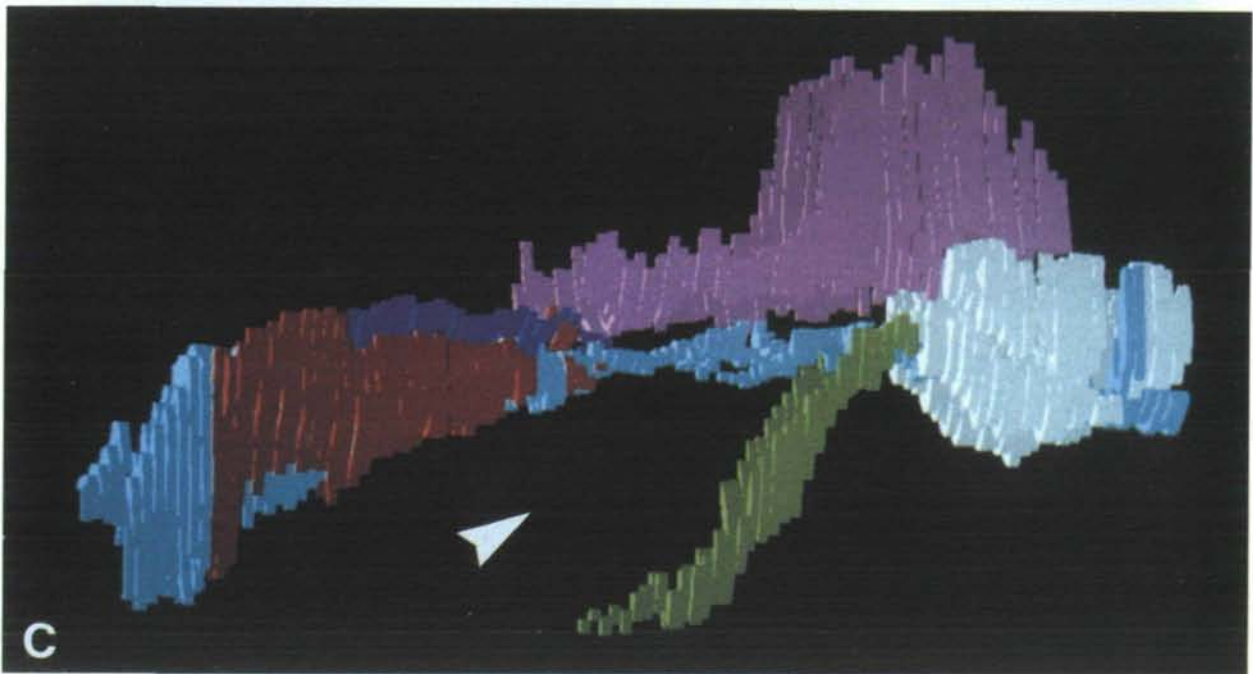
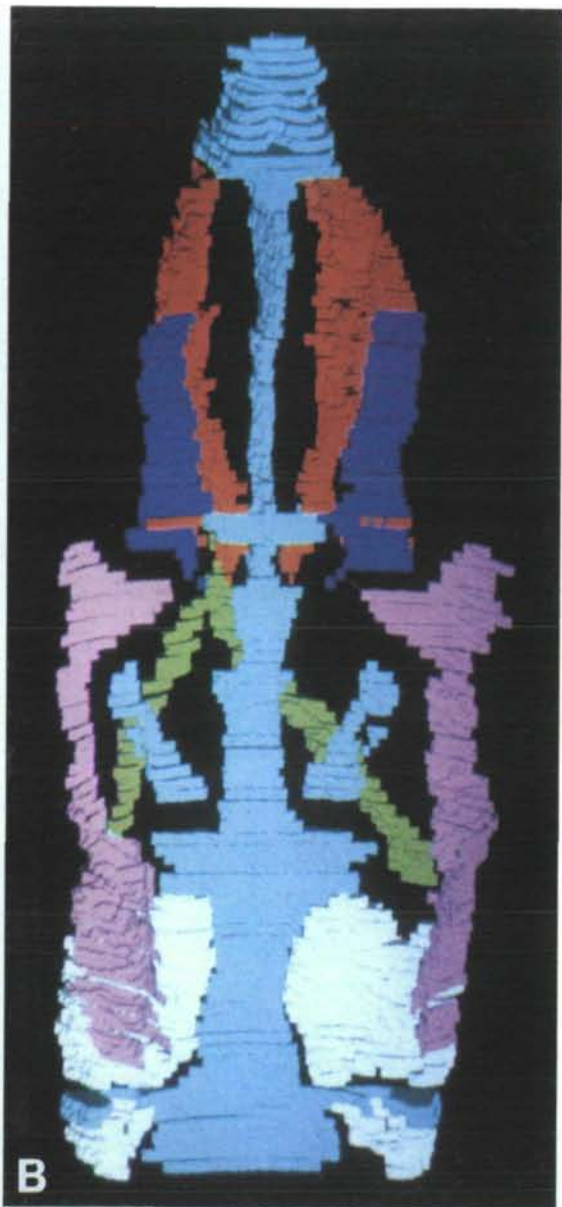
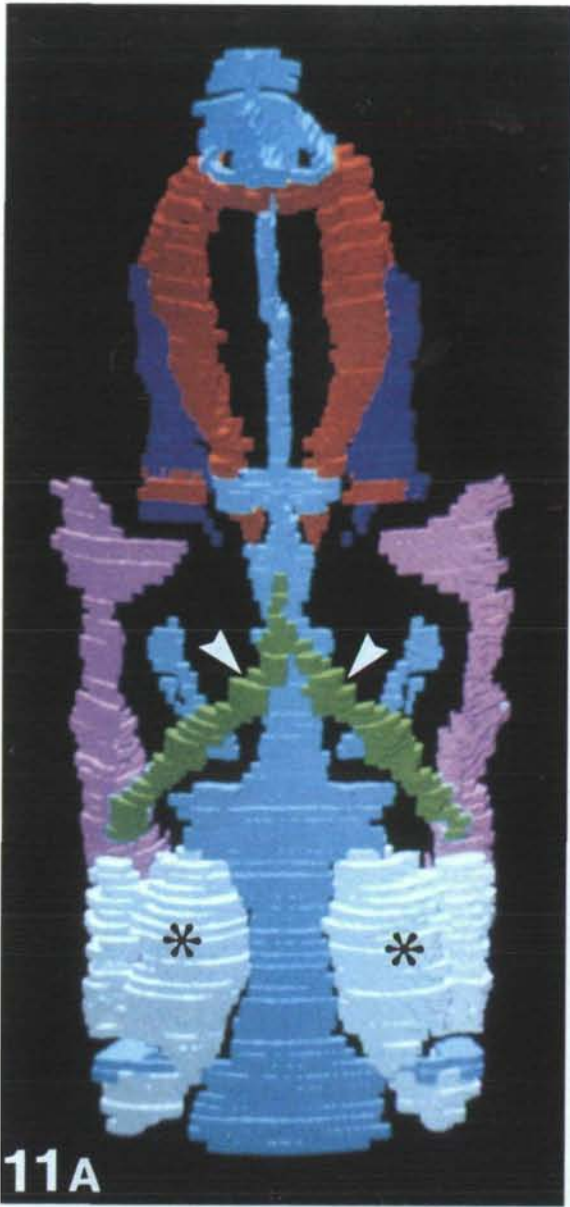
It should also be considered that, if a signalling phenomenon is occurring, then the specificity may not necessarily be a property of the signal itself but may well reside in the responding tissue. Potentially the mesenchymal partner could determine the potential for response and will, by virtue of its developmental history, determine the *nature* of that response. Different mesenchymes might respond in different ways, forming cartilage at certain sites but expressing other differentiative responses elsewhere. The presence of

Anchorin CII on a subpopulation of (avian) mesenchyme cells, or the presence of a mammalian homologue from the Lipocortin/Calpactin family, may constitute the potential for a specific response by certain cells to such matrix-mediated signals (see Introduction).

In conclusion, two aspects of these results must be emphasized. First, there is an extraordinarily precise spatial and temporal correlation between the sites and timing of type II collagen localization during early



**Fig. 10.** Three-dimensional reconstructions of E10 (A,B) and E12 (C,D) anterior neural tube and associated sense organ primordia, with the distribution of type II collagen displayed as yellow beads (B,D). In all figures, the right lateral aspect of the forming brain is seen; dorsal, left; ventral, right and coronal from top. (A,B) E10 without and with type II collagen, respectively. Note distribution of the collagen over the surface of the optic vesicle, otocyst, notochord and on the basolateral aspects of the rhombencephalon. (part of the trunk neural tube – asterisk, and notochord is seen on the right, and with a reversed dorsoventral orientation because of embryonic flexure; type II collagen around the trunk notochord is not shown here). (C,D) E12 without and with type II collagen, respectively. The pattern of distribution seen previously has extended to include the rostrally located olfactory primordia. For the optic cup, localization has become confined to its interior and hence is only glimpsed through the margin of the forming iris (arrowhead). Note that most of the type II collagen is distributed in two major locations, at forebrain and hindbrain axial levels. At later stages, this epithelially associated distribution will be lost and type II collagen will be found almost exclusively within differentiated cartilage. (Trunk structures are not seen in this reconstruction) (op, optic cup; ot, otocyst; n, notochord; ol, olfactory primodium).



**Fig. 11.** Three-dimensional reconstructions of E16 chondrocranium with anatomically adjacent cartilages grouped together and colour-coded for clarity (see below). (A) Ventral perspective showing clearly the V-shaped Meckel's cartilage (arrowheads) and the underside of the otic capsules (asterisks). (B) Dorsal perspective looking down onto the basicranium/basal plate, the aspect upon which the brain would lie; Meckel's cartilage is now obscured. (C) Left lateral perspective, rostral to left, showing Meckel's cartilage dropping away ventrally as it projects rostrally, and defines the gape of the mouth (arrowhead). The supraoccipital arches project dorsally and would enclose the lateral aspects of the mid- and hindbrain. Note how C, particularly, illustrates that the major parts of the chondrocranium (excepting the viscerocranial Meckel's cartilage) are virtually in two halves, anterior/rostral and posterior/caudal, connected only by the rostral extensions of the supraoccipital cartilages and by the parachordals, at the midbrain axial level. (*light blue*: from rostral to caudal comprises the periosteal cartilage, the medial nasal septum, the paired lamina ascendens alae temporalis and the fused parachordals or basal plate; *red*: the paranasal cartilages and sphenothmoid commissures; *dark blue*: the paired alae orbitalis; *lilac*: the supraoccipital cartilages; *white*: the paired otic capsules; *green*: Meckel's cartilage).

craniofacial morphogenesis, the tissue interactions that generate the chondrocranium, and the form of the chondrocranium itself. It is surprising that this relationship remains evident given the extensive redistribution of epithelia and mesenchyme that takes place during development of the head and face (Noden, 1988; Couly and Le Douarin, 1990). Second, the existence of such a relationship not only in the mammal, but also in Aves (Thorogood *et al.* 1986), Amphibia (Hanken, personal communication) and in teleosts (Amanze and Thorogood, unpublished data) suggests a common and evolutionarily conserved developmental role for this molecule in early development. If epithelium-to-mesenchyme signalling does take place, as proposed here, then the 'contents' (i.e. the forming brain and sense organs) induce the 'container' (the cartilaginous neurocranium) and this mechanism is both phylogenetically ancient and fundamentally important in determining the shape of the bony skull that subsequently forms. Further experimental analysis, particularly of the putative signal transduction event, will reveal whether or not such a unifying approach is justified.

We are grateful to Jim Bee and Clementine Hoffman for their comments on the manuscript, and to Patrick Tam and Kathy Cheah for communicating their unpublished results to us. Photographic assistance was provided by Heather Caldwell and technical assistance with TEM by Trevor Courtenay. The work was supported by a grant from the Wellcome Trust to P.T.

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(Accepted 13 December 1990)