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Independent deposition of collagen types II and IX at epithelial–mesenchymal interfaces

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Summary

Previous studies have demonstrated the presence of type II collagen (in mature chickens predominantly a 'cartilage-specific' collagen) in a variety of embryonic extracellular matrices that separate epithelia from mesenchyme. In an immunohistochemical study using collagen type-specific monoclonal antibodies, we asked whether type IX collagen, another 'cartilage-specific' collagen, is coexpressed along with type II at such interfaces. We confirmed that, in the matrix underlying a variety of cranial ectodermal derivatives and along the ventrolateral surfaces of neuroepithelia, type II collagen is codistributed with collagen types I and IV. Type IX collagen, however, was undetectable at those sites. We observed immunoreactivity for type IX collagen only within the notochordal sheath, where it first appeared at a later stage than did collagen types I and II. We also observed type II collagen (without type IX) beneath the dorsolateral ectoderm at stage 16; this correlates with the period during which limb ectoderm has been reported to induce the mesoderm to become chondrogenic. Finally, in older hind limbs we observed subepithelial type II collagen that was not associated with subsequent chondrogenesis, but appeared to parallel the formation of feathers and scales in the developing limb. These observations suggest that the deposition of collagen types II and IX into interfacial matrices is regulated independently, and that induction of mesenchymal chondrogenesis by such matrices does not involve type IX collagen. Subepithelial type IX collagen deposition, on the other hand, correlates with the assembly of a thick multilaminar fibrillar matrix, as present in the notochordal sheath and, as shown previously, in the corneal primary stroma.

Key words: type IX collagen, type II collagen, subepithelial extracellular matrices, monoclonal antibodies, immunohistochemistry, chondrogenesis.

Introduction

In mature chickens, type II collagen has been found only in cartilages, intervertebral disks and the vitreous. In embryos, however, the distribution of type II collagen is much wider, the molecule having been identified as a component of a variety of diverse structures such as the developing cornea (Linsenmayer *et al.* 1977; von der Mark *et al.* 1977), notochord (Linsenmayer *et al.* 1973; Miller & Mathews, 1974; von der Mark *et al.* 1976; Oettinger *et al.* 1985) and neural retina (Newsome *et al.* 1976; von der Mark *et al.* 1977).

More recently, Thorogood *et al.* (1986) observed immunochemically identifiable type II collagen codistributed with collagen types I and IV at the epithelial–mesenchymal interfaces of a number of cephalic organ primordia such as the otic vesicle, the presumptive pigmented epithelium of the optic cup and the ventrolateral neuroepithelium. These interfaces coincide spatially with the later formation of cartilage by the adjacent mesenchyme of neural crest origin. Experiments on a variety of systems have demonstrated an influence of the epithelium on subsequent mesenchymal chondrogenesis (e.g. Holtzer & Detweiler, 1953; Lash, 1968; Benoit & Schowing, 1970; Newsome, 1972; Stewart & McCallion, 1975; Gumpel-Pinot, 1980; McPhee & Van de Water, 1986); further studies on the developing retinal pigmented epithelium (Newsome, 1976) and notochord (Strudel, 1971; Kosher *et al.* 1973; Kosher & Lash, 1975) have implicated the interfacial extracellular matrix as the inductive component. Since the presence in at least some of the cranial interfaces of type II collagen correlated with the period during which the chondrogenic phenotype becomes determined, it has been suggested that this collagen type is involved in the inductive process (Thorogood *et al.* 1986).

Inconsistencies, however, do exist. The developing cornea, for example, initially deposits a subepithelial matrix containing type II collagen (as well as type I), which is then invaded by periocular mesenchymal cells derived also from the cranial neural crest. These cells do not undergo chondrogenesis; rather, they secrete a fibrillar matrix that is unique to the cornea, both in its composition (Anseth, 1961; Ikeda *et al.* 1975; Zak & Linsenmayer, 1983) and morphology (Hay & Revel, 1969).

This diversity of developmental fates of mesenchyme within or adjacent to the various subepithelial matrices must derive from differences in the cell populations and/or the matrices with which they interact. One possible source of variation of the matrices could be the presence or absence of other 'cartilage-specific' molecules which might be coexpressed with type II collagen, such as proteoglycans (e.g. Kosher & Lash, 1975) or other collagen types. Recently Svoboda *et al.* (1988) have demonstrated that early embryonic chicken corneas contain type IX collagen, one of the 'cartilage-specific collagens' (Reese & Mayne, 1981; van der Rest *et al.* 1985; van der Rest & Mayne, 1987). This molecule is found along the surface of type II collagen fibrils from cartilage, with its *N*-terminal domain extending away from the fibrils and potentially interacting with the surrounding matrix (Muller-Glauser *et al.* 1986; van der Rest & Mayne, 1988; Vasios *et al.* 1988; Vaughan *et al.* 1988). In addition, type IX collagen is a proteoglycan (PG-Lt, see Noro *et al.* 1983; Vaughan *et al.* 1985), having a covalently bound chondroitin/dermatan sulphate side chain (Bruckner *et al.* 1985; Huber *et al.* 1986; Konomi *et al.* 1986) which possibly could interact with matrix components or cells.

To ascertain whether developing epithelial–mesenchymal interfacial matrices contain both of these 'cartilage-specific' collagens, we have used monoclonal antibodies against collagen types II and IX (as well as types I, IV and X) to probe sections of staged chicken embryos using immunofluorescence histochemistry, and asked the following questions: (1) do all sites at which type II collagen is deposited also contain type IX, and is their deposition coordinate or independent?; (2) is the presence or absence of type IX collagen in such matrices correlated with subsequent chondrogenesis in the adjacent mesenchyme?; (3) are any morphogenetic events or morphological characteristics related to the deposition and/or removal of subepithelial type IX collagen?

In an earlier report (Fitch *et al.* 1988), we described our observations on the developing cornea and neural retina. We found both ocular tissues to express immunoreactive type IX collagen, but in different temporospatial patterns. In the cornea, type IX collagen is a transitory component of the primary stroma and appears to be deposited independently of type II collagen. The neural retina, on the other hand, appears to deposit coordinately types II and IX into the early vitreous, where both collagens accumulate progressively during development.

Here we report our observations of interfacial matrices that invest a variety of epithelial derivatives of cranial ectoderm and neural epithelium, as well as the notochordal sheath and the subepithelial zone of the developing hindlimb. For comparison, we also describe chondrogenic mesenchyme at a variety of sites.

Materials and methods

Tissue

White Leghorn chicken eggs were obtained from Spafas (Norwich, CT) and incubated for 2–7 days at 38°C. Embryos were removed, rinsed in Hanks balanced saline solution and staged according to Hamburger & Hamilton (1951). Whole embryos of stage 14–28, or isolated limbs or vertebrae of 7–15 days of incubation, were then soaked in 7–8% sucrose in phosphate-buffered saline (PBS) for 5–10 min, embedded in OCT (Miles Laboratories, Elkhart, IN) and frozen in liquid nitrogen. The blocks were stored at −20°C until used. Some embryos were fixed lightly (2–4% paraformaldehyde in PBS, 2–5 min) before being immersed in sucrose. No differences were seen between unfixed and lightly fixed material. 8 μm-thick frozen sections were mounted on 12-spot slides (Shandon Scientific, Sewickley, PA) coated with albumin or polylysine ($M_r \sim 250\,000$; Sigma Chemical Company), dried for 2–4 h and stored at −20°C .

Antibodies

The production and characterization of type-specific monoclonal antibodies against collagen types I (I1B6, Linsenmayer *et al.* 1979; BA1, Linsenmayer *et al.* 1986), II (II6B3, Linsenmayer & Hendrix, 1980; 2B1, described in Fitch *et al.* 1988), IV (IA8, Fitch *et al.* 1982; IIB12 and ID2, Mayne *et al.* 1983), IX (2C2 and 4D6, Irwin *et al.* 1985) and X (AC9, Schmid & Linsenmayer, 1985) have been described previously. All of the antibodies used in this study have been tested for cross- reactivity to the other collagen types and are collagen type-specific; different antibodies within each type-specific group recognize different epitopes on the triple helical molecule (see references cited above and unpublished observations).

All antibodies were stored at 4°C. In the experiments described here, the antibodies were used in the form of either undiluted supernatant from spent hybridoma cultures or ascites fluid diluted 1/300–1/500 with PBS.

Immunofluorescence histochemistry

The pattern of anti-collagen immunoreactivity in sectioned ocular tissues was revealed using an indirect immunofluorescence procedure described previously (Fitch *et al.* 1982). Sections of unfixed material were blocked with 0·1% BSA in PBS for 10–20 min and then covered with a drop of the primary antibody for 1–4 h at room temperature (RT) or overnight at 4°C. Sections of lightly fixed (2–4% paraformaldehyde in PBS, 5–10 min) tissue were quenched for 30–60 min with 0·14 M-Tris buffer containing 0·8% BSA and 0·12 M-glycine, pH 7·0, before their exposure to the primary antibodies. The slides were then washed thoroughly with PBS and incubated with a rhodamine-conjugated goat anti-mouse IgG second antibody (1 h, RT), washed in PBS and mounted in glycerol/PBS (95:5).

In each experiment, usually one or more of the individual monoclonal antibodies against each collagen type was used, along with a mixture of all of the antibodies in each collagen type-specific group. Thus the data from each antibody within a collagen type-specific group, recognizing different epitopes on each collagen molecule, reinforced each other; the type-specific antibody mixtures gave an enhanced immunofluorescent signal. Background immunofluorescence was assessed by the use, as a negative control, of the antibody (AC9) against type X collagen, which is specific for hypertrophic cartilage.

To enhance further the intensity of the fluorescent signal, many of the sections received two rounds of reaction with the primary and secondary antibodies. In this procedure, the second round of reaction included sequential 45-min incubations with primary and secondary antibodies. The details of this method are published elsewhere (Linsenmayer *et al.* 1988).

In some experiments, the tissue sections were first pretreated with dilute acetic acid (0·2 M-HAc, 20 min, RT) or testicular hyaluronidase (Type IV-S, Sigma; 1 mg ml^{-1}, in PBS, 30 min, 37°C) before the application of the primary antibody. These procedures have been shown to expose epitopes masked by their fibrillar organization (Linsenmayer *et al.* 1983) or by matrix glycosoaminoglycans (e.g. von der Mark *et al.* 1976).

Results

Derivatives of head ectoderm and neuroepithelium

In embryos of stage 14–25 of development, we examined several derivatives of cranial ectoderm, including the lens (described in Fitch *et al.* 1988), otocyst and nasal pits, along with the neuroepithelia and their derivatives, including the diencephalon, optic vesicle/retinal pigmented epithelium (RPE) and rhombencephalon. We observed collagen types I, II and IV codistributed in a subepithelial location, consistent with the results of Thorogood *et al.* (1986). In none of these tissues, however, did we observe immunodetectable type IX collagen. These results are illustrated for the stage-16/-17 optic vesicle/RPE/diencephalon (Fig. 1) and the stage-20/-21 nasal pits (Fig. 2), which were not described by Thorogood *et al.* (1986).

In the eye by stage 14 (not shown), we found type-II-collagen-specific immunoreactivity along with that for collagen types I and IV investing the basal surfaces of the optic vesicle and adjacent diencephalon. At stage 16/17, the type-II-collagen-specific immunofluorescence was most intense (Fig. 1B), being found along with types I (not shown) and IV (Fig. 1A) at the interfaces separating the diencephalic neuroepithelium (*ne*, Fig. 1A; large arrows) and the pigmented epithelium of the optic cup (*oc*, Fig. 1A; small arrows) from the subjacent head mesenchyme. Like Thorogood *et al.* (1986), we found the signal for type II collagen to decline selectively from stage 18 onward (not shown). In contrast, none of these sites showed reactivity for collagen types IX (Fig. 1C) or X (negative control, Fig. 1D) at any developmental stage examined. However, in the scleral chondrogenic mesenchyme that subsequently forms around the optic cup, type IX collagen is deposited along with type II beginning at stage 29–30 (not shown).

The invaginating nasal pits at stage 20/21, like the lens and otic vesicles at similar stages of their development (not shown), were lined on their basal surfaces with a matrix that contained collagen types I (Fig. 2A), II (Fig. 2B) and IV (not shown), but not type IX (Fig. 2C).

Our attempts to expose type IX collagen in these sections (and others in which type IX collagen did not codistribute with type II; see below), using our standard procedures for unmasking collagenous epitopes *in situ*, did not unmask any type IX-specific immunoreactivity at such sites.

Notochord/spinal cord

Transverse sections of various axial levels from stage-16 to -40 embryos were observed for the presence of immunoreactive collagens at the surface of the spinal cord and notochord and in the vertebral chondrogenic mesenchyme. At stage 16 (Fig. 3), sections through the midtrunk level (Fig. 3A–D) revealed strong reactivity in the notochordal sheath (large arrow, Fig. 3A) and spinal cord surface (small arrow, Fig. 3A) for collagen types I (Fig. 3A), II (Fig. 3B) and IV (Fig. 3C), but not for type IX (Fig. 3D). Sections through more rostral levels (about 2 mm rostrally – see inset, Fig. 3D), however, clearly revealed type-IX-collagen-specific immunoreactivity in the notochordal sheath (arrows, inset, Fig. 3D), but not around the spinal cord surface. At the most caudal levels (just rostral to the tail bud, Fig. 3E–H) we observed strong reactivity for type IV collagen (Fig. 3G), weak (or sometimes no) reactivity for collagen types I (Fig. 3E) and II (Fig. 3F) and nothing for type IX (Fig. 3H).

At hindlimb levels, where type II collagen was first detected at stage 16, type IX collagen first appeared in the notochordal sheath at stage 20 (not shown), and was detected at all developmental stages thereafter (e.g. stage 26, Fig. 4B; stage 28, Fig. 4D; other stages not shown). In the perinotochordal mesenchyme (sclerotome) at hindlimb levels, type II collagen-specific immunoreactivity was first detectable at stage 26 (arrows, Fig. 4A); similar sections reacted for type IX also showed faint, but clear-cut, immunoreactivity in the chondrogenic mesenchyme (arrows, Fig. 4B). By stage 28, expression of both collagen types in this mesenchyme was widespread (Fig. 4C and D).

Our observations on the circumferential notochordal sheath and spinal cord are summarized as follows. (a) For any collagen type, reactivity in the notochordal sheath and along the ventrolateral surface of the spinal cord first appears in a rostral–caudal temporospatial gradient that parallels the rostral–caudal pattern of development of these structures. (b) At any axial level, immunoreactivity for type IV collagen appears first, followed by that for collagen types I and II. (c) Type IX collagen subsequently becomes detectable in the notochordal sheath several stages after collagen types I and II, but is never found along the spinal cord surface. (d) Type II collagen is gradually lost from the ventrolateral surface of the spinal cord, but persists in the notochordal sheath along with collagen types IX, I and IV as late as 14 days of development (stage 40). (e) In the surrounding vertebral chondrogenic mesenchyme, type IX collagen appears simultaneously with type II.

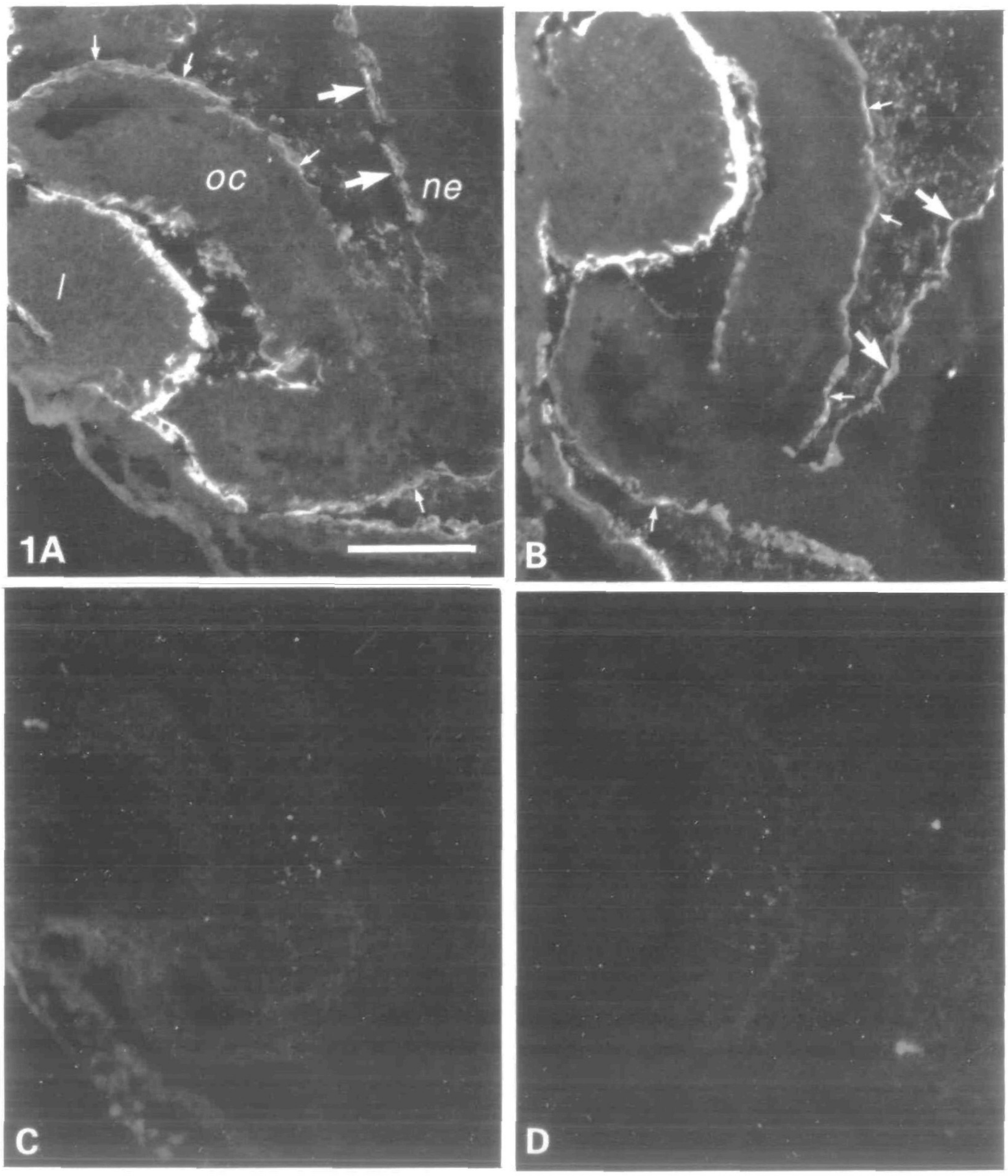

Fig. 1. Sections of stage-16/-17 embryonic chicken eyes reacted with antibodies against collagen types II (A), IV (B), IX (C), or X (negative control, D). Bar in A = 100 μm. The antibodies against type II (A) and IV (B) collagen reacted with the basal surfaces of the neuroepithelium (*ne*; large arrows) and the retinal pigmented epithelium of the optic cup (*oc*; small arrows). These collagens can also be seen codistributed along the basal surfaces of the invaginating lens vesicle (*l*), neural retinal epithelium, and presumptive corneal epithelium (described in Fitch *et al.* 1988). Immunoreactivity for collagen types IX (C) and X (D, negative control) was not detectable.

Hindlimb

In transverse sections through stage-16 embryos at the presumptive hindlimb level, we observed subectodermal immunoreactivity for collagen types I (Fig. 5A), II (arrows, Fig. 5B) and IV (not shown), but not for type IX (Fig. 5C). These collagens formed an apparently continuous sheet from the midline of the embryo laterally, separating the ectoderm from the somitic tissue medially and the somatic mesoderm laterally. Sections from more rostral levels did not appear to contain type II collagen at this flank site (not shown). With outgrowth of the hindlimb, easily visible at stage 18 (Fig. 5D–F), type I collagen persisted throughout the subepithelial zone (Fig. 5D) while clear-cut reactivity for type II was only in the midline region (not shown) and beneath the ectodermal pocket below the limb bud (termed the 'ventral sulcus'; arrows, Fig. 5E). Moving from these sites laterally along the limb bud, type II collagen-specific immunoreactivity faded to essentially undetectable levels. Specific antibody binding for type IX collagen was undetectable at these sites (Fig. 5F). The limb epithelium at this and later stages

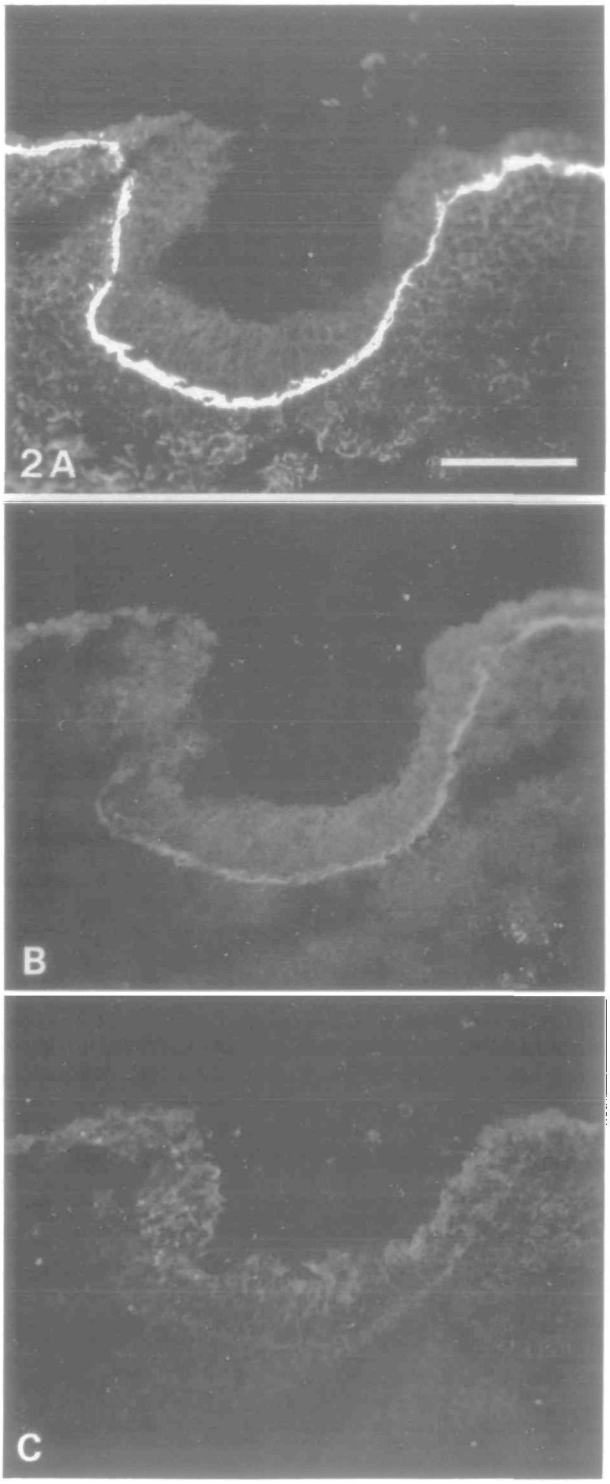

Fig. 2. Stage-20/-21 nasal pits, reacted with antibodies against collagen types I (A), II (B), or IX (C). Bar in A = 100 μm. The subepithelial matrix beneath the nasal pit early in the process of invagination contains immunoreactive type I (A) and II (B) collagens, but not collagen type IX (C).

frequently elicited nonspecific binding (i.e. comparable in intensity to the negative control, not shown) of all of these antibodies (especially evident in Fig. 5D–F and Fig. 6B,C).

Over the ensuing four days, encompassing rapid growth of the hind limb, strong subepithelial immunoreactivity for type I collagen persisted, but reactivity for type II collagen was either very faint or undetectable (not shown). At stage 31 (about 7½ days), however, we observed a reappearance of a strong reaction for type II collagen (Fig. 6B), along with type I (Fig. 6A), in the subepithelial zone of a small restricted region of the proximal hindlimb at the level of the femur. Type II collagen-specific immunoreactivity within this region appeared to be more diffuse than at earlier time points, being deposited well beneath the epithelial basement membrane zone (Fig. 6B) but, unlike type I collagen (Fig. 6A), was not observed in the deeper dermis. The subsequent growth of feather germs in this region was accompanied by a selective loss by 10 days of type II collagen at the base of each feather filament and its retention in interplumar loci (Fig. 6E; compare to Fig. 6D, showing type I collagen beneath and within each feather filament). Type IX collagen was absent from these subepithelial sites throughout the developmental period studied (e.g. Fig. 6C and F). This subepithelial anti-type II collagen immunoreactivity spread distally (and possibly somewhat proximally) with time, so that by 14–15 days of incubation, subepithelial type II collagen could be identified in the scale-forming regions at tibial and metatarsal levels; here again, subepithelial type II collagen was restricted to intervening regions between scales (not shown). At these later stages, the distal spread of subepithelial type II collagen was accompanied by a loss of such reactivity proximally.

As in the developing scleral and vertebral cartilage, the first appearance of type II collagen in the chondrogenic mesenchymal core (at about stage 25; not shown) of the hindlimb was accompanied by that of collagen type IX.

Discussion

In this investigation, we examined collagen deposition at the interfaces that separate certain epithelia from underlying mesenchyme (or mesoderm). In most of the structures described in this report, the subjacent mesenchyme or mesoderm eventually develops into cartilage. There is experimental evidence in some of these cases for an interaction between the interfacial matrix and the adjacent mesenchyme/mesoderm that is essential for the subsequent expression of the chondrogenic phenotype (retinal pigmented epithelium, see Newsome, 1976; notochord, see Strudel, 1971; Kosher *et al.* 1973; Kosher & Lash, 1975). We have confirmed the presence of type II collagen (a 'cartilage specific' collagen in mature chickens) along the basal surfaces of neuroepithelia and a neuroepithelial derivative (the optic cup), invaginating cranial ectoderm and in the notochordal sheath, all of which become invested with cartilage later in development. We also observed type II collagen beneath the presumptive hindlimb ectoderm at a time (stage 16) when the adjacent mesoderm is

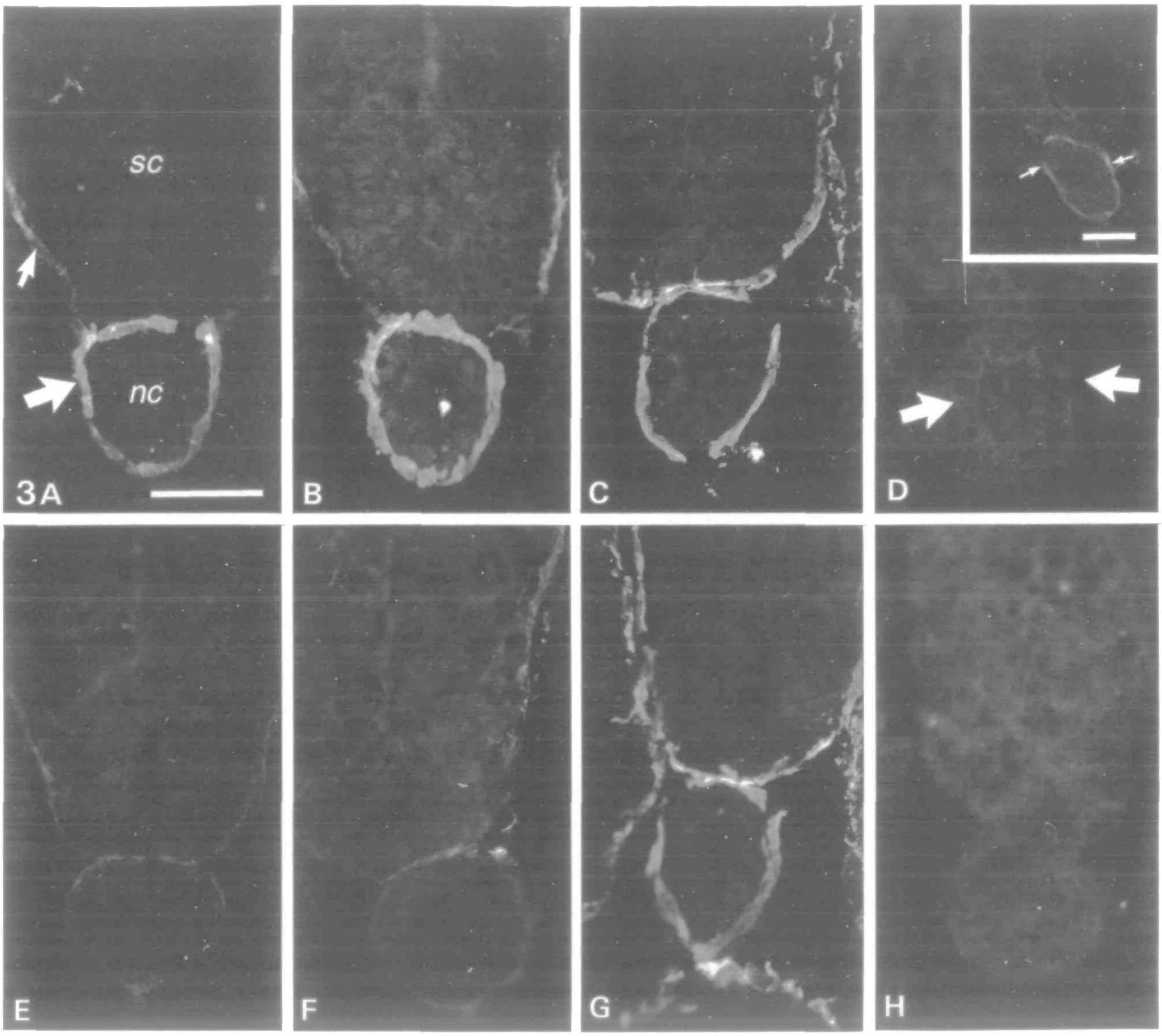

Fig. 3. Transverse sections of ventral spinal cord (*sc*) and notochord (*nc*) from various axial levels of stage-16 embryos, reacted for collagen types I (A,E), II (B,F), IV (C,G), or IX (D,H). Bars in A and D (inset) = 50 μm. Sections shown in A–D are rostral to the presumptive hind limb (i.e. approximately midthoracic level), where the ventrolateral surface of the spinal cord (small arrow in A) and the perinotochordal sheath (large arrows in A and D) contained immunoreactive collagen types I (A), II (B) and IV (C), but not IX (D). At still more rostral levels, however, (inset in D, cervical spinal cord) immunoreactive type IX collagen was visible in the notochordal sheath (arrows) but not along the spinal cord surface. In E–H, sections caudal to the presumptive hind limb are shown. At this level, antibodies against type IV collagen (G) showed a strong reaction with the notochordal sheath and the surface of the neural tube, while immunoreactivity for collagen types I (A) and II (B) was only faintly detectable, and that for type IX collagen (H) was absent.

undergoing an interaction that leads to its subsequent condensation and differentiation as the cartilagenous core of the avian limb (Gumpel-Pinot, 1980)[1]. However, we also observed subepithelial type II collagen at a variety of other sites which, like the embryonic avian cornea, are not associated with cartilage formation, such as the 7- to 15-day hindlimb (this study) and the mesonephros (unpublished observations; Robert Kosher and Michael Solursh, submitted for publication and personal communication).

When these tissues were probed for the presence of type IX collagen (another 'cartilage-specific' type) it was detectable only in the notochordal sheath and in chondrogenic mesenchyme. In the notochord, type IX collagen first appeared several stages later than did collagen types I and II, whereas in chondrogenic mesenchyme these collagens became detectable at the same time.

These results suggest the following. (a) The ex-

[1] Although sections from more rostral levels did not appear to contain type II collagen at this site, we did not determine whether type II collagen might have been present there earlier in development.

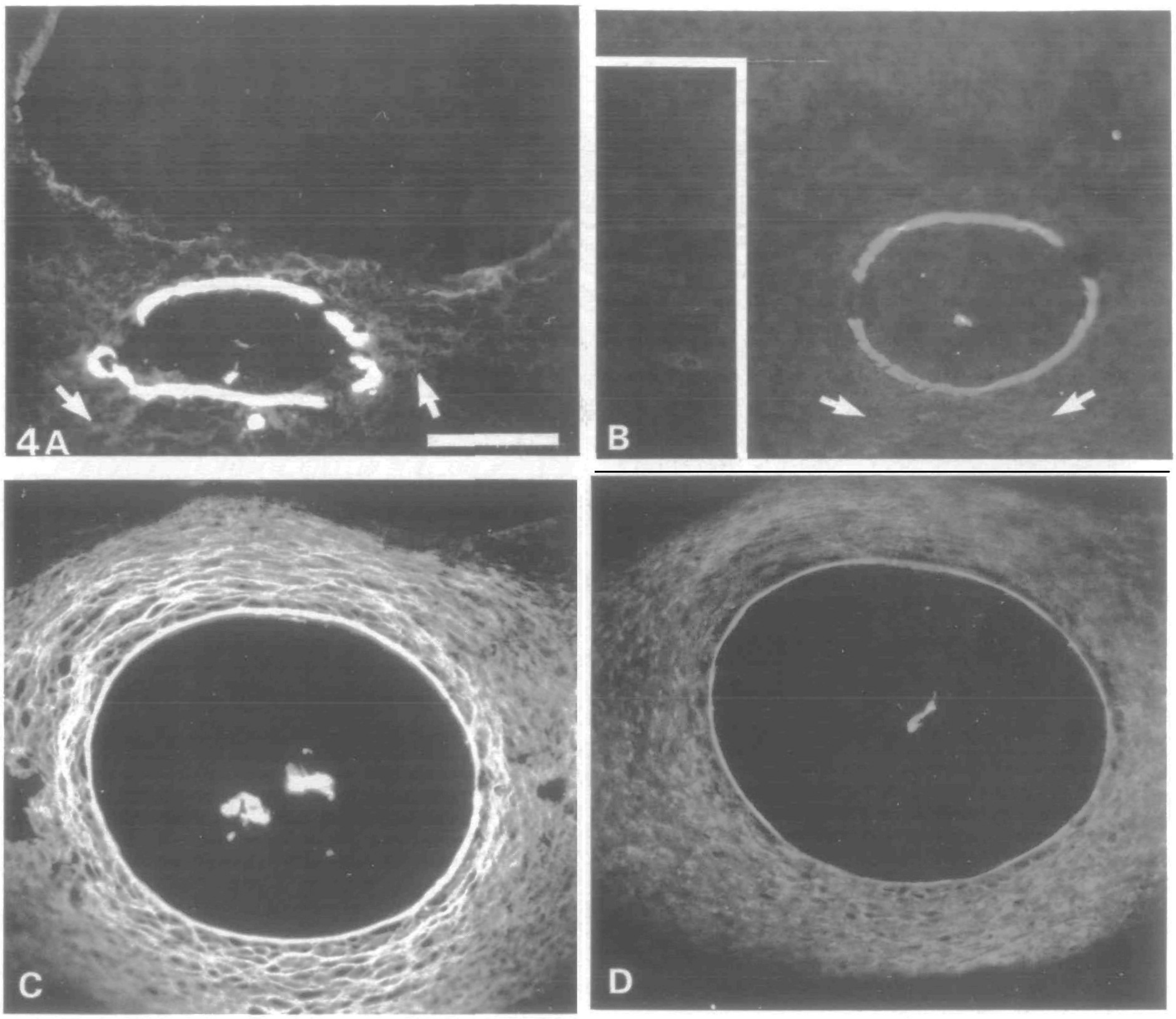

Fig. 4. Sections of stage-26 (A,B) and stage-28 (C,D) embryos at the level of the hind limb, reacted for collagen types II (A,C), IX (B,D) or X (negative control; inset in B). Bar in A = 100 μm. At stage 26, type II collagen (A) became detectable in the perinotochordal chondrogenic mesenchyme (arrows in A) as well as in the notochordal sheath and along the ventrolateral surface of the spinal cord. The notochordal sheath was also reactive for type IX collagen (B), and faint, but clear cut, immunoreactivity for this collagen in the chondrogenic mesenchyme was also observed (B, arrows; compare to absence of type X collagen-specific immunoreactivity, inset). By stage 28, codistributed type II (C) and IX (D) collagens were seen throughout the chondrogenic vertebral mesenchymal condensation as well as in the notochordal sheath.

pression of the 'cartilage collagen' types II and IX (presumably by embryonic epithelia: see Dodson & Hay, 1971; Trelstad *et al.* 1973; Linsenmayer *et al.* 1973, 1977; Smith *et al.* 1976) need not be coordinately linked. (b) Type IX collagen is not deposited at most interfaces where cartilage subsequently forms, and therefore does not appear to be necessary for the inductive process to occur. (c) While the presence of type II collagen at the sites of matrix-mediated induction of chondrogenesis is consistent with its possible involvement in the process, the presence of this molecule at nonchondrogenic sites as well indicates that it is clearly not sufficient: other matrix components and/or a predetermined responsive cell population (e.g. see Benoit & Schowing, 1970) must also be required.

Since we have observed subepithelial type IX collagen only in the notochordal sheath and the primary corneal stroma, we can exclude an inductive role for this collagen. However, these two type IX-containing matrices do share certain structural features that do not apply to the type-II-collagen-containing matrices that lack type IX. Both the primary corneal stroma (Hay & Revel, 1969) and the mature (defined here as post-stage 20) notochordal sheath (e.g. Bancroft & Bellairs, 1976; unpublished observations) are somewhat dense acellular matrices composed of multiple layers of fibrillar material. While the organization of corneal fibrils appears to be more precise than that of the notochordal sheath, the fibrils in the mature notochordal sheath do seem to be arranged in a largely circumferential orientation (unpublished observations). Ruggeri, in his description of notochordal fine structure (1972), found the early notochordal sheath to be rather thin and loosely organized, with extracellular material appearing to

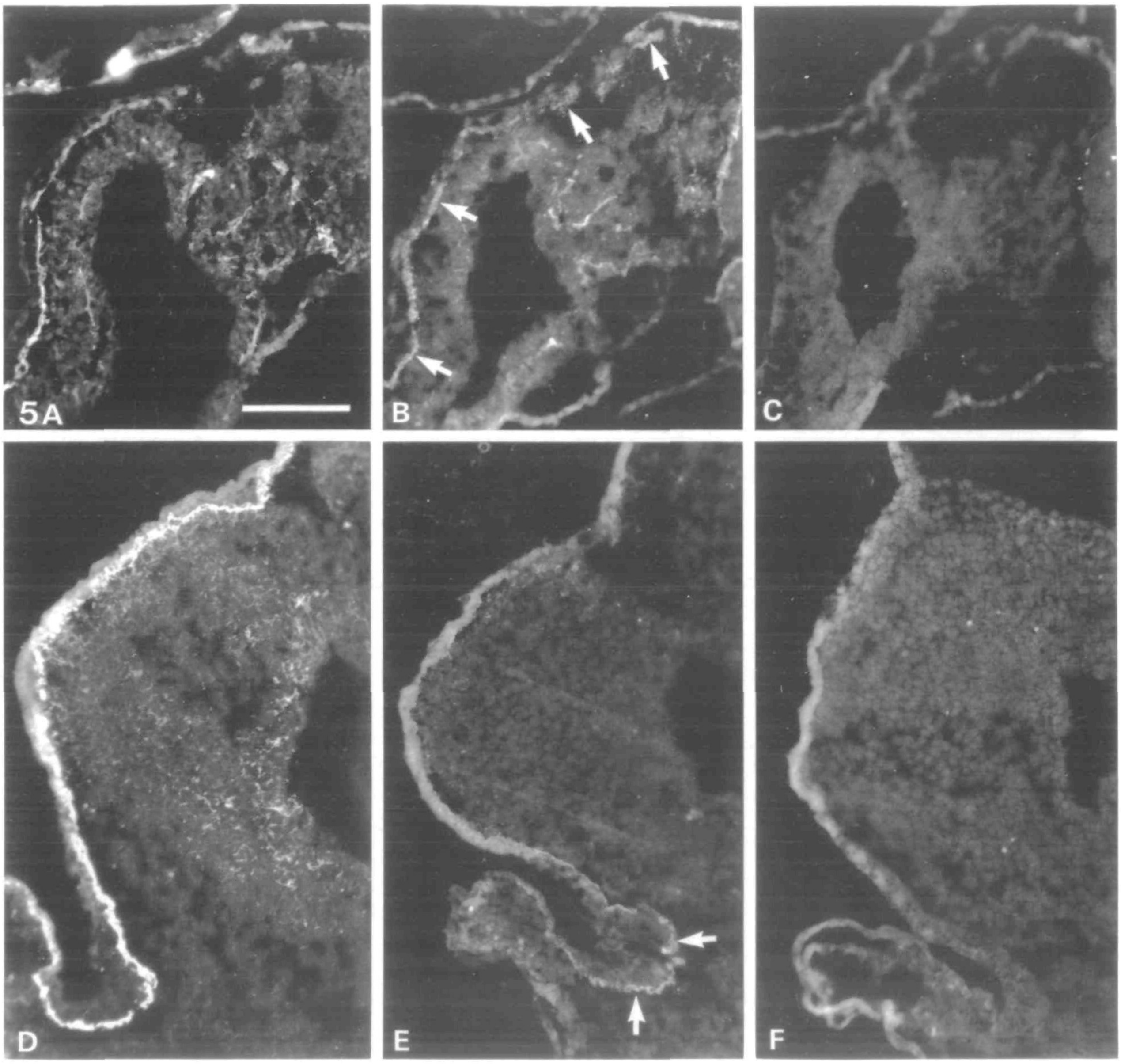

Fig. 5. Stage-16 (A–C) and stage-18 (D–F) embryos sectioned transversely at the level of the hind limb, and reacted with antibodies against collagen types I (A,D), II (B,E), and IX (C,F). Bar in A = 100 μm. At stage 16 at the level of the presumptive hind limb, immunoreactivity for type I (A) and II (B) collagens was found codistributed beneath the ectodermal epithelium (arrows in B). Immunoreactivity for type IX collagen (C) was undetectable. By stage 18, when limb outgrowth is well under way, subectodermal type I collagen (D) remained abundant, while type II collagen (E) was found associated only with the 'ventral sulcus' (E, arrows); in the limb, subepithelial type II collagen was considerably reduced or absent. Type IX collagen (F) remained negative.

diffuse away from the notochordal surface. Likewise, Bancroft & Bellairs (1976) noted that, in young embryos, the perinotochordal matrix closely resembled that associated with the basal surface of the spinal cord, both being thin mats of randomly oriented fibrillar material (see Bancroft & Bellairs, 1976; figures 11 and 12). The present study shows that, at the early developmental stages described by these investigators, both the neural sheath and the notochordal sheath lack type IX collagen. With further development, however, only the notochordal sheath thickens substantially (our unpublished observations). This assembly of a thicker multilayered matrix correlates well with the deposition of type IX collagen within the notochordal sheath. A similar sequence also occurs in the corneal primary stroma, which from its inception contains type II collagen and, as it thickens, acquires type IX collagen (Fitch *et al.* 1988). These observations fit well with the proposed function for type IX collagen in cartilage, where it is found associated with type II collagen fibrils and may link fibrils to other matrix components (Vaughan *et al.* 1988; van der Rest & Mayne, 1988).

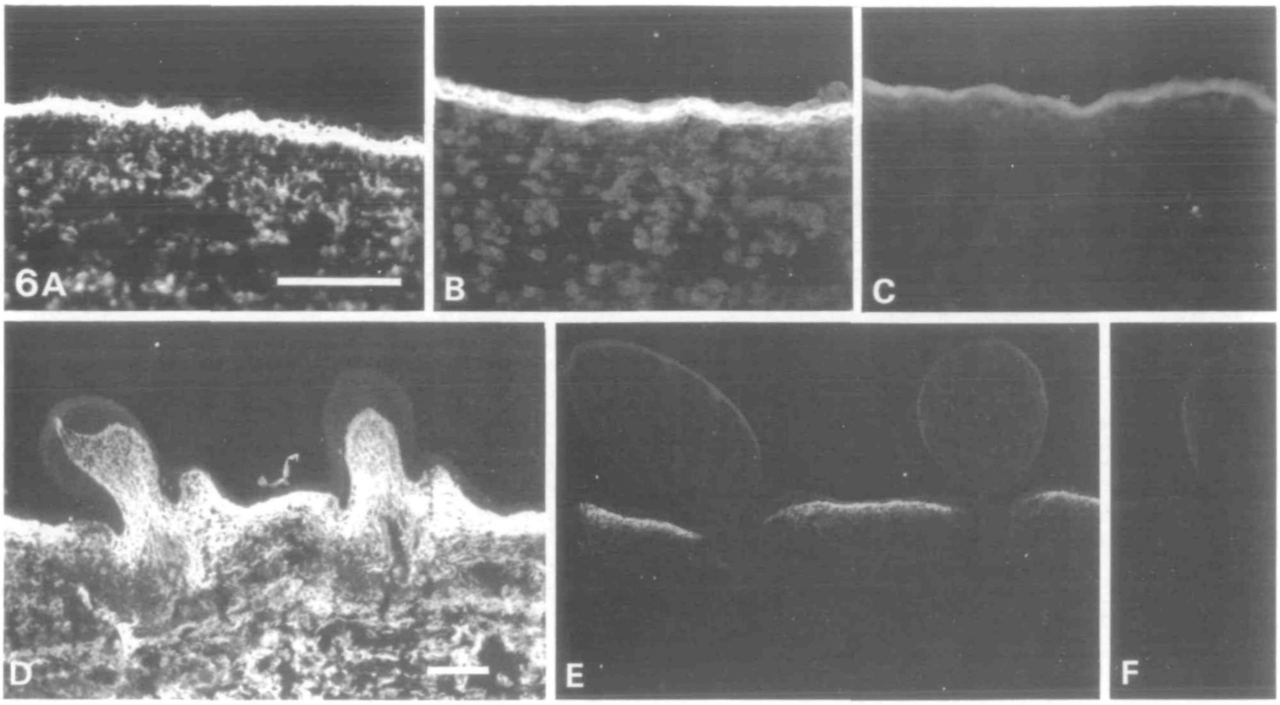

Fig. 6. Sections of 7½-day (stage 31; A–C) and 10-day (D–F) hindlimbs, reacted for collagen type I (A,D), II (B,E), and IX (C,F). Bars in A and D = 100 μm. At stage 31, a continuous subepithelial matrix contained type I collagen (A) and, in a restricted region of the proximal hindlimb, type II (B). Beneath the subepithelial zone, the underlying mesenchyme was immunoreactive for type I collagen but not for type II. These tissues did not react for type IX collagen (C). In 10-day limbs, type I collagen (D) was abundant within the feather buds, in the subepidermal matrix between them, and in the deeper tissues as well. Type II collagen (E) was restricted to the subepithelial matrix between the feather buds, and was absent at the roots of, and within, the feather buds themselves. Again, type IX collagen (F) was negative.

Work is under way to determine whether type IX collagen in the corneal primary stroma and notochordal sheath is also associated with the interstitial fibrils composed of collagen types II and I (Hendrix *et al.* 1982; Birk, Fitch & Linsenmayer, unpublished observations).

Recent studies on type IX collagen mRNA from early corneas have indicated that the corneal molecule differs substantially from that of cartilage in lacking a large noncollagenous domain at its *N*-terminus (Svoboda *et al.* 1988). It is possible that this difference in molecular structure is responsible at least in part for the different supramolecular organizations of the cartilage *versus* corneal matrices. The similarities in matrix organization of cornea and notochord suggest that they may express the same form of the type IX molecule. Experiments designed to test this hypothesis are in progress.

Lastly, in the developing hindlimb, subectodermal type II collagen is deposited early (stage 16) and then becomes greatly attenuated or absent for several days. Strong immunoreactivity for this collagen then reappears in a small region of the proximal hindlimb at stage 31 (about 7½ days) and spreads distally with time. Type II collagen is codistributed with type I collagen subepithelially but not in the deeper dermis, where type I collagen is reported to be codistributed with type III (Mauger *et al.* 1982). Type II collagen, which is initially continuous under the epithelium (at stage 31), disappears beneath the developing feather buds (and scales), but is retained as a transitory component of the intervening matrix. The subepithelial matrix, including its collagenous component, has been implicated in the establishment and maintenance of the pattern of feather and scale formation (Stuart & Moscona, 1967; Goetinck & Sekellick, 1972; Goetinck & Corlone, 1988), and our preliminary observations suggest that the spatiotemporal pattern of deposition of subepithelial type II collagen may closely parallel this pattern. Further studies on closely staged hindlimbs and in glabrous skin are warranted.

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