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Tissue-specific expression of the fibril-associated collagens XII and XIV

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SUMMARY

Interstitial collagen fibrils form the supporting scaffold of all connective tissues. The synthesis of this framework is subject to a precise spatial and temporal regulation in order to meet the mechanical needs of every tissue type. A subgroup of non-fibrillar collagens termed FACIT seems to play a role in this regulation by providing specific molecular bridges between fibrils and other matrix components. Collagens XII and XIV represent such FACIT molecules and occur preferentially in tissues containing banded type I collagen fibrils. We have used the techniques of indirect immunofluorescence and in situ hybridization to investigate the expression patterns of the two molecules during chicken embryonic development. We detected specific differences in these patterns, which may be related to the respective functions of the two proteins within the connective tissues. Collagen XIV was expressed at very few sites in the 6-day-old embryo, but occurred in virtually every collagen I-containing tissue (skeletal muscle, cardiac muscle, gizzard, tendon, periosteum, nerve) by the end of embryonic development. In contrast, collagen XII was fairly abundant in the 6-day-old embryo but was, at later stages, restricted to only a few dense connective tissue structures (bone, tendon, gizzard). Thus, our results suggest that collagen XII and collagen XIV serve different functions during embryonic development although their structures are highly similar.

Key words: collagen XII, collagen XIV, extracellular matrix, chicken embryo, immunohistochemistry, in situ hybridization

INTRODUCTION

The precise architecture of the extracellular matrix is largely responsible for the extensive diversity observed among different connective tissues. Unique combinations of collagens, proteoglycans and adhesive glycoproteins provide each tissue with specific mechanical and functional properties. Interstitial collagen fibrils build up the insoluble framework common to each matrix type (for review, see van der Rest and Garrone, 1991). These fibrils consist to a large extent of type I or II collagen molecules arranged in a staggered manner that results in a characteristic cross-striated pattern. The tissue-specific modulation of fibrillar diameter and three-dimensional array appears to be achieved by interactions with other extracellular matrix components. The existence of heterotypic fibrils containing minor interstitial collagens (types III, V, XI) in addition to the aforementioned collagen types may partly explain the wide diversity of the fibrillar framework (Keene et al., 1987; Birk et al., 1988; Mendler et al., 1989). Recently, a new class of collagens has been identified. These molecules are unable to form fibres by themselves, but are likely candidates that may control the architecture of interstitial fibrils (for reviews, see van der Rest et al., 1990; Shaw and Olsen, 1991).

The prototype of this new subfamily is collagen IX. This molecule is known to associate covalently with the surface of type II collagen fibrils (van der Rest and Mayne, 1988), thereby providing them with new functional properties. Collagen IX is composed of three distinct polypeptide chains, which consist of alternating collagenous (COL1 to COL3) and non-collagenous (NC1 to NC4) domains. Additional members of the subfamily were identified on the basis of structural similarities with collagen IX, involving in particular the COL1 domain, the NC4 domain as well as some conserved cysteine residues within the NC1 domain. Collagens XII and XIV represent such molecules. They are both homotrimers and contain two short collagenous segments (COL1, COL2) and three non-collagenous domains (NC1 to NC3). While the NC1 and NC2 domains are relatively short, the amino-terminal NC3 domain is extremely large and reveals a complex modular structure with a high potential for functional interactions (Yamagata et al., 1991; Wälchli et al., 1993; Gerecke et al., 1993; see also Fig. 1, below). A current model states that collagens XII and XIV associate with the surface of interstitial collagen fibrils via their conserved COL1 domain. In this model, the COL2 domain sticks out from the fibril and projects the large NC3 domain into the extracellular space. It is possible that these molecules act as molecular bridges among interstitial fibrils or between fibrils and other components of the extracellular matrix. Immunoelectron microscopical studies have indeed demonstrated a close association of collagens XII and XIV with striated type I collagen fibrils (Schuppan et al., 1990; Keene et al., 1991). Based on this model, the name FACIT (Fibril-Associated

Collagens with Interrupted Triple-helices) has been proposed for the new subfamily (Gordon et al., 1989). The recently described collagens XVI and Y (Pan et al., 1992; Yamaguchi et al., 1992; Yoshioka et al., 1992; Myers et al., 1993) may represent additional members of the FACIT subclass of collagens.

Collagens XII and XIV exhibit a very similar overall structure. Despite their structural resemblance, the two proteins possess slightly distinct biochemical properties as shown by differential extraction of the two collagens from biological material (Watt et al., 1992; Aubert-Foucher et al., 1992). They are both found preferentially in connective tissues that contain type I collagen fibrils. Nevertheless some differences have been reported for the distribution of the two proteins (Sugrue et al., 1989; Castagnola et al., 1992; Watt et al., 1992). In order to gain a better understanding of the specific roles of collagens XII and XIV, we set out to search systematically for differences in their expression patterns and to compare their distribution with that of collagen I. By in situ hybridization and immunohistochemistry, we observed a strict codistribution of collagens I and XIV in most tissues. Collagen XII on the other hand revealed a more restricted distribution and was found preferentially in tightly packed connective tissue structures. Furthermore, we found a striking temporal difference in the expression of the two proteins during embryonic development. Collagen XII was widely expressed in 6-day-old chicken embryos whereas collagen XIV was confined to very few locations at this early stage.

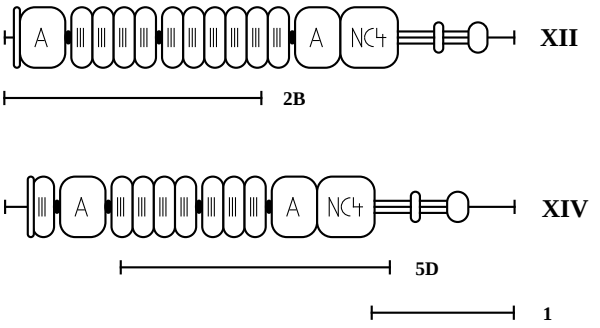


Fig. 1. Modular structures of collagen XII (short form) and collagen XIV. The positions of the cDNA clones 2B, 5D and 1 relative to the mRNAs of collagens XII and XIV are denoted by horizontal bars. The collagenous segments are shown by three horizontal lines. The modules similar to the A motifs of von Willebrand factor, to the type III repeats of fibronectin and to the NC4 domain of collagen IX are indicated. Short linker segments are shown by black ovals. The signal peptide as well as two short non-collagenous segments are not specifically marked. The untranslated regions of the mRNAs are shown by single lines.

MATERIALS AND METHODS

Immunological procedures

Antibodies

Polyclonal antibodies against chicken collagen XIV (Trueb and Trueb, 1992a) and against chicken collagen XII (Koch et al., 1992) were prepared in rabbits by standard procedures (Harlow and Lane, 1988). The antisera were purified by affinity chromatography to avoid any cross-reaction. For this purpose, two columns were utilized, one containing collagen XIV coupled to Affigel 10 (Bio-Rad, Richmond, CA), the other containing collagen XII coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden). Each antiserum was applied to the corresponding affinity column. After extensive washing with Tris-buffered saline, specifically bound antibodies were eluted with 0.1 M glycine-HCl, pH 2.5. The pH of the effluent was immediately neutralized by adding 1 M Tris-HCl, pH 8.0. In order to further eliminate any cross-contaminating antibodies, the antisera were passed through the second column. The purified antibodies were tested by enzyme-linked immunosorbent assays (ELISA) and by immunoblotting as described previously (Koch et al., 1992).

Immunohistochemistry

Tissue samples from 6-, 10- and 17-day-old chicken embryos were frozen in liquid nitrogen and cut into 5-8 μm sections. The sections were placed onto clean glass slides, allowed to dry for 2 hours at ambient temperature and fixed with acetone. After blocking unspecific sites for 5 minutes in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), the sections were incubated for 1 hour with the primary antibody diluted in 1% BSA/PBS. Unbound antibodies were washed off with PBS. Bound antibodies were detected by incubation for 30 minutes with a secondary fluorescein-labelled anti-rabbit IgG antibody (Zymed Laboratories, San Francisco, CA). After extensive washing, the tissue sections were examined with a Zeiss Axiovert microscope equipped with epifluorescence optics.

In situ hybridization

cDNA clones

cDNA probes specific for the mRNA of the α1 chains of collagens I, XII and XIV were used for in situ hybridization. The α1(I) probe (196 bp) corresponds to the first exon of the chicken α1(I) gene and contains 114 bp of the 5′ untranslated region, 66 bp coding for the signal peptide of the procollagen chain and 16 bp coding for the first $5\ ^1/_3$ amino acids of the amino-terminal propeptide (Dietz et al., 1993). The cDNA clone for collagen XII (Fig. 1) extends from the 5′ untranslated region to the region encoding the ninth fibronectin type III repeat (FN III) of the short form of chicken collagen XII (nucleotides 1-3175; Trueb and Trueb, 1992b). Two independent cDNA clones for collagen XIV were used (Fig. 1) and both clones yielded virtually identical results. Clone 5D (3.4 kbp) encompasses nucleotides 1516 to 4959 of the chicken collagen XIV mRNA (Wälchli et al., 1993) encoding the segment located between the second FN III repeat and the triple-helical region of the polypeptide. Clone 1 (1.8 kbp) contains nucleotides 4717 to 6456 of the collagen XIV mRNA. It thus covers the sequence coding for the collagenous helix as well as the entire 3′ untranslated region. The specificity of the probes was demonstrated by northern blotting experiments (Trueb and Trueb, 1992a).

Pretreatment of sections

Cryosections were prepared as described above with the difference that they were placed on TESPA (3-aminopropyltriethoxysilane)-coated slides, allowed to dry for only 2-5 minutes and fixed in 4% paraformaldehyde/PBS for 15 minutes. The sections were subjected to in situ hybridization under sterile conditions following previously described procedures with minor modifications (Peltonen et al., 1988; Hayashi et al., 1986). The samples were incubated in 0.2 M HCl, 0.3 M NaCl for 20 minutes, rinsed twice for 2 minutes in 2× standard saline citrate (SSC) and incubated for 5 minutes at 37°C with 1 μg/ml of proteinase K (Boehringer, Mannheim) in 2 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4. The samples were treated first for 3 minutes, then for 10 minutes with 2 mg/ml glycine in PBS, rinsed with distilled water and acetylated for 10 minutes in a freshly prepared solution of 0.25% acetic anhydride in 0.1 M tri-

ethanolamine, pH 8.0. The sections were then dipped twice into distilled water, dehydrated by successive incubations in 70%, 95% and 100% ethanol and subsequently air-dried.

Hybridization

The sections were heated at 90°C for 5 minutes, cooled rapidly on ice and prehybridized for 3-16 hours at 42°C. The prehybridization solution contained 50% formamide, 0.6 M NaCl, 1 mg/ml BSA, 200 μg/ml salmon sperm DNA, 10% dextran sulfate, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10 mM dithiothreitol (DTT), 0.5 mM ethylenediamine tetraacetic acid (EDTA) and 10 mM Tris-HCl, pH 7.4. Before being spread over the pretreated sections, the prehybridization solution had been heated for 10 minutes at 95°C and cooled on ice. Hybridization was carried out overnight at 42°C using a solution of the same composition, into which 100-150 ng/ml of the ^{35}S-labelled specific cDNAs were added. These cDNAs had been labelled with ^{35}S-dCTP (Amersham, UK) by the random-primed oligolabelling method (Feinberg and Vogelstein, 1983) to a specific activity higher than 10^8 cpm/μg. The radioactive probes obtained exhibited a size of 200 to 400 nucleotides according to the manufacturer of the oligolabelling kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). Linearized pUC13 DNA was used for control hybridizations and revealed a uniform, low-density distribution of silver grains, similar to the background observed in areas devoid of any tissue.

Post-hybridization washes

After hybridization the samples were washed at room temperature twice for 5 minutes with a solution containing 0.5× SSC, 1 mM EDTA and 10 mM DTT, twice for 5 minutes with 0.5× SSC, 1 mM EDTA, and once for 10 minutes with 50% formamide containing 0.15 M NaCl, 0.5 mM EDTA, 5 mM Tris-HCl, pH 7.4. The next steps were performed in a shaking water bath at 55°C and included two washes with 0.5× SSC followed by two washes with 0.2× SSC, each for 5 minutes. After a last wash in 0.2× SSC at room temperature, the sections were dehydrated with 70% and 95% ethanol containing 0.3 M ammonium acetate, and finally air-dried.

Autoradiography

The slides were dipped into Kodak NTB-3 nuclear track emulsion melted at 42°C and diluted 1:1 with 0.6 M ammonium acetate. They were allowed to dry horizontally at room temperature for 4 hours and were exposed in a desiccant-containing box at 4°C for 5-11 days. The samples were developed with Kodak Dektol developer at 15°C for 2 minutes, rinsed shortly in 1% acetic acid and fixed with Kodak Fixer at 15°C for 10 minutes. After counterstaining with Gill's hematoxylin no. 3 for 1-2 minutes (Sigma Diagnostics, St Louis, MO) they were dehydrated and mounted with Eukitt (Gribi AG, Belp, Switzerland). Finally, the slides were examined and photographed with a Zeiss Axioplan microscope equipped with dark-field optics.

RESULTS

The tissue distribution of collagens XII and XIV was investigated at three developmental stages of chicken embryogenesis by in situ hybridization and by immunohistochemistry. Since collagens XII and XIV are believed to be associated with the surface of interstitial collagen fibrils, the resulting patterns were compared with the distribution of collagen I. In the first set of experiments, we examined well-differentiated tissues, such as muscle, long bones and peripheral nerves, of late chicken embryos (17 and 10 days of incubation). The results suggested a difference in the temporal expression of collagens XII and XIV during embryogenesis. We therefore investigated in the second step the distribution patterns found in early embryos (6 days) before the final differentiation of the individual tissues has occurred.

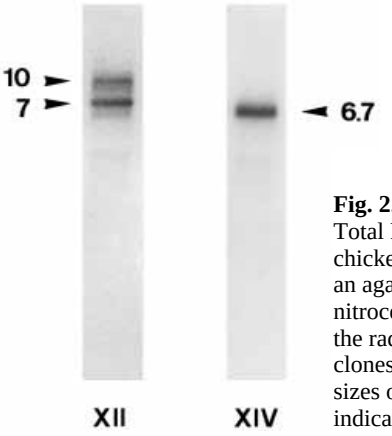


Fig. 2. Northern blot analysis. Total RNA from 17-day-old chicken embryos was resolved on an agarose gel, transferred to nitrocellulose and hybridized to the radioactively labelled cDNA clones 2B (XII) or 5D (XIV). The sizes of positive bands are indicated (in nucleotides $\times 10^{-3}$).

The specificity of the cDNA probes and of the antibodies used in this study was demonstrated by northern blotting and ELISA, respectively. On a northern blot performed with total RNA from 17-day-old chicken embryos (Fig. 2), the cDNA probe for collagen XII revealed two major mRNA species of 7000 and 10,000 nucleotides corresponding to the two splice variants described previously (Trueb and Trueb, 1992b). A probe specific for collagen XIV stained a single mRNA band of 6700 nucleotides. No cross-reactivity could be detected between the two probes. Likewise our purified antibodies against collagens XII and XIV did not display any cross-reactivity when tested by ELISA (Fig. 3). Furthermore, they recognized on immunoblots no other proteins from the culture media conditioned by skin fibroblasts (not shown).

Skeletal, cardiac and smooth muscle

The extracellular matrix of skeletal muscle can be divided into three functionally distinct layers: the epimysium, which surrounds each intact muscle, the perimysium, which delineates smaller groups of muscle fibres, and the endomysium, which is found around individual myofibres. Collagen I is known to be a major constituent of all three layers (Light and Champion, 1984). In situ hybridization with a probe for the α1(I) chain showed indeed that collagen I mRNA was highly abundant in the connective tissue septa associated with the muscle fibres as well as in the walls of small blood vessels (Fig. 4A,B). The distribution of the mRNA for collagen XIV closely followed that observed with the collagen I probe as demonstrated on sections of 17-day-old (Fig. 4C) as well as 10-day-old chicken embryos (not shown). In contrast, mRNA for collagen XII could not be detected in skeletal muscle of 17-day-old embryos with the exception of a weak signal in the wall of small blood vessels (Fig. 4E). In situ hybridization of tissue sections from 10-day-old embryos, however, demonstrated the presence of low, but distinct amounts of this mRNA species (Fig. 4G).

On the other hand, immunohistochemistry showed a nice codistribution of the two FACIT collagens in the epimysium, the perimysium, the endomysium and the wall of small blood vessels (Fig. 4D,F,H). In contrast to the observation made by in situ hybridization, collagen XII was clearly detectable at the protein level in skeletal muscle of 17-day-old embryos. Hence, the synthesis of the mRNA for this collagen seems to have

ceased by this developmental stage whereas the protein is still present within the extracellular matrix.

The connective tissue of the myocardium is organized in the same three interconnected layers as the extracellular matrix of skeletal muscle. In cardiac muscle from 17-day-old embryos, collagen I mRNA was found in the larger septa (epi- and perimysium) and in the adventitia of small muscular arteries (Fig. 5A,D). Collagen XIV was detected at both the mRNA and the protein levels at locations similar to collagen I. A difference, however, may be worth mentioning: collagen XIV was more prominent in the smaller connective tissue septa, but less abundant in the adventitia of small blood vessels (Fig. 5B,E). Interestingly, collagen XII was virtually absent from the myocardial extracellular matrix. No signal above background could be detected for this collagen species by in situ hybridization or by immunohistochemistry at the developmental stages examined (Fig. 5C,F). Nevertheless, expression of collagen XII was observed in the dense connective tissue of the cardiac valves (not shown) in accordance with earlier reports (Callahan and Sugrue, 1991).

Chicken gizzard displays a characteristic rhomboid pattern of thick connective tissue strands interspersed among the smooth muscle fibres. At the 17-day stage, these trellis-like septa showed high expression of collagens I, XII and XIV as revealed by in situ hybridization. All three mRNA species appeared to be produced mainly by the fibroblasts dispersed between the smooth muscle layers of the gizzard wall (Fig. 5G,H,I,J). Staining with antibodies against collagen XII and XIV revealed a strict codistribution of the two FACIT proteins (Fig. 5K,L), which is in contrast to the observations made above with skeletal and cardiac muscle. No significant difference was observed between 10- and 17-day-old embryos in the distribution of the three collagen types discussed in this report, although the interstitial fibres were more randomly organized at the 10-day stage. Bright staining for collagen I, XII and XIV transcripts was also observed by in situ hybridization in the closely packed fibre bundles of the thick tendinous layer that surrounds the gizzard muscular coat. Furthermore, the three mRNA species were detected in the submucosa on the inner side of the muscular coat, in the lamina propria and in the basal part of the glandular epithelium (not shown).

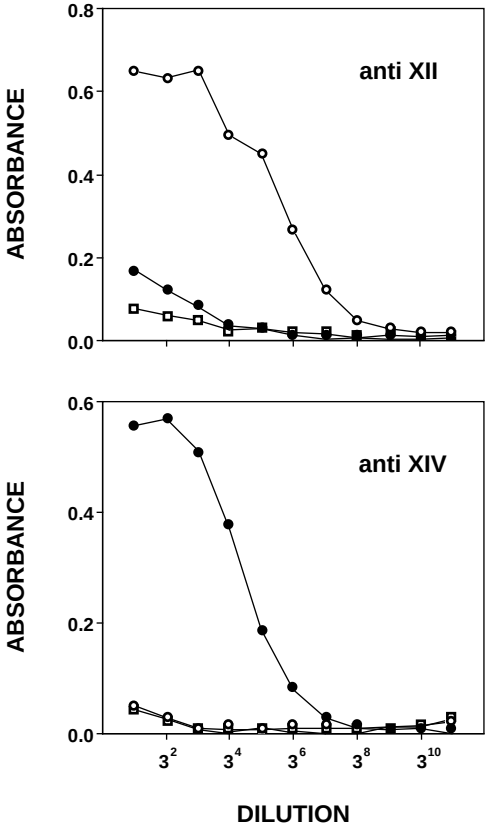


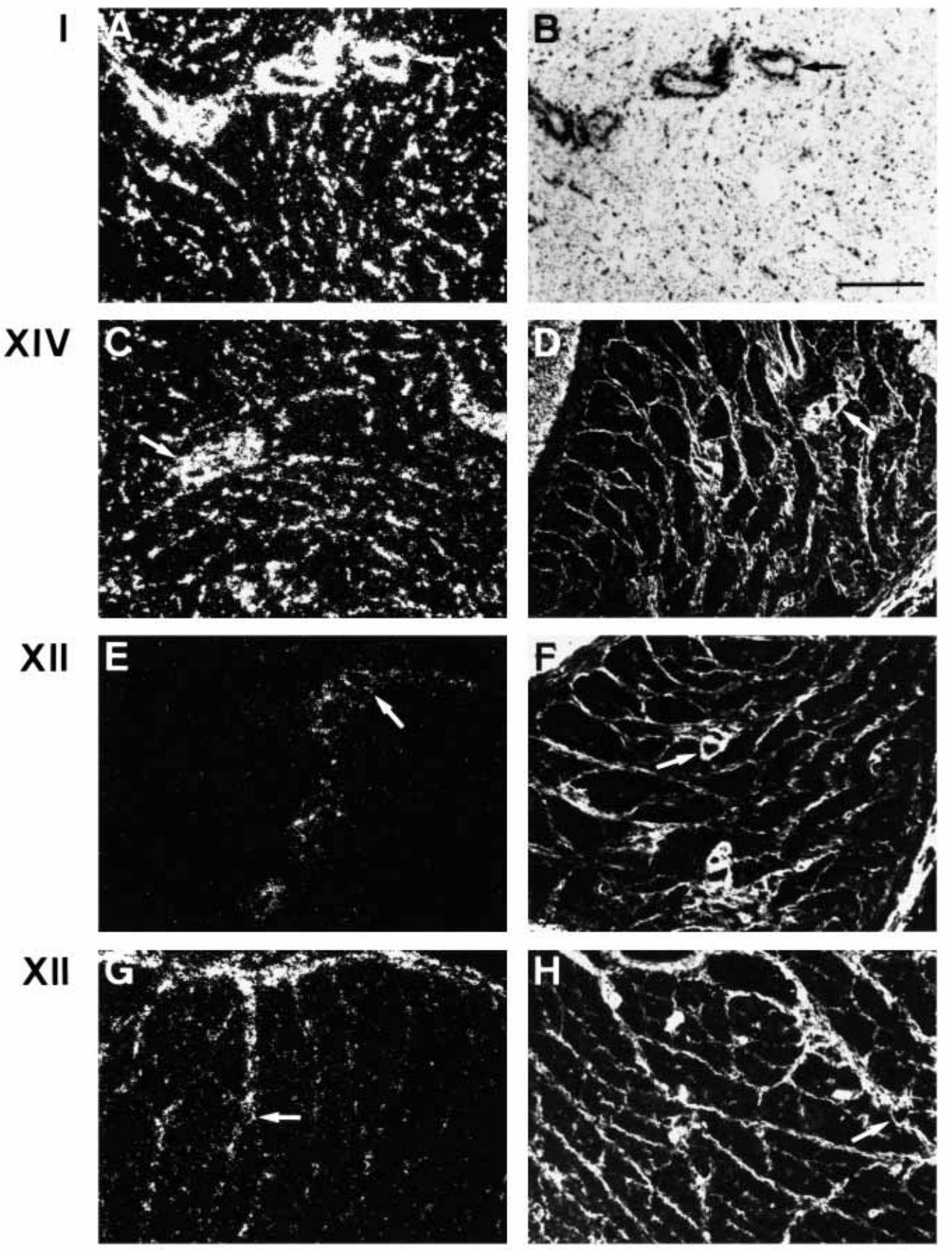
Fig. 3. Characterization of antisera against chicken collagens XII and XIV by ELISA. Purified antisera against collagen XII (anti-XII) or XIV (anti-XIV) were tested in serial threefold dilutions on microtiter plates coated with chicken collagen XII (○), chicken collagen XIV (●) or chicken tenascin (□).

Developing long bones and associated structures

Ossification of avian long bones begins after differentiation of the perichondrium, which surrounds the cartilage models, into the periosteum. The periosteum comprises an outer fibrous layer and an inner osteoblastic layer responsible for bone deposition. While perichondral ossification proceeds, the cartilage is gradually removed and replaced by bone marrow. In situ hybridization of cross-sectioned ribs from 17-day-old chicken embryos revealed an intense signal with the cDNA probe for collagen I. Staining occurred throughout the two layers of the periosteum and extended into the osteoid lining the marrow cavities (Fig. 6A,D). This observation is in accordance with earlier data obtained by immunofluorescence experiments (von der Mark et al., 1976). The expression of collagen XIV mRNA was restricted to the fibrous layer of the periosteum (Fig. 6B). On the other hand, collagen XII transcripts were observed in the fibrous periosteal layer as well as in the first rows of osteoblasts producing perichondral bone (Fig. 6C). In 10-day-old embryos, where ossification of the ribs has just begun (Romanoff, 1960), the localization of the mRNAs for collagens I, XII and XIV was consistent with that found at the 17-day stage.

When the sections were analysed by immunohistochemistry, collagen XIV was preferentially detected in the outer periosteal layer (Fig. 6E). A faint signal was also observed in the osteoid lining the marrow cavities. Collagen XII occurred in both periosteal layers, in the bone matrix and in the osteoid rim of marrow cavities (Fig. 6F). Similar results were obtained with cross-sectioned femur. While these findings are in agreement with data reported for collagen XII by Nakahara et al. (1990), they contradict our observations made by in situ hybridization where endosteal osteoblasts seemed to produce no mRNA for collagen XII or XIV. The discrepancy between the results

Fig. 4. Distribution of collagens I, XII and XIV in skeletal muscle of 17-day-old (A-F) and 10-day-old (G,H) chicken embryos by in situ hybridization (ish) and by immunofluorescence (if). Serial sections were hybridized with cDNA probes for collagen I (A, dark-field; B, bright-field), collagen XIV (C) and collagen XII (E,G) or stained with antibodies against collagen XIV (D) and collagen XII (F,H). Note the absence of collagen XII mRNA from the connective tissues of 17-day-old skeletal muscle (E). Small blood vessels are denoted by arrows. Bar, 200 μm.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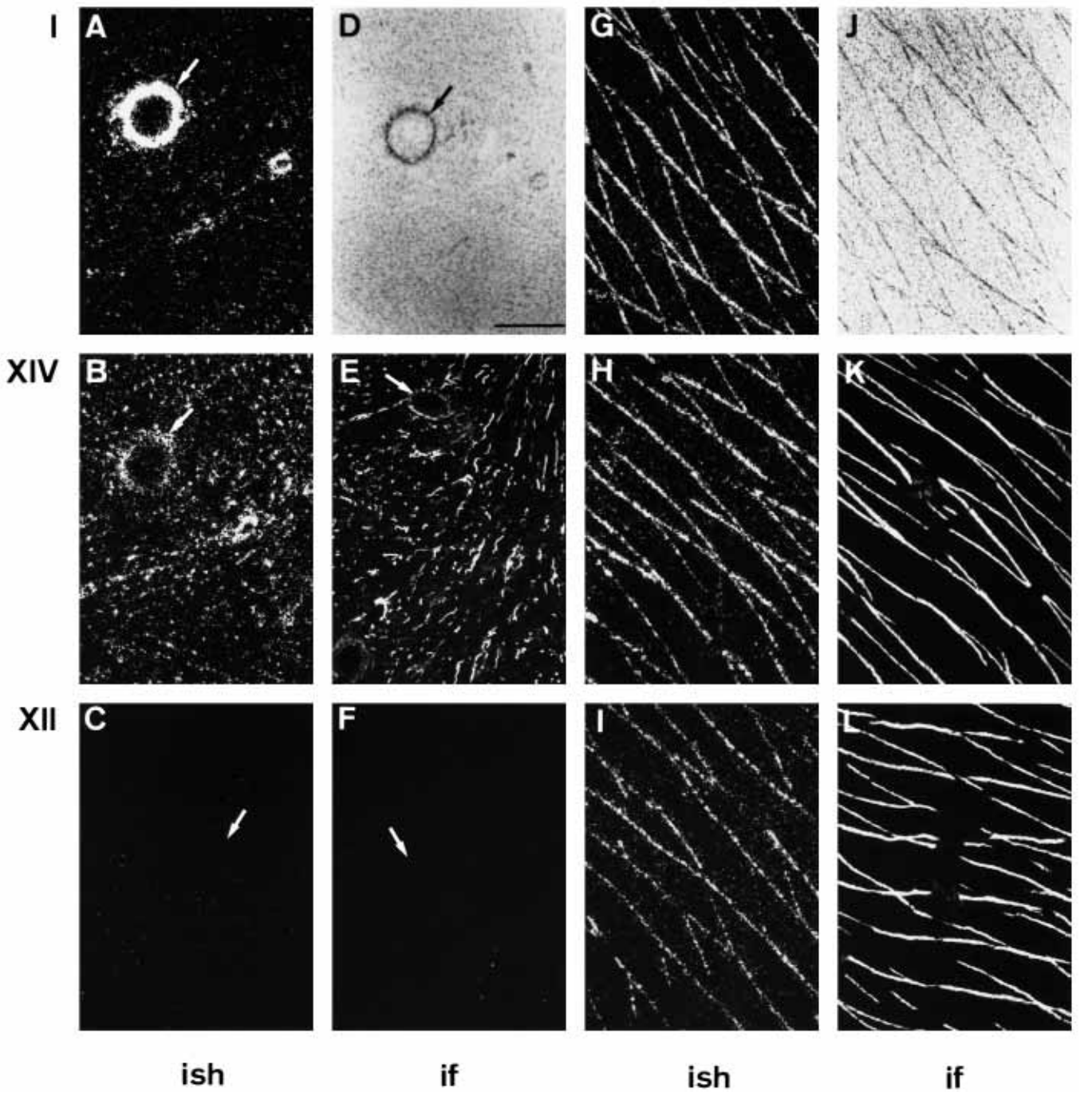

Fig. 5. Distribution of collagens I, XII and XIV in cardiac muscle (A-F) and gizzard smooth muscle (G-L) of 17-day-old chicken embryos by in situ hybridization (ish) and by immunofluorescence (if). Serial sections were hybridized with cDNA probes for collagen I (A,G, dark-field; D,J, bright-field), collagen XIV (B,H) and collagen XII (C,I) or stained with antibodies against collagen XIV (E,K) and collagen XII (F,L). Note the absence of collagen XII from the connective tissues of cardiac muscle (C,F). Small blood vessels are denoted by arrows. Bar, 200 μm.

obtained by the two methods may be explained by an accumulation of the respective proteins during bone development. Thus, at late developmental stages the accumulated proteins can be detected with our antibodies, whereas the corresponding mRNAs have decreased below detection level.

Diaphyseal cartilage, which is prominent in developing long bones from 10-day-old embryos but almost entirely removed at the 17-day stage, did not appear to express collagen I, XII or XIV, since no staining was obtained by in situ hybridization or immunohistochemistry.

Tendons were uniformly stained with our cDNA probes for collagens I, XII and XIV (Fig. 6G,H,I,J) as well as with our antibodies against the two FACIT proteins (Fig. 6K,L). A particularly strong signal was obtained with antibodies against collagen XII, suggesting that this collagen must be fairly abundant in chicken tendons.

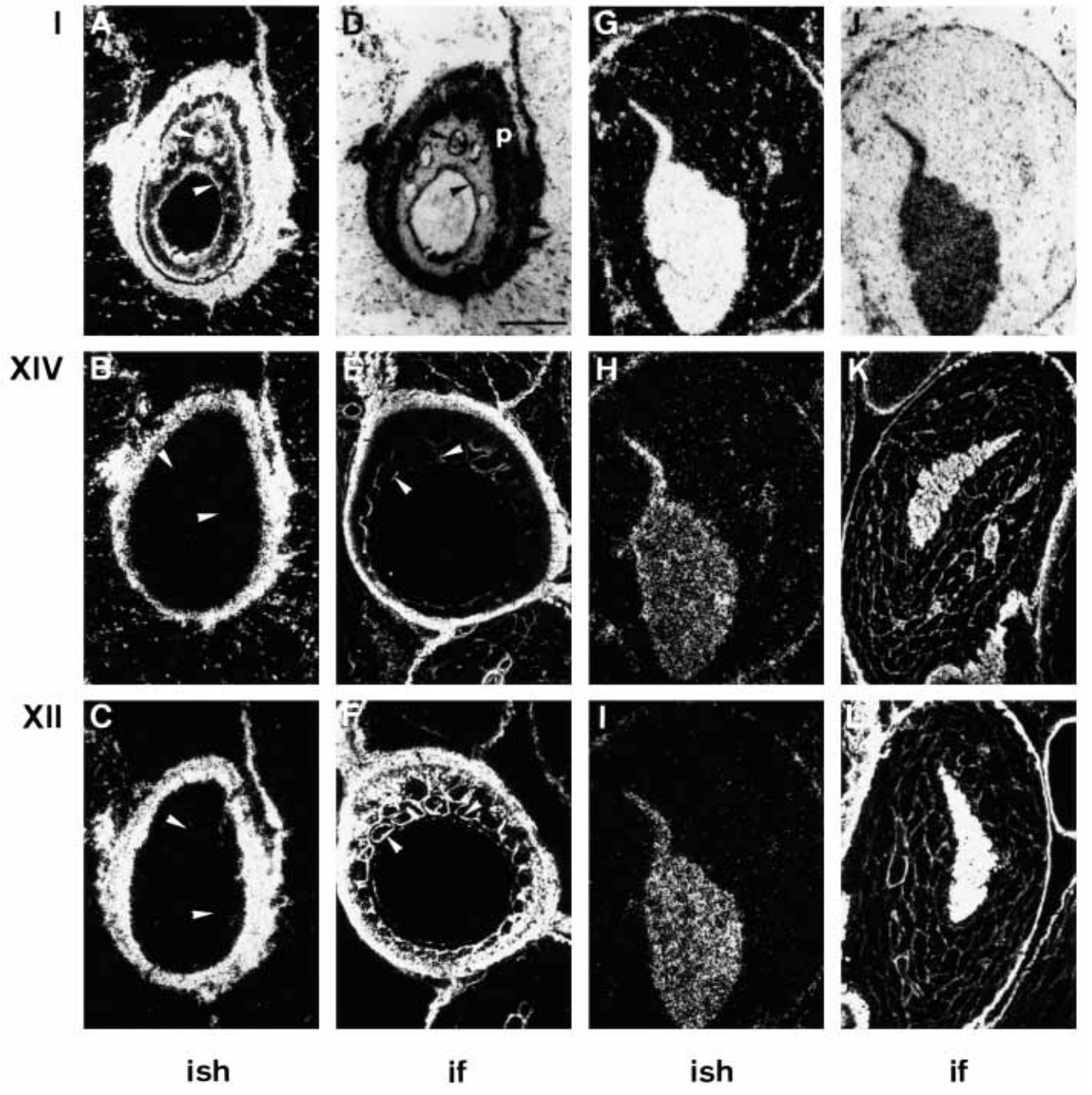

Fig. 6. Distribution of collagens I, XII and XIV in cross-sections of ribs (A-F) and hind-limb tendons (G-L) of 17-day-old chicken embryos by in situ hybridization (ish) and by immunofluorescence (if). Serial sections were hybridized with cDNA probes for collagen I (A,G, dark-field; D,J, bright-field), collagen XIV (B,H) and collagen XII (C,I) or stained with antibodies against collagen XIV (E,K) and collagen XII (F,L). Note the staining observed for collagens XII and XIV by immunohistochemistry in the osteoid lining marrow cavities (E,F). The osteoid rim of marrow cavities is denoted by arrowheads. p, periosteum. Bar, 200 μm.

Nerves

The connective tissue of peripheral nerves is composed of three compartments: the highly collagenous epineurium, which provides the nerve trunk with mechanical strength, the tight perineurium, which acts as a diffusion barrier, and the endoneurium, which surrounds each axon/Schwann cell unit. In situ hybridization of hind-limb cross-sections from 17-day-old embryos showed that collagen I mRNA was present in each of these successive layers (Fig. 7A,B) in accordance with previous immunofluorescence studies (Shellswell et al., 1979). The mRNA for collagen XIV appeared to colocalize with that for collagen I in the epineurium and the perineurium (Fig. 7C). However, the perineurial staining was strong and punctate in the case of collagen I but more diffuse in the case of collagen

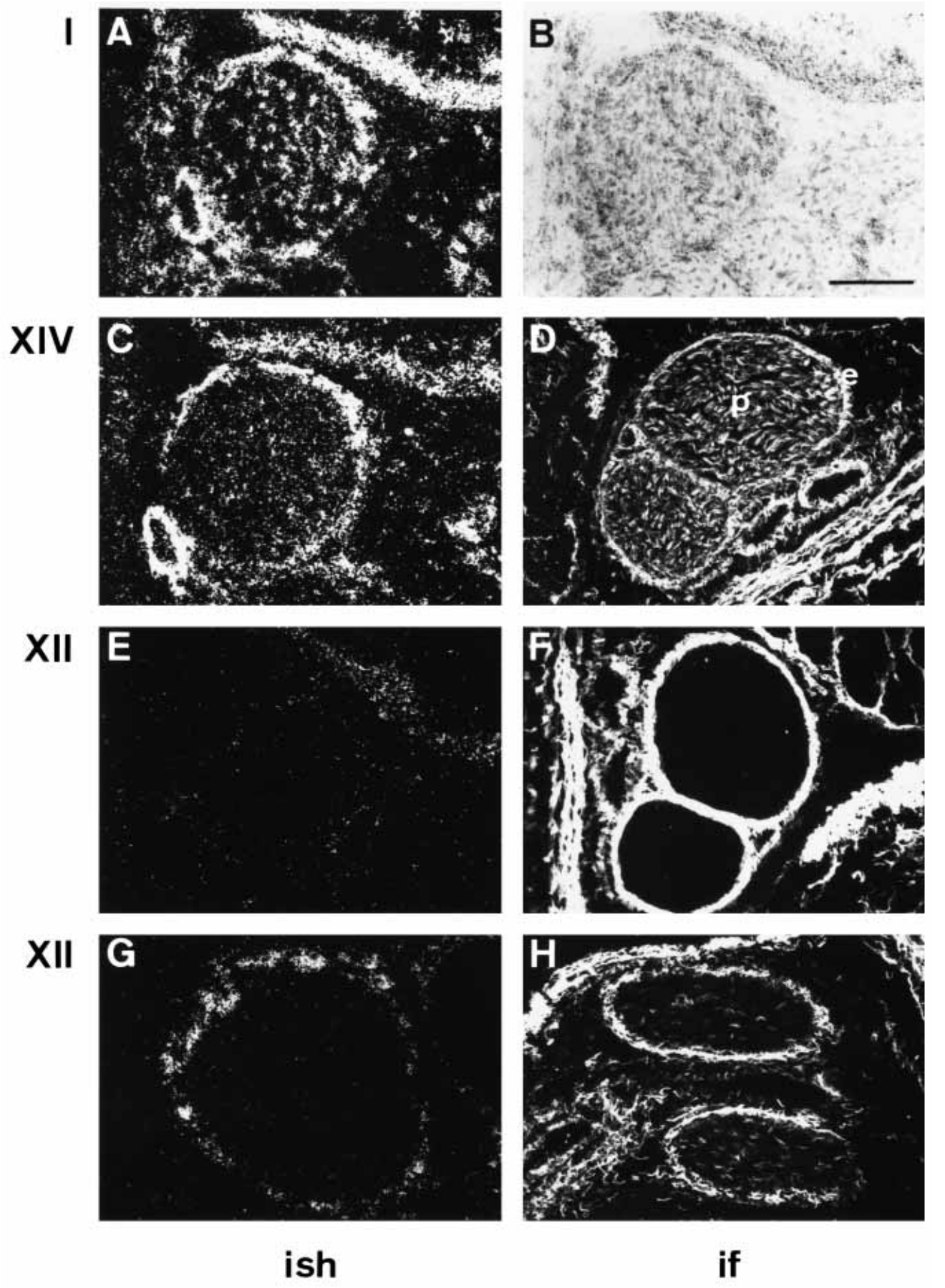
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Fig. 7. Distribution of collagens I, XII and XIV in cross-sections of peripheral nerves of 17-day-old (A-F) and 10-day-old (G,H) chicken embryos by in situ hybridization (ish) and by immunofluorescence (if). Serial sections were hybridized with cDNA probes for collagen I (A, dark-field; B, bright-field), collagen XIV (C) and collagen XII (E,G) or stained with antibodies against collagen XIV (D) and collagen XII (F,H). Note the absence of collagen XII from the perineurium at the 17-day stage (E,F) and the low staining observed at the 10-day stage (G,H). e, epineurium; p, perineurium. Bar, 100 μm.

XIV. Immunofluorescence experiments with antibodies against collagen XIV revealed a nice undulating pattern in the epineurium and the perineurium, suggesting that this protein is closely associated with the connective tissue fibres (Fig. 7D). In contrast, collagen XII was expressed to a much smaller extent than its FACIT counterpart. At the mRNA level it was totally absent in nerves from 17-day-old embryos (Fig. 7E). At the 10-day stage only a faint signal could be detected in the epineurium (Fig. 7G). However, at the protein level a prominent signal was observed for collagen XII in the epineurium at both stages and in some perineurial fibres at the 10-day stage (Fig. 7F,H). Thus, expression of the mRNA for this FACIT collagen seems to have ceased early in the embryonic development of peripheral nerves, but antibodies can still detect the accumulated protein at later stages.

Six-day-old chicken embryo

In situ hybridization of longitudinal sections from 6-day-old chicken embryos showed a fairly uniform distribution of collagen I mRNA in the mesenchyme surrounding the dorsal root ganglia and the cartilage of sclerotomes (Fig. 8A). Ganglia and cartilage themselves were negative as were the dorsal and ventral roots. The myotomes were weakly labelled by our collagen I-specific probe and appeared as a series of stripes alternating with the ventral part of the sclerotomes. Nevertheless the strongest signal was detected as a thick band in the dermatome underneath the ectoderm.

Striking differences in the staining pattern were observed when the hybridizations were repeated with probes specific for collagen XII or XIV. Transcripts for collagen XIV were detected in the ectoderm as a clear and distinct signal (Fig. 8B). Two independent cDNA clones for this collagen yielded the same ectodermal staining, confirming the specificity of the signal. All other areas were negative except for some faint staining of the mesenchymal tissues joining the dorsal portions of the myotomes. In contrast to these findings, collagen XII transcripts could not be detected in the ectoderm, but they were found in the underlying dermatome. Furthermore, staining was observed in an undulating band of mesenchymal tissues connecting the dorsal myotomal segments (Fig. 8C). Sclerotomes and myotomes were irregularly stained with a pronounced signal in the region of the sclerotomes situated ventrally to the dorsal root ganglia. The future ribs appeared as labelled stripes ventroposterior to each myotome.

In situ hybridization of cross-sections from the thoracic region of the spinal cord confirmed the uniform distribution of collagen I mRNA in the mesenchymal tissues around the notochord and the neural tube (Fig. 8D,G). Notochordal cells were labelled in a weak, punctate fashion. Neural tissues were negative, as were the cartilaginous portions of the sclerotomes. Collagen XIV transcripts were confined to very few regions (Fig. 8E). A faint signal was detected in some areas of the developing vertebrae. The most striking observation, however, was the expression of collagen XIV by a discrete group of cells in the ependymal layer that lines the central canal of the neural tube. The positive cells appeared to be restricted to the region of the sulcus limitans. This surprising finding could be corroborated by immunofluorescence experiments (Fig. 8H). Antibodies against collagen XIV stained the same region of the neural tube as our cDNA probes. Although the signal was rather weak, it could clearly be detected on the original photograph. It is therefore likely that a subpopulation of neuroglial cells are capable of producing collagen XIV. Additional tissues that were weakly stained with our antibodies included the cell layers ventral to the notochord and some mesenchymal tissues associated with the developing vertebral body.

Collagen XII was detected on the cross-sections in the myotomes and in the developing vertebrae, both at the mRNA (Fig. 8F) and at the protein level (Fig. 8I). It was also found in the perinotochordal matrix and in the connective tissues associated with small blood vessels as well as in the dorsal and ventral roots. The dorsal root ganglia were negative, as was the notochord itself. Furthermore, an expression pattern complementary to the one observed for collagen XIV was revealed in the neural tube, especially by in situ hybridization: the ependymal zone was negative but the surrounding mantle layer, corresponding to the future gray substance, was slightly positive.

DISCUSSION

Interstitial collagen fibrils form the supporting scaffold of most extracellular matrices. Although these fibrils consist essentially of type I or II collagen molecules, they are able to fulfil quite diverse functions in different tissues and at distinct developmental stages by varying their spatial arrangement and their fibrillar diameter. It appears now that the formation of heterotypic fibrils containing minor interstitial collagens (types III, V, XI) in addition to the major interstitial collagen types as well as interactions with non-fibrillar collagens (types IX, XII, XIV) may allow them to meet this challenge.

This study focuses on the differential distribution of the non-fibrillar collagens XII and XIV and on the colocalization of these molecules with collagen I. Collagens XII and XIV exhibit a very similar overall structure; nevertheless, there are some important differences in their domain structures and in their amino acid sequences. It seems therefore conceivable that the two molecules serve slightly different purposes. Consequently, their expression might follow distinct spatial and temporal patterns depending on the specific needs of each tissue type. From all the tissues examined here, some indeed reveal striking differences in the level of synthesis of the two collagen species. Skeletal and cardiac muscles show high expression of collagen XIV in the connective tissue septa associated with muscle fibres. Neither muscle type, however, expresses any collagen XII in 17-day-old chicken embryos. The same observation applies to the connective tissue of peripheral nerves: epineurium and perineurium are rich in collagen XIV, but virtually devoid of collagen XII. On the other hand there are tissues that contain collagen XII but lack

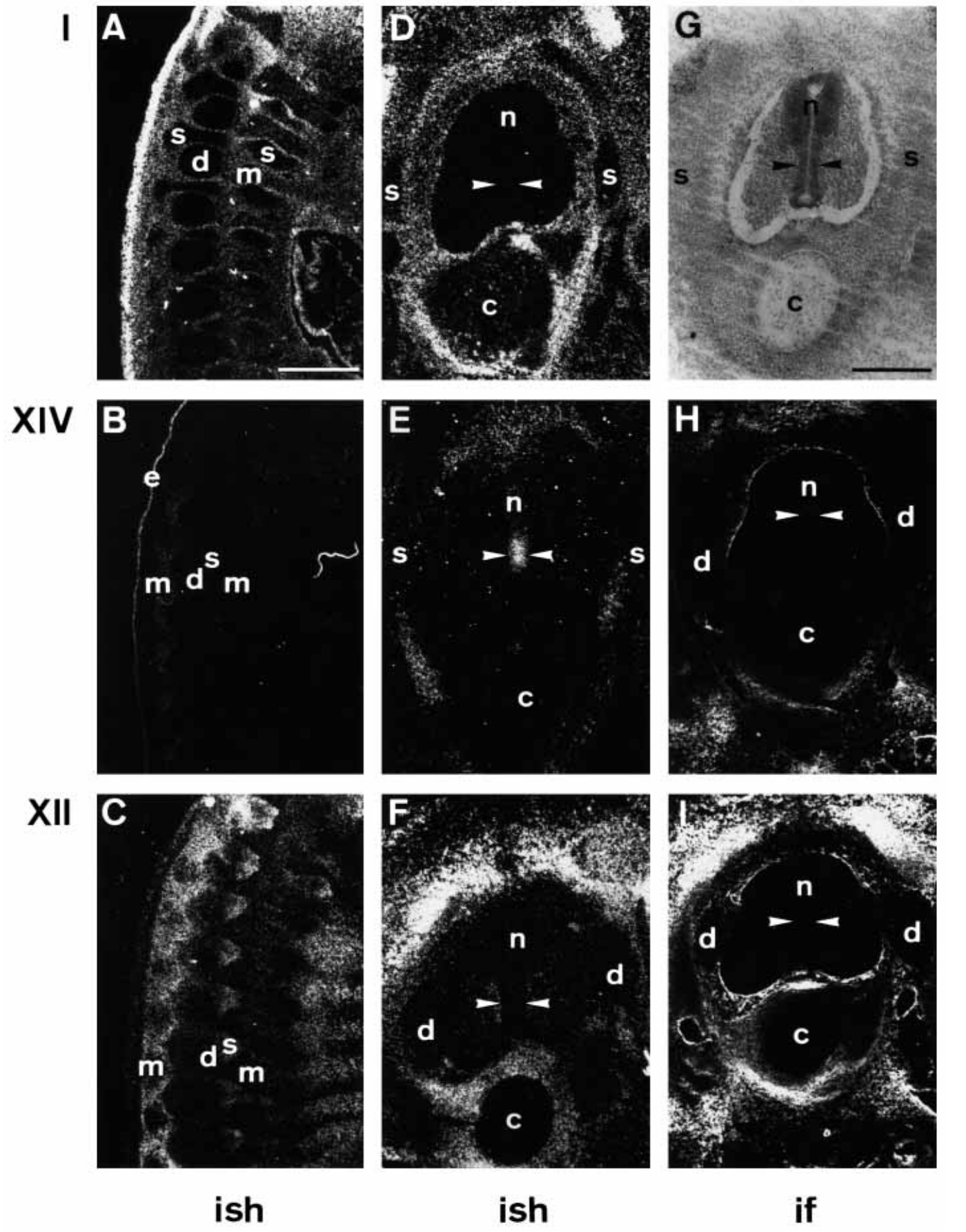
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Fig. 8. Distribution of collagens I, XII and XIV in the 6-day-old chicken embryo by in situ hybridization (ish) and by immunofluorescence (if). Sagittal sections were hybridized with cDNA probes for collagen I (A), collagen XIV (B) and collagen XII (C). Posterior is to the top and dorsal is to the left. The three sections shown here have been cut at a slightly different depth relative to the trunk axis, in the order (A)-(B)-(C) from the centre to the periphery of the embryo. Note the low level of collagen XIV mRNA. Cross-sections at the level of the thoracic vertebrae were hybridized with cDNA probes for collagen I (D, dark-field; G, bright-field), collagen XIV (E) and collagen XII (F) or stained with antibodies against collagen XIV (H) and collagen XII (I). Dorsal is to the top. Note the complementary distribution of collagen XII and XIV transcripts within the neural tube (E,F). The ependymal zone is indicated by arrowheads. d, dorsal root ganglion; e, ectoderm; m, myotome; n, neural tube; c, notochord; s, sclerotome. Bars, 800 μm (A-C); 400 μm (D-I).

collagen XIV. In developing long bones, collagen XII is abundant in both the outer (fibrous) layer and the inner (osteogenic) layer of the periosteum, whereas collagen XIV occurs exclusively in the outer layer.

The combination of in situ hybridization with immunohistochemical experiments makes it possible to study the temporal biosynthetic course of long-lived proteins like collagens that accumulate in the extracellular matrix. In this regard collagens XII and XIV exhibit a clearly different regulation. Collagen XII is highly expressed in 6-day-old embryos, particularly in the extracellular matrix associated with the developing spinal cord. As development proceeds, it gradually disappears from some, but not all, tissues. This is well illustrated with skeletal muscle, which still produces mRNA for collagen XII at the 10-day stage but not at the 17-day stage; yet the deposited protein can be detected in skeletal muscle from 17-day-old embryos by immunohistochemistry. Shortly before hatching, only dense connective tissue structures exposed to high mechanical stress such as tendons, ligaments and gizzard are actively producing collagen XII. In contrast, collagen XIV is barely detectable in the 6-day-old embryo. During development it appears to take over the role of collagen XII and in the 17-day-old embryo it represents the predominant FACIT protein in virtually all tissues.

The specific association of collagens XII and XIV with interstitial collagen I fibrils remains a controversial topic. Studies indicating such an interaction were performed by Schuppan et al. (1990) as well as Keene et al. (1991). These authors showed by immunoelectron microscopy that both molecules are located on the surface of type I collagen fibrils from skin and tendon. However, a direct interaction with collagen I could not yet be demonstrated. In vitro protein binding assays revealed some affinity of collagen XIV for heparin and collagen VI, but absolutely no affinity for pepsin-extracted collagen I (Brown et al., 1993). Nevertheless, collagens XII and XIV must be firmly anchored in the extracellular matrix, since a high ionic strength is required to achieve extraction from biological material and since the two proteins are virtually absent from serum (Watt et al., 1992; Brown et al., 1993). Our data obtained with 17-day-old embryos demonstrate the presence of collagen XII, collagen XIV, or both, in every tissue containing collagen I. Moreover, the staining patterns found for the two molecules strictly follow the distribution of collagen I. Immunofluorescence studies with our antibodies against collagens XII and XIV even show a distinct labelling of undulating interstitial fibrils, as is particularly well illustrated with peripheral nerves. The assumption that the two FACIT proteins are associated exclusively with type I collagen fibrils is in line with our observations made with cartilage. The collagenous matrix of hyaline cartilage consists mostly of type II collagen fibrils. All studies performed so far with chicken embryos have failed to detect either of the two FACIT proteins in hyaline cartilage (Sugrue et al., 1989; Yamagata et al., 1991; Trueb and Trueb, 1992a). The only exception is the cartilage matrix close to the developing articular surface, which could be stained by our probes for collagens I, XII and XIV (not shown). This region is known to synthesize collagen I at the overlap between epiphyseal and fibrous articular cartilage (von der Mark et al., 1976). The situation appears to be more complicated in species other than chicken. Watt et al. (1992) were able to isolate substantial amounts of collagens XII and XIV from fetal bovine epiphyseal cartilage after extraction with high salt buffer. Furthermore, studies performed with mouse embryos showed the presence of collagen XII in fibrous cartilage but not in hyaline cartilage (Oh et al., 1993). It remains therefore to be elucidated whether collagen XII or XIV may also associate with structures other than type I collagen fibrils.

The technique of in situ hybridization allows us to address the question as to which cell type produces a certain mRNA species. The cellular origin of the various collagen types in skeletal muscle is not yet well understood. In vitro studies indicate that myoblasts as well as fibroblasts contribute to connective tissue formation in muscle, since both cell types are capable of synthesizing different collagens (Sasse et al., 1981). On the basis of the similar expression patterns observed for collagen I and for our two FACIT proteins, we assume that all three molecules are synthesized by the same cell type. Although the resolution of our photographs does not allow a definitive answer, we believe that fibroblasts located within the connective tissue of muscle fibres represent the major site of production of collagens I, XII and XIV. This assumption is consistent with studies performed with rat heart tissues and isolated cardiac muscle cells. In situ hybridization revealed the presence of mRNA for collagen I in fibroblasts of the interstitium, but not in cardiomyocytes (Eghbali et al., 1989). On the other hand, experiments performed with developing long bones, nerves and liver suggest that also more specialized cell types are able to synthesize collagens XII and XIV. Osteoblasts of the periosteum and the lining of bone marrow cavities, endo- and perineurial cells of peripheral nerves, fat-storing cells and sinusoidal endothelial cells of the liver parenchyma (Knittel et al., 1992) all represent sites of collagen XII or XIV synthesis.

Two very intriguing observations suggesting that even epidermal and neuroglial cells may produce collagen XIV should also be mentioned in this context. In the 6-day-old embryo, mRNA transcripts for collagen XIV could clearly be detected in the ectoderm. This signal was specific, since two independent cDNA probes for collagen XIV yielded the same result. Furthermore, epidermal staining was also observed by in situ hybridization in the skin of 10-day-old embryos, but not in that of 17-day-old embryos (not shown). At the protein level, however, neither we nor other researchers could detect any collagen XIV with specific antibodies in the epidermal region. Additional studies will therefore be required to reconcile this apparent contradiction and to resolve the question of whether

epidermal cells can indeed produce collagen XIV. The other striking observation made with 6-day-old embryos was the expression of collagen XIV mRNA by a subpopulation of cells in the ependymal epithelium of the neural tube. These cells are derived from neuroepithelial cells, which are mitotically active during early embryonic development and give rise to the neurones and the supporting tissues of the nervous system (Romanoff, 1960). Since the same region was also stained with antibodies against collagen XIV and since a similar signal was still observed at the 10-day stage (not shown), we must assume that a subpopulation of neuroglial cells is indeed able to produce collagen XIV. As the neural tube is totally negative for collagen I, these observations suggest that - at least in the 6-day-old embryo - our FACIT proteins may be expressed without concomitant expression of collagen I.

Nevertheless, the major sources of collagens XII and XIV in most tissues are obviously fibroblasts. In this regard it is worth mentioning that dramatic changes occur in the transcription of these molecules upon cultivation of the cells in vitro. When kept in culture, neither fibroblasts obtained from chicken tendons (Trueb and Trueb, 1992a) nor fibroblasts prepared from entire chicken embryos (unpublished observation) synthesize any collagen XIV. Actually, only very few cell lines, including rhabdomyosarcoma cells and osteosarcoma cells, maintain the ability to produce collagen XIV in vitro (Schuppan et al., 1990; Brown et al., 1993). On the other hand collagen XII is expressed in large amounts by cultured fibroblasts. However, under culture conditions transcription of collagen XII is switched from the short to the long form (Oh et al., 1993). A most interesting issue would therefore be to elucidate the mechanisms responsible for the changes observed upon cultivation. One hypothesis would postulate that fibroblasts require an unidentified growth factor or another signal originating from the interaction with other cell types or with components of the extracellular matrix in order to produce collagens XII and XIV in the correct amount within each tissue. An answer to the questions raised here would provide an important clue about the possible role of these FACIT proteins in modulating the architecture of the extracellular matrix.

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