Alternatively Spliced Type II Procollagen mRNAs Define Distinct Populations of Cells during Vertebral Development: Differential Expression of the Amino-Propeptide

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Abstract. Type II collagen is a major component of cartilage providing structural integrity to the tissue. Type II procollagen can be expressed in two forms by differential splicing of the primary gene transcript. The two mRNAs either include (type IIA) or exclude (type IIB) an exon (exon 2) encoding the major portion of the amino (NH_2)-propeptide (Ryan, M. C., and L. J. Sandell. 1990. *J. Biol. Chem.* 265:10334–10339). The expression of the two procollagens was examined in order to establish a potential functional significance for the two type II procollagen mRNAs. First, to establish whether the two mRNAs are functional, we showed that both mRNAs can be translated and the proteins secreted into the extracellular environment. Both proteins were identified as type II procollagens. Secondly, to test the hypothesis that differential expression of type II procollagens may be a marker for a distinct population of cells, specific procollagen mRNAs were localized in tissue by in situ hybridization to oligonucleotides spanning the exon junctions. Embryonic vertebral column was chosen as a source of tissue undergoing rapid chondrogenesis, allowing the examination of a variety of cell types related to cartilage. In this issue, each procollagen mRNA had a distinct tissue distribution during chondrogenesis with type IIB expressed in chondrocytes and type IIA expressed in cells surrounding cartilage in prechondrocytes. The morphology of the cells expressing the two collagen types was distinct: the cells expressing type IIA are narrow, elongated, and "fibroblastic" in appearance while the cells expressing type IIB are large and round. The expression of type IIB appears to be correlated with abundant synthesis and accumulation of cartilagenous extracellular matrix. The expression of type IIB is spatially correlated with the high level expression of the cartilage proteoglycan, aggrecan, establishing type IIB procollagen and aggrecan as markers for the chondrocyte phenotype. Transcripts of type II collagen, primarily type IIA, are also expressed in embryonic spinal ganglion. While small amounts of type II collagen have been previously detected in noncartilagenous tissues, the detection of this new form of the collagen in relatively high abundance in embryonic nerve tissue is unique. Taken together, these findings imply a potential functional difference between type IIA and type IIB procollagens and indicate that the removal of exon 2 from the pre-mRNA, and consequently the NH_2-propeptide from the collagen molecule, may be an important step in chondrogenesis. In addition, type II procollagen, specifically type IIA, may function in noncartilage tissues, particularly during development.

Type II collagen is the predominant collagenous component of cartilage composing >50% of the extracellular matrix. Like type I collagen, the most abundant collagen in vertebral organisms, it is an interstitial collagen[1] synthesized as a procollagen monomer, assembled into molecular trimers and processed extracellularly to remove NH_2- and COOH-terminal extension propeptides. The interstitial collagen monomer contains a non-interrupted Gly-X-Y protein domain of ~1,014 amino acids, flanked by the globular propeptides. The COOH-terminal propeptide of ~275 amino acids is connected to the main triple helix by a short telopeptide, is highly conserved between interstitial collagens, and is thought to be involved in initiation of trimer formation. The NH_2-terminal propeptide shows more structural and sequence diversity among the interstitial collagens, but generally consists of a short globular domain followed by a cysteine-rich domain, a Gly-X-Y domain of 40–60 residues, and a short connecting telopeptide (Sandell and Boyd, 1990). It

1. Interstitial collagens, also called Group 1 collagens, are defined as the major fibril-forming collagens by Miller, E. J. and S. Gay (1987. *Methods Enzymol.* 144:3–40) and include types I, II, III, V, and XI.

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has been suggested that the propeptides, particularly the NH_2-propeptide, are involved in feedback regulation of collagen synthesis and in the formation of fibrils in the extracellular matrix (Wiestner et al., 1979; Wu et al., 1986; Fleischmajer et al., 1983).

Traditionally, the presence of type II collagen has served as a marker for cartilage, however, small amounts of type II collagen have been detected at the epitheliomesenchymal interfaces during morphogenesis of the cartilaginous neurocranium and in other noncartilaginous tissues of developing chicken embryos (Linsenmayer et al., 1977; von der Mark et al., 1977; Newsome et al., 1976; Smith et al., 1976; Linsenmayer and Little, 1978; Kosher and Solursh, 1989). In prechondrogenic limb mesenchymal cells, type II collagen mRNA has been detected prior to the onset of overt cartilage differentiation and before accumulation of detectable amounts of type II collagen (Kravis and Upholt, 1985; Kosher et al., 1986). Kosher et al. (1986) suggested that low level expression of type II collagen gene by chondrogenic progenitor cells may represent a molecular manifestation of the state of determination of the cells, however, the role of type II collagen in prechondrocytic and nonchondrocytic cells is not known.

Recently, two type II procollagen transcripts were found to be produced from the single COL2A1 gene (Ryan and Sandell, 1990). We reported the finding of an exon (exon 2) present in the human COL2A1 gene (Ryan et al., 1990) that was not present in type II collagen cDNA clones from human chondrocytes (Baldwin et al., 1989) or rat chondrosarcoma (Kohno et al., 1984). This exon encodes the collagenase resistant, cysteine-rich domain of the NH_2-propeptide. The protein domain contains 69 amino acids, including 10 cysteine residues, is conserved between interstitial collagens chains α1(I), α1(II), α1(III), α2(V), and is homologous to a domain present in the extracellular matrix proteins thrombospondin and von Willebrand factor (Ryan and Sandell, 1990). Further investigation of type II procollagen mRNA transcripts revealed that this domain is expressed in embryonic and adult chondrocyte mRNA populations and is subject to alternative splicing in the pre-mRNA (Ryan and Sandell, 1990). Northern blot analysis using oligonucleotide probes spanning the boundaries between exon 1 and exon 2 (type IIA) or exon 1 and exon 3 (type IIB) indicated that type IIB mRNA was the predominant form expressed in RNA isolated from cartilage cells although both mRNAs were present in the RNA populations examined. We suggested that the expression of exon 2 may be a marker for a distinct population of cells and have designed this study to test that hypothesis.

The expression of the two procollagens was examined in medium from cultured juvenile costal chondrocytes (shown previously to express both type II procollagen mRNAs). These cells translated and secreted both type II procollagens at relatively high levels. Identification of the cells synthesizing the two type II procollagens was accomplished by in situ hybridization to mRNA using oligonucleotide probes designed to identify specific mRNAs.

Materials and Methods

Cell Cultures

Procollagens were isolated from medium of human costal chondrocytes. Costal cartilage was obtained from ribs removed during pectus excavatum repair. Chondrocytes were isolated by dispersion with proteases as follows. Cartilage slices were incubated at 37°C with hyaluronidase (1 mg/ml in PBS; bovine testicular from Sigma Chemical Co., St. Louis, MO, for 10 min, followed by 0.25% trypsin (GIBCO/BRL, Gaithersburg, MD) for 45 min). The cartilage was then chopped in small fragments and incubated at 37°C with collagenase (3 mg/ml in serum-free culture medium, clostridial peptidase from Worthington Biochemical Corp., Freehold, NJ) for 24 h for articular cartilage or 48 h for costal cartilage. The dispersed cells were then washed with Ca^{++}– and Mg^{++}–free PBS and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS with medium changes every 3–4 d, as described previously (Goldring et al., 1986). Costal chondrocytes from two different individuals (C-29: 16-yr-old female, 16 d of culture; C-30: 6-yr-old female, 8 d of culture) were used for the experiments described in this study. The cells were labeled 24 h after the last medium change for a further 24 h in serum-free Dulbecco's modified Eagle's medium (5 ml per 10-cm dish) supplemented with 50 μg/ml ascorbate and 50 μg/ml β-aminoproprionitrile fumarate and containing 25 μCi/ml of [^{3}H]proline (>20 Ci/mmol; Amersham Corp., Arlington Heights, IL) and 50 μCi/ml of [^{35}S]cysteine (1071 Ci/mmol; Amersham Corp.).

Isolation of Procollagens

The culture medium containing [^{3}H]proline labeled proteins was adjusted to 5 mM EDTA, and 1 mM *N*-ethylmaleimide. Proteins were precipitated by the addition of 300 mg/ml of ammonium sulfate which was stirred overnight at 4°C. The precipitate was collected by centrifugation at 15,000 rpm, 4°C, for 30 min in an SS 34 rotor (Sorvall Instruments, Newton, CT) using an RC 5C centrifuge (Sorvall Instruments). The precipitate was suspended in 0.05 M Tris-HCl, pH 7.5, 0.25 M NaCl, 0.1% Triton X-100. The samples were treated with 0.25% diisopropyl fluorophosphate for 4 h on ice and then dialyzed against the same buffer. Samples were run over a Sepharose-gelatin column (Pharmacia Fine Chemicals, Piscataway, NJ) to remove fibronectin. When indicated, aliquots were digested with purified bacterial collagenase (Morris et al., 1983) or pepsin. Labeled proteins were visualized by fluorography after separation on SDS-PAGE (Burgeson et al., 1976) using Amplify (Amersham Corp.). Cyanogen bromide peptide mapping of gel bands was performed according to the method of Sokolov et al,. (1989) using the 16-h incubation. Human type I procollagen was prepared from the medium of primary skin fibroblasts incubated with [^{3}H]proline (Amersham Corp.) for 24 h. The medium was precipitated with ammonium sulfate and treated with diisopropyl fluorophosphate as described above. The sample was then fractionated under nondenaturing conditions on a Sepharose-6B column (Pharmacia Fine Chemicals) and the fractions containing type I procollagen pooled and frozen. [^{3}H]Proline-labeled mixture of chick types II and XI collagen was obtained from organ culture of chick sterna as previously described (Morris and Bachinger, 1987). Labeling was for 60 min and purification proceeded only to passage over DEAE cellulose to remove proteoglycan. Unless otherwise indicated, all reagents were purchased from Sigma Chemical Co.

In Situ Hybridization

Tissue used in this study was human fetal vertebral column, 57-d gestation, (Carnegie Stage 23), provided by the Central Laboratory for Human Embryology at the University of Washington. The age was determined from crown to rump length by the method of Shepard (1975). Tissue was frozen in O.C.T. compound (Miles Laboratories Inc., Elkhart, IL) and sectioned with a cryostat, or fixed in 4% paraformaldehyde, embedded in Epon, and sectioned on a microtome. For in situ hybridization, frozen sections were used with in situ hybridization procedures described by Lewis et al., (1985). In brief, slides with a single section of vertebral tissue (6–10 μm) were warmed to room temperature. Sections are postfixed in 4% paraformaldehyde, treated with acetic anhydride (0.25% in 0.1 M triethanolamine), dehydrated, delipidated, and air dried. An ^{35}S-labeled oligonucleotide probe was added to hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 300 mM NaCl, 10 mM Tris, 1 mM EDTA, 1× Denhardt's (0.02% each of BSA, Ficoll, and polyvinylpyrollidone), 0.5 mg yeast tRNA/ml, and 10 mM dithiothreitol. A 45-μl aliquot (containing 2.5 pmol of probe/ml) was applied to the slide. Specific activity estimates ranged from 1.0 to 1.2 × 10^7 cpm/pmol. Sections were coverslipped and slides were incubated overnight in moist chambers at 37°C. After the overnight incubation, coverslips were removed and sections washed four times in 1× standard saline citrate (1× SSC = 150 mM NaCl, 15 mM sodium citrate) for 15 min at 50°C. In some cases, stringency was increased by elevating wash temperatures. Slides were washed twice for 1 h at room temperature. The sections were dehydrated through a graded series of alcohols containing 300 mM ammonium acetate and exposed to Hyperfilm BMax (Amersham

Corp.) for 3 d. Slides were dipped in NTB 2 emulsion (Eastman Kodak Co., Rochester, NY), diluted 1:1 with 600 mM ammonium acetate and exposed for 8–12 d. The emulsion was developed in D-19 (Eastman Kodak Co.), diluted 1:1 with distilled water at 16°C. Sections were counterstained with cresyl violet acetate and coverslipped. Autoradiographs were analyzed with the aid of a MCID Image Analysis System (Imaging Research, St. Catharines, Ontario, Canada).

Oligonucleotide Probes

Probes specific for type IIA and type IIB collagen have been described (Ryan and Sandell, 1990) and contain the following. For type IIA, 12 nucleotides of exon 1 and 12 nucleotides of exon 2, 5'-TGCCAGCCTCCTGGACATCCTGGC-3'; for type IIB, 12 nucleotides of exon 1 and 12 nucleotides of exon 3, 5'-CTCCTGGTTGCCGGACATCCTGGC-3'. Other oligonucleotides used in this report were the following. All type II collagen mRNAs, a 21mer oligonucleotide of exon 8, 5'-GCCTTCTGATCAAATCCTCCA-3'; aggrecan (nucleotides 1,096–1,120) of cDNA reported by Baldwin et al. (1989), 5'-CTCGTGCCAGATCATCACCACACAT-3'; α1(I) procollagen, exon 1/exon 2 junction, 5'-TGATTGGTGGGATGTCTTCGTCTT-3' (d'Alessio et al., 1988). Oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) and purified by passage through a 5% acrylamide gel; only the full-sized oligomers were eluted. Oligonucleotides were labeled with 5'-[α-thiol-^{35}S]-ATP (New England Nuclear, Boston, MA) using terminal deoxynucleotidyl transferase (Bio-Rad Laboratories, Gaithersburg, MD).

Results

Translation and Secretion of Two Procollagens

Since mRNAs generated by alternative splicing of a gene are not necessarily translated into functional proteins, it was necessary to determine whether the two type II collagen mRNAs could produce protein products. A diagram of the COL2A1 gene, type IIA and type IIB procollagen mRNAs, and the predicted procollagens is presented in Fig. 1. To determine whether two procollagens could be translated from the two mRNAs, media from cultures of freshly isolated juvenile costal chondrocytes were analyzed. These cells were chosen for analysis because we had previously demonstrated that they express both type IIA and type IIB collagen mRNAs (Ryan and Sandell, 1990) and do not contain detectable levels of type I collagen mRNA. Upon incubation of these cells with [^{3}H]proline, collagens and fibronectin were the most prominent radiolabeled components due to the relatively high content of proline in these matrix proteins.

Characterization of the two procollagen molecules and processing intermediates secreted by these chondrocytes demonstrated that they have the classical characteristics of specific procollagens including sensitivity to degradation by bacterial collagenase, pepsin sensitivity of the propeptide domains, predicted migration patterns on SDS-PAGE with and without reduction, and a cyanogen bromide peptide pattern characteristic of type II collagen. Fig. 2 *A* shows the pattern of collagen chains separated by SDS-PAGE under reducing conditions. Previous analysis of sequence implied from cDNA and genomic clones have shown that the NH_2-propeptide of type IIA (Ryan and Sandell, 1990; Su et al., 1989), the C-propeptide (Sandell et al., 1984) and triple helix (Sandell, et al., 1983; Upholt and Sandell, 1986) of type II collagen are similar to those of the α1 chain of type I collagen in amino acid content and peptide length with one exception: the NH_2-propeptide of type II procollagen contains a longer Gly-X-Y domain (30 amino acids) than type I procollagen. In analogy to α1(I) collagen, four forms of the type IIA chain were expected: pNC procollagen containing both

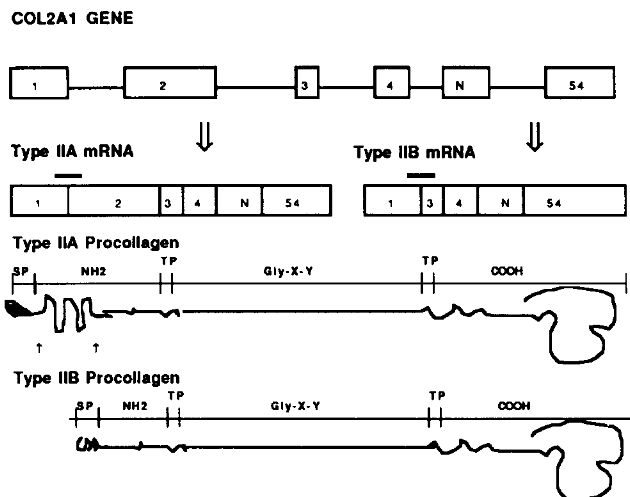

Figure 1. Diagram of the COL2A1 gene, type IIA and type IIB procollagen mRNAs, and type IIA and type IIB procollagens. Exons are indicated as open boxes. Oligonucleotide probes specific for type IIA or type IIB procollagen mRNA are indicated by bars above the respective mRNAs and described in Methods. In the procollagen molecules, SP is the signal peptide; NH_2 is the amino-terminal propeptide; TP is the telopeptide, Gly-X-Y indicates the triple helical domain, and COOH is the carboxy-terminal propeptide. The regions indicated by a straight line are triple helical and regions indicated by the curved lines are globular portions of the protein. The differentially spliced domain is indicated by arrows in the type IIA procollagen.

NH_2- and COOH-propeptides, pC collagen containing only the COOH-propeptide, pN collagen containing only the NH_2-propeptide, and the fully processed α1(II) chain. With the presence of two type II procollagens, an additional pNC (without exon 2) and pN (without exon 2) procollagen is predicted. Samples from two separate costal chondrocyte cultures were analyzed. Passage through a gelatin-Sepharose column removed fibronectin. Here, five collagenase-sensitive bands were observed, one alpha chain and four bands of procollagens. Under these reduced conditions, we predict that the pN (+ exon 2) procollagen cannot be resolved from the pC procollagen.

Subsequent analyses of the juvenile costal chondrocytes were performed on sample C-30. To determine which of the collagenase-sensitive bands 1–5 were procollagens, the sample was digested with pepsin under conditions whereby only the globular domains of the procollagens would be removed. Fig. 2 *B* shows the predicted pepsin resistance of the mature collagen alpha chain and the pepsin sensitivity of the four more slowly migrating bands: all predicted procollagens (lane *2*) were reduced to the size of alpha chains (lane *3*). To exclude the possibility that any of these bands could be derived from type III procollagen, an interstitial collagen that contains interchain pepsin-resistant disulfide bonds, the digested sample was electrophoresed without prior reduction of disulfide bonds (Fig. 2 *B*, lane *4*). The lack of trimeric molecules (>300,000 D) after pepsin digestion and electrophoresis under nonreducing conditions indicated that there was no type III procollagen in the sample.

The analysis presented in Fig. 2 *C* was designed to visualize the pN forms of type II procollagens. To resolve pN

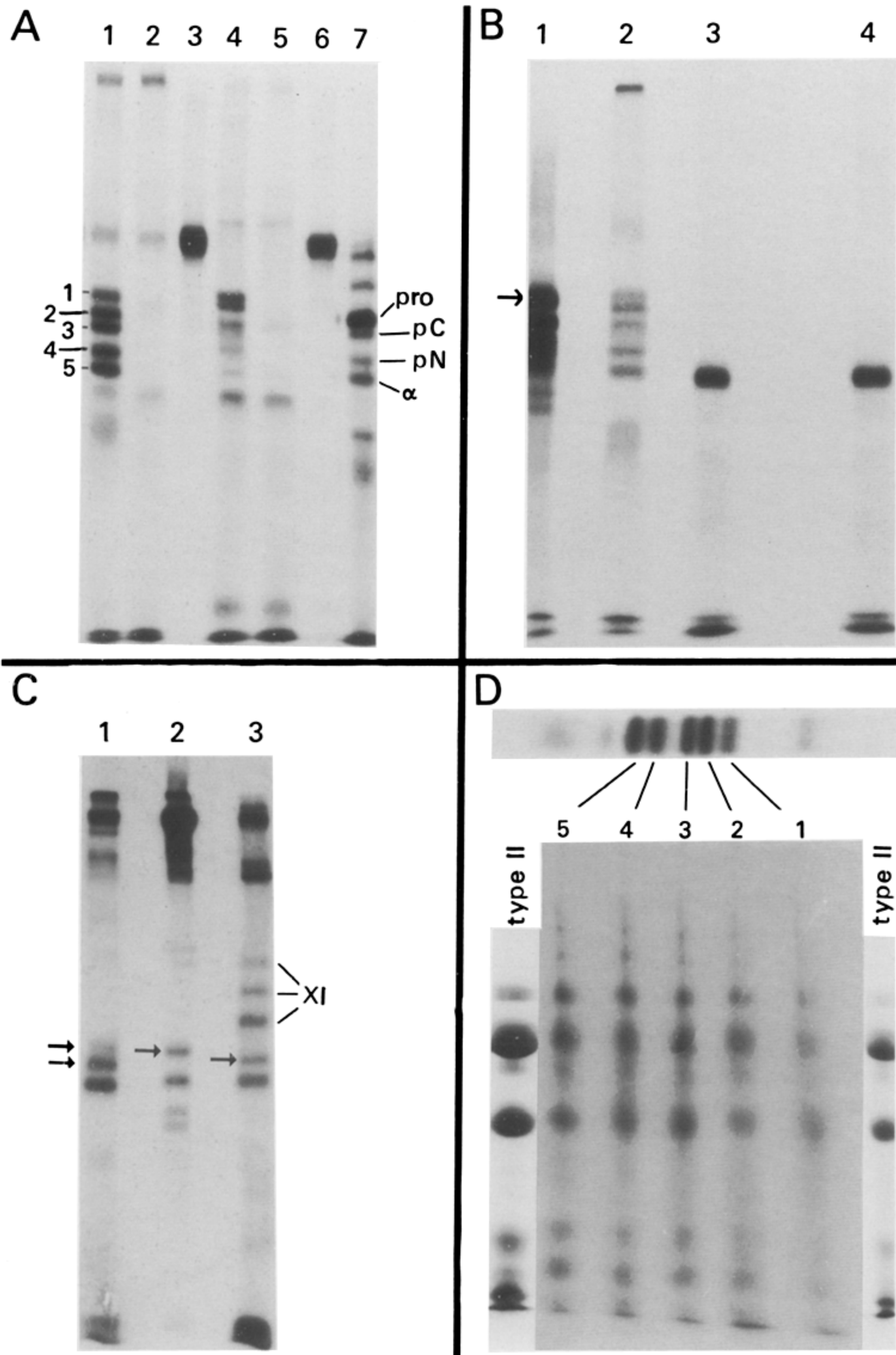

Figure 2. Identification of two type II procollagens. (*A*) [^{3}H]-Proline-labeled components of costal chondrocyte culture medium. Samples were passed through a gelatin-Sepharose column and analyzed by SDS-PAGE on a 5% gel before and after digestion with bacterial collagenase. Lane *1*, sample C30; lane *2*, sample C30 + bacterial collagenase; lane *4*, sample C29; lane *5*, sample C29 + bacterial collagenase. Lanes *3* and *6* are aliquots of material bound to gelatin sepharose (primarily composed of fibronectin) from each of the respective samples. Lane *7*, chick type II procollagen; each of the procollagen forms are indicated and the upper and lower bands are type XI and type IX collagen chains, respectively. All samples were reduced with β-mercaptoethanol before electrophoresis. (*B*) Analysis of human type II procollagen by SDS-PAGE after pepsin digestion. Pepsin digestion of type II procollagen removes propeptides and yields a single band corresponding to the α chain. Sample C30, shown in *A*, was digested with pepsin as described in Materials and Methods and analyzed by SDS-PAGE on a 5% gel. Lane *1*, isolated human type I procollagen. Lane *2*, sample C30. Lane *3*, sample C30 digested with pepsin. Lane *4*, sample C30 digested with pepsin and run without reduction of disulfide bonds. The arrow indicates the migration of pNC α1(I) and type IIA procollagens. (*C*) Analysis of human type II procollagen by SDS-PAGE without prior reduction of disulfide bonds. To more clearly analyze the pNα chains, sample C30 was electrophoresed on a 5% gel without reduction of disulfide bonds. Because they contain interchain disulfide bonds, procollagen and pC collagen (and type IX where present) migrate near the top of the gel. Lane *1*, sample C30; arrows indicate two possible forms of pNα and below these is the α chain. Lane *2*, human type I procollagen; arrow indicates the pNα(I) chain and below this are the α1, pNα2, and the α2 chains. Lane *3*, chick type II procollagen; arrow in lane *1* indicates the pNα chain which lacks the cysteine-rich domain and below this is the α chain. The chick sternal cartilage sample contains type XI, identified as previously described (Thom and Morris, 1991). (*D*) CNBr peptide mapping of type II procollagen chains. Sample C30 was resolved by SDS-PAGE on a 5% gel which is shown horizontally at the top. Electrophoretic migration was from right to left. Band 1 is the α chain, band 2 is pNIIB, band 3 is pCα and pNIIA, band 4 is pNCIIB and band 5, the slowest migrating, is the presumptive pNCIIA. After fluorography, each of the five bands was excised from the gel, digested with CNBr (see Materials and Methods), and analyzed by SDS-PAGE on a 10% gel. The CNBr digestion pattern of pepsin extracted human type II collagen is shown flanking the radiolabeled samples. These samples were electrophoresed on an identical SDS-PAGE and stained with Coomassie blue.

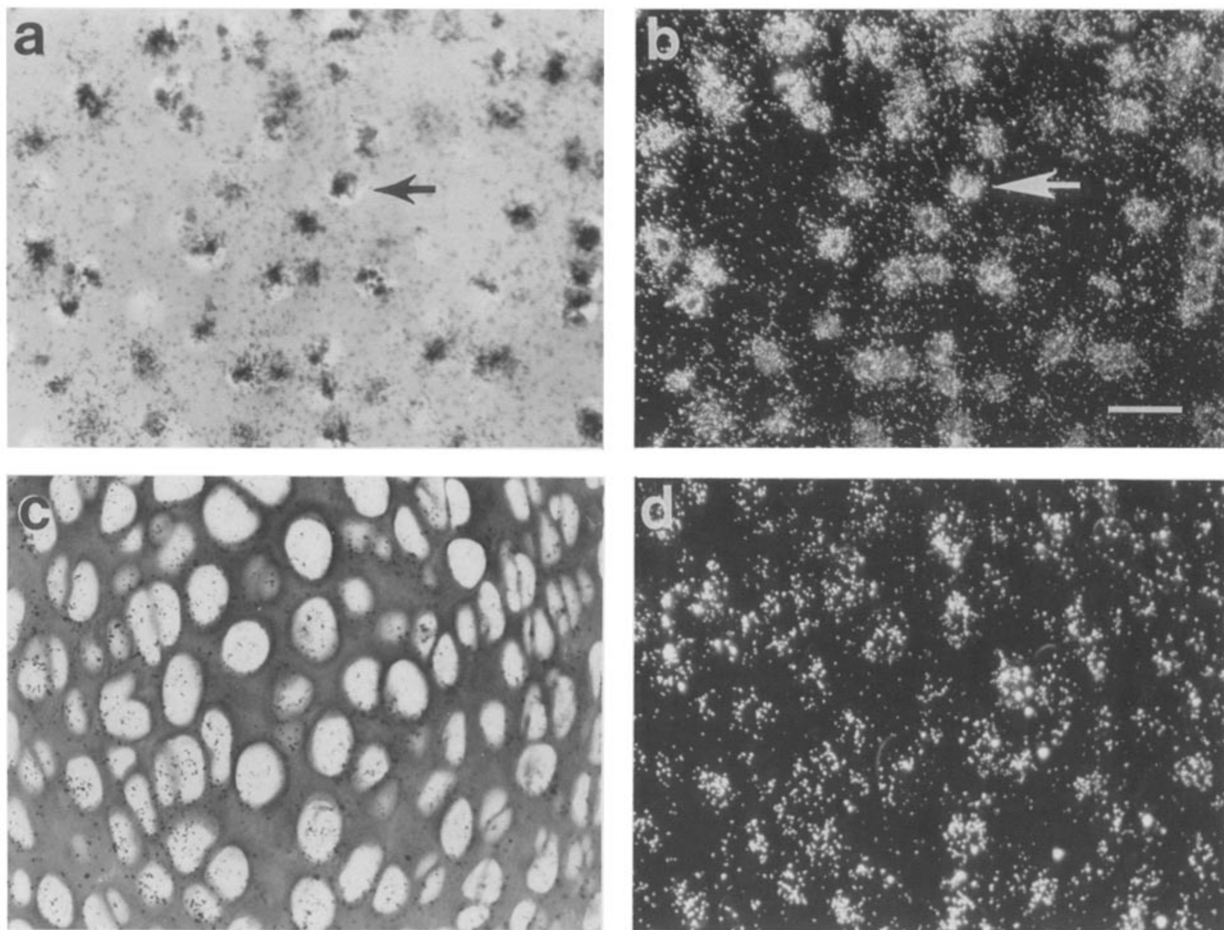

Figure 3. Expression of type IIB mRNA in cartilage. Sections of newborn bovine articular cartilage (*a* and *b*) and human fetal digit cartilage in the zone of chondrification (*c* and *d*) observed by both bright-field and dark-field microscopy. In *a* and *c*, positive hybridization is seen in all cells sectioned and the cells can be aligned with the grains visualized by dark field in *b* and *d* (for example, see arrows). Upon hybridization with the probe specific for type IIA, no positive hybridization was observed. In the dark-field photographs (*b* and *d*), the extent of background hybridization can be observed between cells in the extracellular matrix. Exposure time was 1 wk. Bar, 16 μm.

procollagens, advantage was taken of the fact that the COOH-propeptide contains interchain disulfide bonds and therefore procollagens containing this region, the pNC and pC (>375,000 D), form covalent trimers. These large trimers, without disulfide bond reduction, would migrate near the top of the 5% SDS-PAGE gel. Under nonreducing conditions, the pN procollagens still migrate in the middle of the lane and are consequently more readily resolved. The costal chondrocyte sample is compared to human type I procollagen and chick sternal type II. Unlike the SDS-PAGE gels shown in Fig. 2, *A* and *B*, multiple high molecular mass bands can be observed near the top of the gel. The pN procollagens are indicated by arrows and the mature alpha chain migrates ahead of the procollagens. In the type I collagen sample, the two predicted bands, α1(I) and pNα(I) procollagen are observed. In the chicken sternal chondrocyte sample, two chains are also predicted and observed. Note that the pNα1(I) chain (lane *2*) migrates more slowly than the chick sternal pNα1(II) (lane *3*) presumably because the pNα1(I) chain contains cysteine-rich domain while the chicken pNα1(II) chain does not. Chicken sternal chondrocytes express type IIB procollagen because, as we have shown previously and will show subsequently in this publication, the predominant form of type II collagen in cartilage mRNA is type IIB. In the costal chondrocyte sample, two procollagen bands were observed which migrated at sizes consistent with those of type IIA and type IIB pN procollagens.

To confirm that these procollagens were all forms of the type II collagen, cyanogen bromide peptide analysis of each gel band was performed (Fig. 2 *D*). Each band (labeled 1–5 as in Fig. 2 *A*) yielded identical peptide maps characteristic of type II collagen. The same pattern of peptides is observed in both type IIA and type IIB procollagens due to a cleavage at the methionine at amino acid three of the Gly-X-Y domain. The NH_2-propeptides cannot be detected. The five bands can be now identified as: band 1, pNC of type IIA; band 2, pNC of type IIB; band 4, pN of type IIB; band 5, the mature α chain. Band 3 (Fig. 2 *D*, and lane *3*) is presumably a mixture of pC and pN (+ exon 2) and shows only one cyanogen bromide peptide pattern.

Chondrocytes Synthesize Type IIB Procollagen

Previous results using Northern blot analysis of RNA indicated that type IIB procollagen was the major collagen ex-

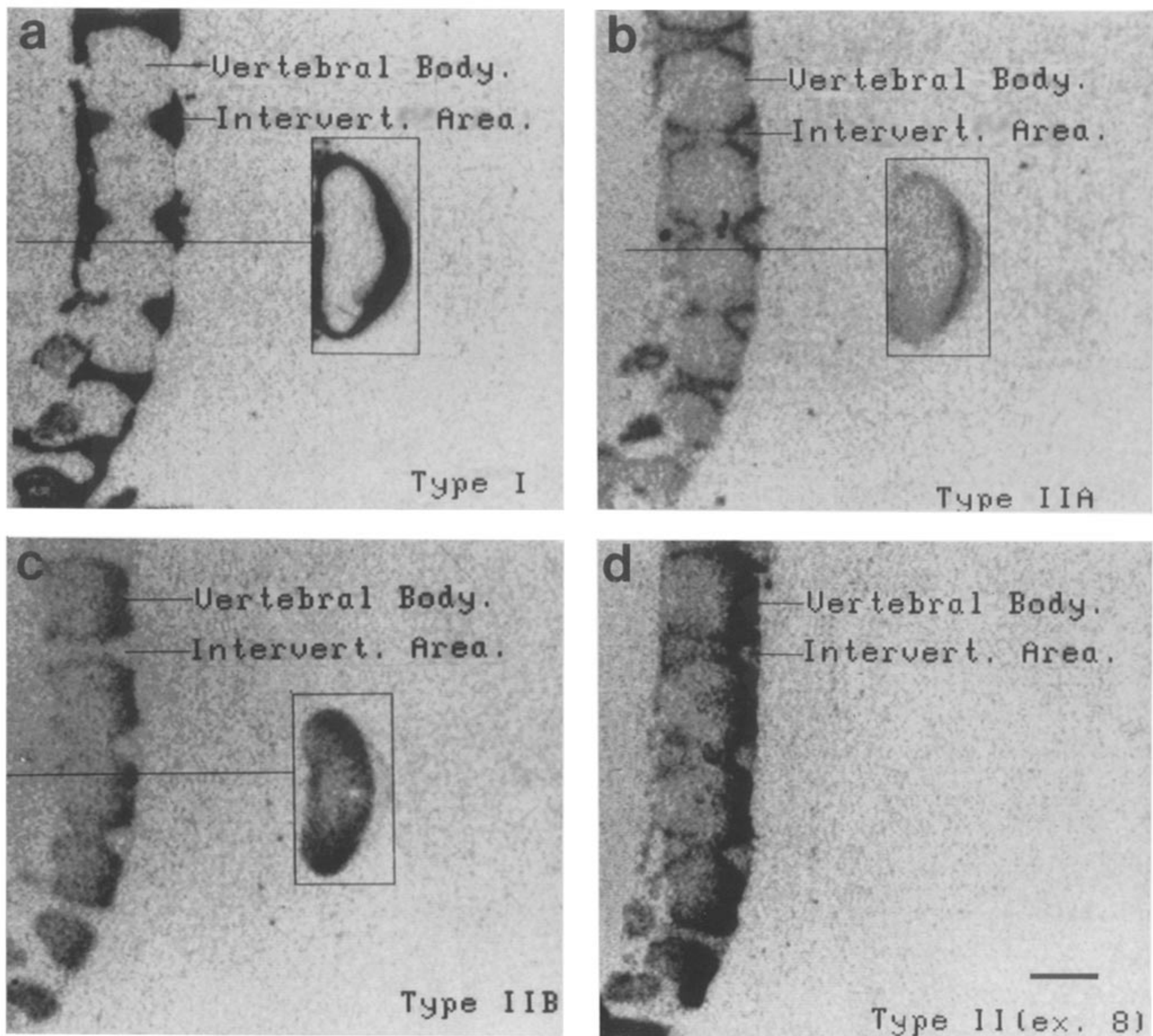

Figure 4. Tissue expression of type I, type IIA, and type IIB collagen mRNA in vertebral tissue observed by autoradiography of tissue sections. Mid-sagittal sections of vertebral column were hybridized with collagen-specific oligonucleotide probes: type I collagen (*a*); type IIA collagen (*b*); type IIB (*c*); and all type II collagens (*d*). In *a–c*, the insets represent a cross-sectional view hybridized with the same probes. Dorsal side is on the right and the spinal cord has been removed. Autoradiographs were analyzed with the aid of a MCID Image Analysis System without contrast enhancement. Type I procollagen is localized to the intervertebral area (*a*), while type IIA (*b*) apparently surrounds the intervertebral area. Type IIB (*c*) is observed only within the centrum/vertebral body. In *d*, a probe specific for exon eight of type II collagen shows the total distribution pattern of both type IIA and type IIB. Autoradiographs were exposed to film for 1-5 d. Bar, 0.6 mm.

pressed in cultured bovine articular chondrocytes, cultured human costal chondrocytes, and vertebral chondrocytes (Ryan and Sandell, 1990) and fetal bovine auricular and cultured fetal vertebral chondrocytes (Sandell, L. J. unpublished observations). Using oligonucleotide probes spanning the junction of exon 1 and exon 2 (type IIA) or exon 1 and exon 3 (type IIB), the expression of type II collagens was investigated by in situ hybridization to mRNA. These exon-spanning probes are the same probes previously used in Northern blot analysis: they are described in Materials and Methods and indicated in the diagram in Fig. 1. The results shown in Fig. 3 demonstrate the abundant expression of type IIB procollagen in fetal bovine articular cartilage (Fig. 3, *a* and *b*) and fetal human digit cartilage (Fig. 3, *c* and *d*). These tissues have morphological characteristics of cartilage, with rounded lacunae, abundant hyaline extracellular matrix and staining of the matrix with cresyl violet. Note that the cells show strong hybridization with the oligonucleotide probe, while the extracellular matrix shows only background hybridization. These tissues showed no hybridization with probes for type IIA procollagen or type I procollagen.

Localization of Type II Procollagen Expression in Specific Populations of Cells of the Developing Vertebral Column

The presence of both type IIA and type IIB collagen mRNAs

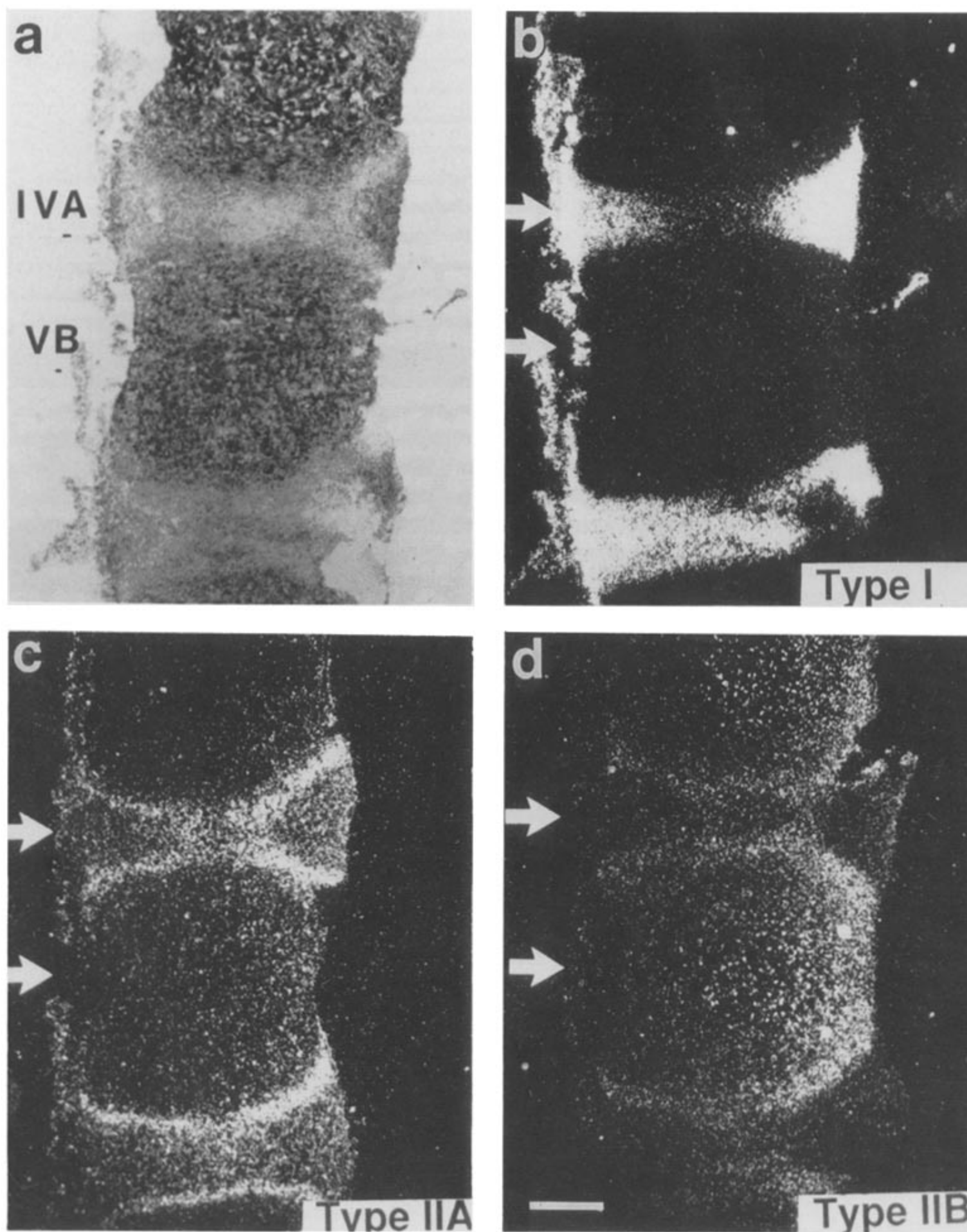

Figure 5. Serial sections of vertebral tissue hybridized with type I and type II collagen probes, stained with cresyl violet and observed by bright-field optics (*a*) and dark-field optics (*b–d*). The tissue architecture can be seen in *a*. The centrum/vertebral body (*VB*) contains rounded cells producing abundant extracellular matrix that stains pink with cresyl violet. The intervertebral area (*IVA*) is made up of cells that are fibroblastic in appearance and stain blue with cresyl violet. No extracellular matrix staining is observed in the IVA. Type I collagen mRNA is found in the intervertebral area and surrounding mesenchymal tissue in fibroblastic cells (*b*). Type IIA mRNA hybridized to fibroblast-like cells surrounding the intervertebral area (*c*) and type IIB hybridized with cells in the vertebral body. Exposure times were: 3 d (*b*); 2 wk (*c*); and 1 wk (*d*). Bar, 0.2 mm.

has been demonstrated in populations of total RNA isolated from fetal skeletal tissue and cultured costal chondrocytes. In preparations of isolated RNA, Northern blots probed with exon-spanning oligonucleotides indicated that there was relatively more type IIB collagen mRNA in fetal skeleton than type IIA collagen mRNA (Ryan and Sandell, 1990). Since the fetal skeleton is undergoing chondrogenesis and contains cells at various stages in the developmental process, the cellular localization of type IIA and type IIB collagen mRNAs was examined by in situ hybridization. The same exon-spanning oligonucleotide probes described previously were used. At this time of development (57 d), the vertebra were in a late stage of chondrification and the cells in the center of the more developed cranial vertebra were likely hypertrophic. Type I collagen mRNA, characteristic of mesenchymal cells was present in the intervertebral area (Fig. 4 *a*). Localization of type II mRNAs revealed that type IIA was localized in cells surrounding the cartilage (Fig. 4 *b*) in the prechondrogenic area that will eventually become the articular endplate of the vertebral body while type IIB was present primarily in cartilagenous tissue of the centrum (the precursor of the vertebral body) where extracellular matrix was accumulated around the cells (Fig. 4 *c*). Although most of the tissue has been lost in the freezing procedure, some type IIA can also be seen in the perichondrium remaining on the dorsal side of the vertebral body. The expression of collagens was not radially symmetrical across the tissue (seen in the cross sections) and appears to be more abundant on the ventral side of the vertebral body (sagittal section) at this plane of sectioning. Fig. 4 *d* shows the tissue hybridized with a probe for exon 8 of the COL2A1 gene. This exon is present in both type IIA and type IIB procollagens and hybridizes with all type II procollagen–synthesizing tissues as expected.

While the distribution of these collagen mRNAs was observed in the film autoradiographs, more resolution was obtained with emulsion autoradiography (Fig. 5). Cells in the

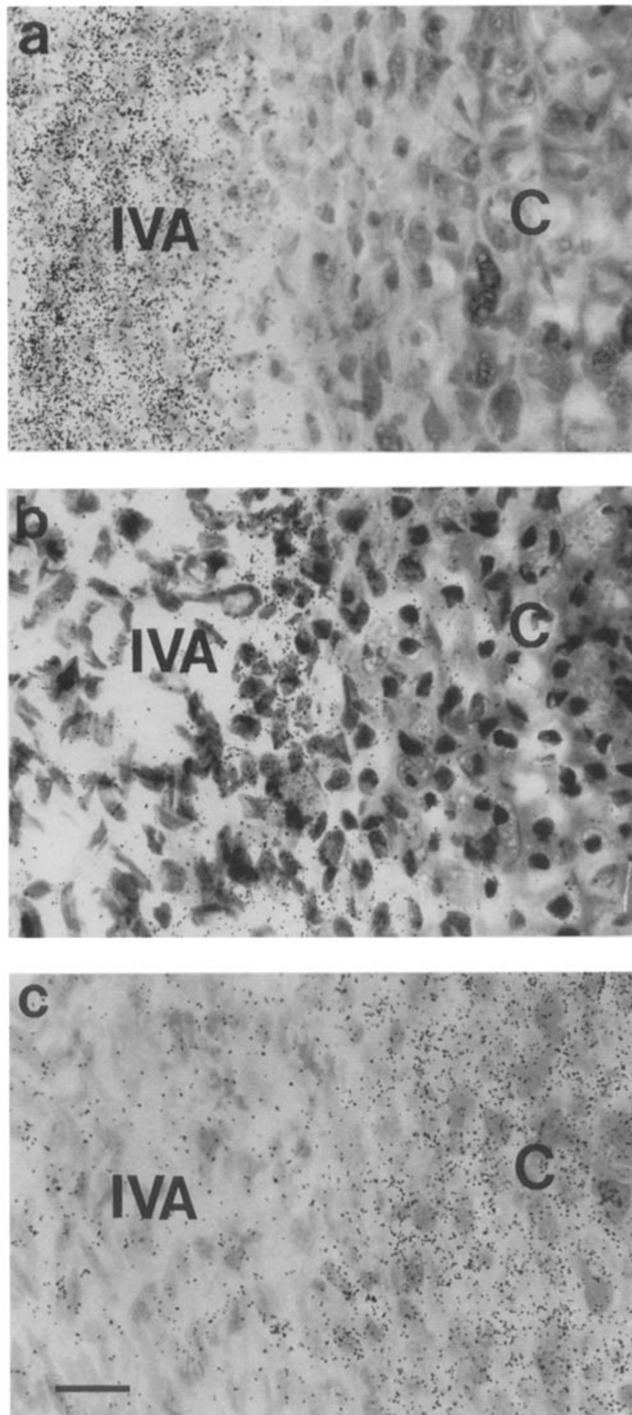

Figure 6. Expression of type I and type II collagens in serial sections of vertebral column. Type I procollagen mRNA (*a*), type IIA (*b*) and type IIB (*c*). Intervertebral tissue is on the left and vertebral tissue on the right. Type type I probe hybridizes only in the intervertebral area to cells fibroblastic in appearance while the type IIA hybridizes to a population of cells between the intervertebral area and the vertebral body. Type IIB collagen is localized to the rounded cells in the vertebral body. Exposure times were 3 d (*a*), 2 wk (*b* and *c*). Bar, 16 μm.

vertebral bodies (Fig. 5 *a*, *VB*) are rounded and the tissue is cartilagenous in morphology, while the cells surrounding the vertebral body and in the intervertebral area (*IVA*) have an elongated, fibroblastic character. Type I procollagen mRNA was synthesized by a distinct population of cells in the intervertebral area while type IIB procollagen mRNA was synthesized in cells of the vertebral body. Between the intervertebral area and the cartilagenous zone, a population of cells synthesized type IIA procollagen mRNA. Some hybridization to type IIA is also apparent in the IVA. From this data, it is not possible to determine whether a transitional population of cells synthesizes both type IIA and type IIB procollagen.

The cellular expression of types I, IIA, and IIB collagen mRNAs is shown in Fig. 6. This area is the junction between the cartilagenous tissue of the centrum/vertebral body and the intervertebral area. The progression from cells synthesizing type I to those synthesizing type IIA collagen to those synthesizing type IIB collagen can be seen. The particular morphology of the cells can also be observed. There appears to be an abrupt change in gene expression between the type I procollagen-synthesizing cells of the intervertebral area and the cells beginning to express type II procollagen. The cells expressing type IIA collagen accumulate very little extracellular matrix while the cells synthesizing type IIB collagen are surrounded by stainable cartilagenous matrix. From Fig. 6 *b*, it can be seen that the expression of type IIA is confined to a subset of cells that do not synthesize type I procollagen. However, it remains to be determined whether the same cells can synthesize both type IIA and type IIB procollagens. Certainly, the cells in the cartilagenous tissue synthesize only type IIB procollagen. From these results a three-dimensional model can be established where there is a cartilagenous core structure made up of cells synthesizing type IIB procollagen. The cartilagenous core is surrounded by tissue synthesizing type IIA procollagen.

Coexpression of a Cartilage Proteoglycan Aggrecan with Type IIB Procollagen

In light of the differential expression of type IIA and type IIB procollagen mRNAs, we determined whether mRNA for the other predominant cartilage extracellular matrix protein, the chondroitin sulfate proteoglycan, was expressed in a distinct pattern. Fig. 7 shows vertebral tissue hybridized with an oligonucleotide encoding a region of the core protein of the large cartilage aggregating proteoglycan, recently named aggrecan (Doege et al., 1990).

Expression of Type IIA Procollagen in Nonchondrocytic Cells

During the analysis of type II procollagen expression in the developing vertebral column, distinct expression of type II collagen mRNA was observed in the cells forming the facet joint and in the spinal ganglion. Fig. 8, *a–c* are cross sections of the vertebral column in a plane through the spinal ganglion. The spinal ganglion shows positive hybridization to the type IIA procollagen mRNA probe. Sagittal sections on a plane through the spinal ganglion and facet joint are also shown (Fig. 8, *d* and *e*). The beginning of facet joint formation between the vertebral processes can be seen at the right

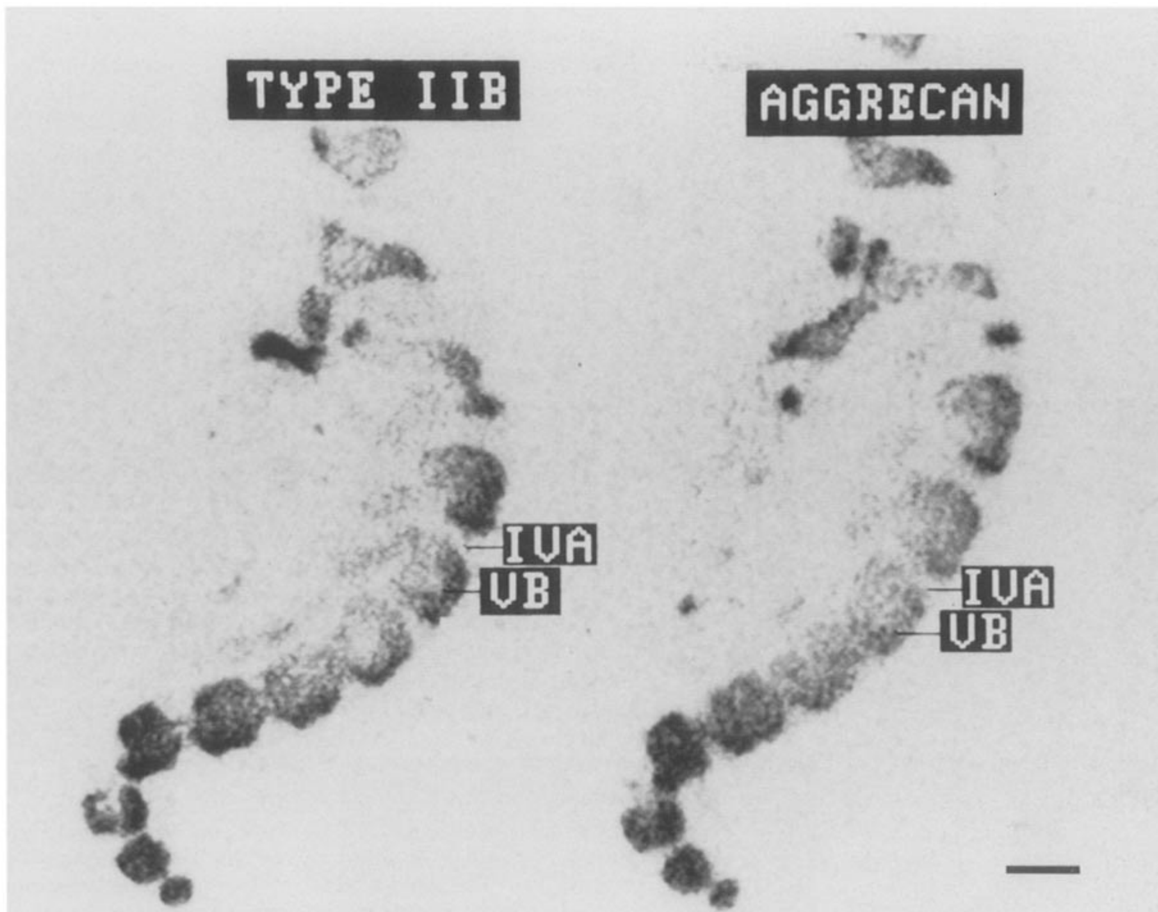

Figure 7. Autoradiograph of the localization of aggrecan mRNA and type IIB procollagen. Both probes hybridize to cells in the vertebral body and other cartilagenous structures at the hip of the embryo. Film was exposed for 3 d. Bar, 0.5 mm.

of the ganglion in this photograph. In Fig. 8 *e*, note the particularly high concentration of type IIA procollagen mRNA at the interface between the vertebral processes in the developing facet joint. Type IIB is not detected in these cells, but is abundant in the adjacent cartilage. Again, the cells synthesizing type IIA had morphological features characteristic of fibroblasts.

Discussion

Alternative splicing of pre-mRNA is now known to be of widespread importance for the generation of protein isoforms. By this mechanism functions ranging from intracellular and extracellular localization to enzyme activity can be modulated by expression of the appropriate protein domain. Alternative splicing is also used quantitatively to regulate gene expression by generating truncated open reading frames, or by regulating mRNA stability or translational efficiency via variability in the untranslated regions. For type II procollagen, we provide evidence that differential splicing of pre-mRNA regulates the expression of a protein domain of the procollagen molecule: that is, type IIA procollagen contains the cysteine-rich 69–amino acid domain of the NH_2-propeptide and type IIB procollagen does not. We further show that the expression of type IIA and type IIB procollagens is tissue-specific in developing vertebrae. Type IIB procollagen mRNA is the predominant type II collagen mRNA in cartilage and is coexpressed with mRNA for the cartilage-specific proteoglycan, aggrecan. The removal of exon 2 during processing of pre-mRNA is correlated with a change in cell morphology from spindle shaped to rounded and the increased accumulation of extracellular matrix. This differential expression of the NH_2-propeptide in a tissue-specific manner raises the following intriguing questions. What is the function of the NH_2-propeptide in type II and other interstitial collagens? Is there a role for type IIA procollagen in development? What are the factors that regulate the differential splicing of type II procollagen mRNA? Because of the relatively large alternatively spliced domain of type II procollagen (69 amino acids), its homology with other interstitial collagens, and the simplicity of the splicing pattern (exon 2: in or out), this system provides an excellent model for study of the regulation of alternative splicing and role of the NH_2-propeptide in interstitial collagens.

Synthesis of Type IIA and Type IIB Procollagens

Both type IIA and type IIB procollagens are synthesized and secreted by juvenile costal chondrocyte cultures. Previously, we had shown that these cultures synthesized substantial amounts of both type IIA and type IIB procollagen mRNA (Ryan and Sandell, 1990). The high level of synthesis of type

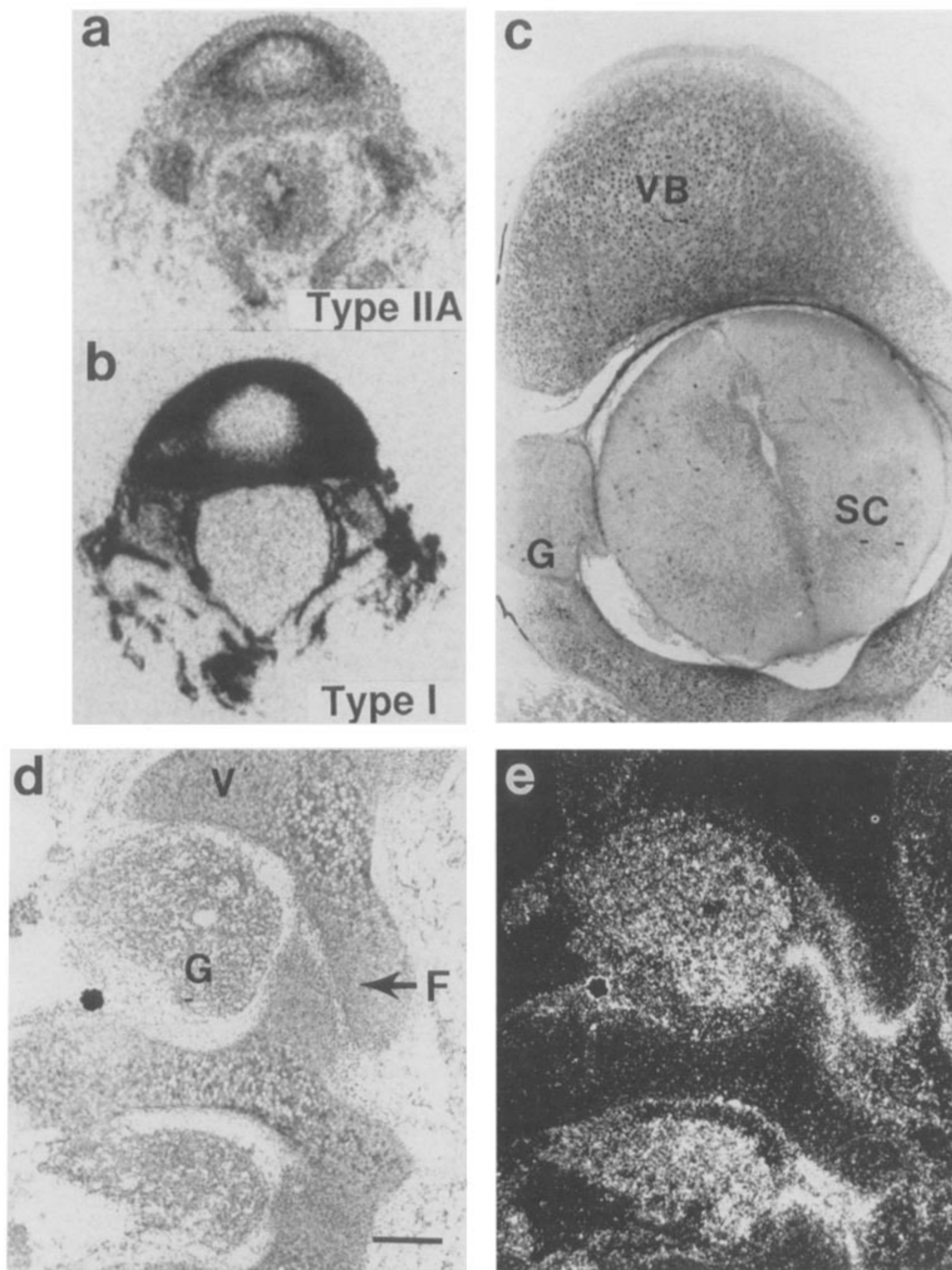

Figure 8. Autoradiograph showing differential expression of type I (*a*) and type IIA (*b*) in serial cross sections of a vertebral column. A section of the same region embedded in Epon for better resolution is shown in (*c*). Vertebral body (*VB*); spinal ganglion (*G*); spinal cord (*SC*). The type IIA probe hybridizes with a region of the vertebral body and the spinal ganglion; the type I probe hybridizes with the surrounding tissue, but not with the ganglion. *d* (bright field) and *e* (dark field) show sagittal sections through the spinal ganglion, vertebral process and facet joint hybridized with type IIA collagen probe. The type IIA probe hybridizes to the ganglion and to the cells surrounding the cartilagenous vertebral processes, particularly in the developing facet joint. Exposure times were 1 wk (*a*), 3 d (*b*), 2 wk (*e*). Bars, 0.2 mm.

IIA by these cells was somewhat surprising due to the predominant expression of type IIB in cartilage. However, these cells were derived from the pectus excavatum operation in which growing rib tips were removed, digested to free cells from the matrix, and cultured. In the cultures, the presence of type IIA could be accounted for by the cells undergoing chondrogenesis in the tissue or, on the other hand, in culture, these cells may be subject to less stringent controls permitting the synthesis type IIA procollagen mRNA. Evidence for the presence of both type IIA and type IIB procollagen in costal cartilage is provided by in situ hybridization studies with fetal ribs where both probes for type IIA and type IIB hybridized to the tissue (Sandell, L. J., and J. Sugai, unpublished observations).

From these studies, we do not know whether the type IIA and type IIB procollagens form homotrimers or heterotrimers. However, because type IIA and type IIB procollagens are synthesized primarily by different cell, and procollagen trimers are formed intracellularly, we assume that both type IIA and type IIB procollagens usually exist as homotrimers. A great deal of evidence indicates that the $\alpha3$ chain of type XI collagen is a product of the COL2A1 gene, implying that a type II procollagen chain can be incorporated into more than one type of collagen molecule (Burgeson and Hollister, 1979). When both type XI and type II collagen were isolated from the same cartilage tissue and separated on SDS-PAGE, the intact $\alpha3$ chain appeared to be ~10,000 D larger than the intact type II collagen chain (Wu, J., and D. Eyre, University of Washington, personal communication). This $\alpha3$ chain in bovine fetal cartilage is now known to be

identical to the type IIB procollagen shown in this study: the $\alpha3$ chain is larger than the α chain derived from type II collagen because the $\alpha3$ chain is not cleaved by the processing proteinase that removes the NH_2-propeptide from the type II molecule and, therefore, the amino acid sequence begins at the site of signal peptide cleavage. At this time, we do not know whether the chondroblasts or other type IIA collagen–containing tissues also express type XI collagen and consequently, we do not know whether type IIA collagen can also be incorporated into the type XI molecule.

Function of the NH_2-Propeptide

We have established the fact that two type II procollagen chains, differing in the presence or absence of the cysteine-rich domain of the NH_2-propeptide can be transcribed, translated, and secreted in chondrocyte cultures. As shown in Fig. 1, the NH_2-propeptide is characterized by a short globular domain followed by the interchain disulfide region, then a Gly-X-Y domain. In the type I collagen molecule, there are three chains: two $\alpha1(I)$ and one $\alpha2(I)$. The $\alpha1(I)$ chain contains the cysteine-rich domain while the $\alpha2(I)$ chain, due to a truncated exon in the COL1A2 gene, does not contain the cysteine-rich domain (see Sandell and Boyd, 1990, for a detailed comparison). Consequently, in overall structure of the procollagen, the type IIA chain resembles the $\alpha1(I)$ polypeptide and the type IIB chain resembles the $\alpha2(I)$ polypeptide. Cleavage of the NH_2- and COOH-terminal extension peptides generally occurs extracellularly by specific neutral proteases presumably prior to collagen fibrillogenesis. In collagens type I and II, the removal of the propeptides is concomitant with fibrillogenesis. In types III (Fleischmajer et al., 1981) and V collagen (Fessler et al., 1981), there is evidence for retention of the NH_2-propeptide in the mature collagen molecule. The propeptide may function after cleavage from the procollagen as the trimeric type I NH_2-propeptide has been isolated from bovine bone (Fisher et al., 1983) and is present in serum indicating that the NH_2-propeptide may have a role independent of its function in the intact procollagen.

Two major functions have been suggested for the NH_2-propeptide: (*a*) it plays a role in fibrillogenesis and (*b*) it plays a role in feedback inhibition of collagen synthesis. For example, Fleischmajer et al. (1985) reported that the diameter of collagen type I fibers was decreased from 35–40 to 20–30 nm by the presence of pN procollagen and that polymerization of the thin fibrils involved polymerization of pN collagen. The effect is thought to be physiologically relevant as they found significant amounts of pN collagen in chicken embryonic skin and showed that the ratio of pN procollagen to processed collagen varies with age, presumably due to different levels of N-proteinase activity. The role of the NH_2-propeptide in feedback regulation of collagen synthesis has been suggested by studies on dermatosporactic sheep and the human disease Ehlers-Danlos Syndrome Type IV. In both of these diseases, the NH_2-propeptide is retained in the mature collagen molecule and increased synthesis of both type I and type III collagens was observed. When isolated NH_2-propeptides of type I and type III collagen were added directly to cultured fibroblasts, collagen synthesis was inhibited (Weistner et al., 1979). Other in vitro studies support a function for the propeptides in regulation of biosynthesis that may be mediated by a cell surface receptor (Weistner et al., 1979; Paglia et al., 1981; Horlein et al., 1981; Wu et al., 1986, 1991; Fleischmajer et al., 1990). In addition, there may be as yet uncharacterized interactions of the collagen NH_2-propeptide with other extracellular matrix molecules.

Chondrogenesis

The vertebral column is derived from mesenchymal cells that arise from the sclerotomes of the somites. Each sclerotome consists of two regions: loosely arranged cells cranially and densely packed cells caudally. It is commonly thought that the annulus fibrosis of the intervertebral disc is derived from the densely packed cells which move cranially to align with the center of the myotome (Moore, 1988). The mesenchymal cells of the centrum are derived from the remaining densely packed cells, which fuse with the loosely arranged cells of the caudal sclerotome. Consequently, the centrum develops from two adjacent sclerotomes. Chondrification begins in two lateral centers of the centrum during the sixth week of embryogenesis and continues until the centers fuse to form the cartilagenous centrum. The chondrocytes in the center of the centrum/vertebral body begin to hypertrophy and ossification begins soon thereafter. The cells surrounding the centrum/vertebral body that express exon 2 are considered to serve as chondrogenic zones for the centra (O'Rahilly and Gardner, 1978). Consequently, the splicing out of exon 2 may mark a new stage in chondrogenesis. This event could be responsible for determining whether type II mRNA containing cells proceed to chondrogenesis or proceed along another developmental pathway. The factor(s) necessary to remove exon 2 may be part of the chondrogenic phenotype and the control of this switch may be causal in chondrogenesis. On the other hand, the factors necessary for control may be present in the prechondrocytes directing inclusion of exon 2. The mesenchymal and fibroblast-like cells previously found to contain type II collagen mRNA (Hayashi et al., 1986) likely express the type IIA procollagen form that contains the NH_2-propeptide, while the chondrocytic cells express the alternatively spliced type IIB form. These cells, called the zone of progenitor cells in mouse neonatal condyles, incorporate [^{3}H]thymidine at a higher rate than surrounding cell populations (Shurtz-Swinski et al., 1989). The distinct cellular localization of the two different type II procollagens indicates that they may play different functional roles in distinct tissues. The expression of the NH_2-propeptide is correlated with a low level of extracellular matrix deposition, while removal of exon 2 is correlated with increased synthesis and accumulation of the characteristically abundant cartilage matrix. The expression of type IIA procollagen mRNA present in the cartilage surrounding the vertebral body persists at least up to 122 d of gestation (Sandell, L. J., and J. Sugai, unpublished observations). This localization of type IIA procollagen during chondrogenesis is remarkably similar to the expression of the 6.4-kb form of cytotactin mRNA, also an alternatively spliced species (Prieto et al., 1990), in embryonic chicken vertebral column.

Type IIA procollagen is also particularly abundant in the regions destined to form the articular cartilage of joint tissue both in the cartilage surrounding the vertebral bodies (Fig.

4) and in the cartilage joining the dorsal vertebral processes (Fig. 8). Type IIA procollagen distribution compared to type IIB procollagen distribution indicates that type IIA may be particularly localized in interface regions. Although a highly speculative suggestion, it is possible that type IIA procollagen expressed by these noncartilagenous cells functions to identify the region of joint formation.

These results have important implications for the regulation of the chondrocyte phenotype. It has previously been assumed that the phenotype was established by regulation at the level of transcription. Evidence showing increased accumulation of type II collagen mRNA and core protein as chondrogenesis proceeded in vitro (Vuorio et al., 1982) supported the theory that as mesenchymal cells differentiated into chondrocytes, both type II collagen and proteoglycan gene expression increased coordinately. Upon closer examination, however, it was found quite consistently that the expression of type II mRNA preceded overt chondrogenesis (Kravis and Upholt, 1985; Kosher et al., 1986) and that some nonchondrocytic cells surrounding chondrogenic tissue contained type II procollagen mRNA (Hayashi et al., 1986). We would predict that the expression of type II collagen in fibroblastic cells (Hayashi et al., 1986) as well as the prechondrogenic expression of mRNA established by Kosher et al., (1986) and Kravis and Upholt (1985) is due to the expression of type IIA. Therefore, the expression of aggrecan may be coordinated with a change in the mRNA splice pattern of type II collagen in addition to an increase in transcription. It will be necessary to define the isoform of type II collagen synthesized in a variety of developmental and repair systems as all previous studies on type II collagen mRNA and type II collagen, in which the collagen was identified by cyanogen bromide mapping or type II-specific antibodies, would not have detected a difference between type IIA and type IIB procollagens.

Alternative splicing of pre-mRNA appears to play a role in development. Expression of alternatively spliced forms of fibronectin are correlated with development (ffrench-Constant and Hynes, 1988) and wound healing (ffrench-Constant et al., 1989), however, the function of these alternatively spliced forms and their existence as fibronectin molecules have not been determined. In chondrocytes, two examples of alternative exon usage have been described that are relevant to the cartilage phenotype. First, type IX collagen, originally thought to be cartilage specific, is expressed in developing cornea, although a different promoter is used and a specific protein domain is removed (Nishimura et al., 1989; Muragaki et al., 1990). Secondly, Adams and colleagues (Bennett et al., 1989; Bennett and Adams, 1990) have shown that the COL1A2 gene is expressed in chondrocytes, but again, a different promoter is employed yielding a protein product that is not collagenous. It has been suggested that both the alternative COL1A2 product (Bennett and Adams et al., 1990) and the type I collagen NH_2-propeptide (Wu et al., 1986) may function to inhibit collagen synthesis at a pretranslational level. Taken together, a pattern of events is beginning to emerge which determines the cartilage phenotype. These events are likely controlled by regulatory elements in the nucleus either acting on the DNA directly or on processing of pre-mRNA. Investigation of these nuclear events will broaden our understanding of chondrogenesis, cartilage repair processes, and skeletal dysplasias.

Expression of Type II Procollagen mRNA in Nonchondrogenic Embryonic Tissue

The importance of a change in type II procollagen form is revealed by the expression of type IIA procollagen in nonchondrocytic tissues and expression of type IIB procollagen in chondrocytes. A cell may not have the ability to exclude exon 2 by alternative splicing either due to the lack of a specific splicing factor or repression of alternative splicing and, as a consequence, proceed along with a completely different developmental route. Indeed, von der Mark and colleagues (von der Mark et al., 1976) have shown that type II collagen accumulates in the cell-free region adjacent to the embryonic notochord into which somitic sclerotomal cells expand prior to differentiating into vertebral cartilage. We have observed type IIA procollagen mRNA, in situ, in the notochordal remnants of the developing intervertebral disk nucleus pulposus. The expression of type II procollagen in noncartilagenous portions of the embryo and spinal ganglion was surprising. However, in early embryogenesis, the notochord induces the overlying ectoderm to form the neural plate. The neural ectoderm differentiates into the central nervous system and spinal cord and cells from the neural ectoderm migrate to form the spinal ganglia, the medulla of the adrenal gland, pigment cells, and other connective tissues of the head and neck. Possibly, in contrast to mature tissues, type II procollagen plays a different, more ubiquitous role during development. Indeed, Thorogood et al. (1986), have found type II collagen at the epitheliomesenchymal interface and suggested that type II collagen may be instructive in future differentiation of the cartilagenous neurocranium. Finally, the recent findings of Kosher and Solursh (1989) showing immunoreactive type II collagen in a variety of embryonic basement membranes may be related to the expression of type IIA at the interfaces of developing vertebral column. Taken together, these results suggest the possibility that type II collagen expression in these areas is actually type IIA procollagen and chondrogenesis is dependent on the removal of exon 2 to synthesize type IIB procollagen. These results indicate that type II procollagen may play a more widespread role than previously suspected, particularly in developing tissues.

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References

Baldwin, C. T., A. M. Reginato, C. Soth, S. A. Jimenez, and D. J. Prockop. 1989. Structure of cDNA clones coding for human type II procollagen. *Biochem. J.* 262:521-528.

Bennett, V. D., and S. L. Adams. 1990. Identification of a cartilage-specific promoter within intron 2 of the chick α2(I) collagen gene. *J. Biol. Chem.* 265:2223-2230.

Bennett, V. D., I. M. Weiss, and S. L. Adams. 1989. Cartilage-specific 5′ end of chick α2(I) collagen mRNAs. *J. Biol. Chem.* 264:8402-8409.

Burgeson, R. E., and D. W. Hollister. 1979. Collagen heterogeneity in human cartilage: identification of several new collagen chains. *Biochem. Biophys. Res Commun.* 87:1124-1131.

Burgeson, R. E., F. A. Adli, I. I. Kaitila, and D. W. Hollister. 1976. Fetal membrane collagens: identification of two new collagen alpha chains. *Proc. Natl. Acad. Sci. USA.* 73:2579-2983.

d'Allesio, M., M. Bernard, V. Benson-Chanda, M. L. Chu, L. Dickson, D.

Weil, and F. Ramirez. 1988. Complete nucleotide sequence of the region encompassing the first twenty-five exons of the human pro-α1(I) collagen gene (COL1A1). *Gene (Amst.)*. 67:105–115.

Doege, K., C. Rhodes, M. Sasaki, J. R. Hassell, and Y. Yamada. 1990. Molecular biology of cartilage proteoglylcan (aggrecan) and link protein. *In* Extracellular Matrix Genes. L. J. Sandell and C. D. Boyd, editors. Academic Press, Inc. Orlando, FL. 137–155.

Fessler, L. I., C. A. Kumamoto, M. E. Meis, and J. H. Fessler. 1981. Biosynthesis of procollagen [pro alpha $1(V)_2$ pro alpha 2(V)] by chick tendon fibroblasts and procollagen pro alpha $1(V)_3$ by hamster lung cell cultures. *J. Biol. Chem.* 256:9640–9645s.

ffrench-Constant, C. L., and R. O. Hynes. 1988. Alternative splicing of fibronectin is temporally and spatially regulated in the chicken embryo. *Development.* 106:375–388.

ffrench-Constant, C., L. Van De Water, H. Dvorak, and R. O. Hynes. 1989. Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. *J. Cell Biol.* 109:903–914.

Fisher, L. W., P. G. Robey, N. Tuross, A. Otsuka, D. Tepen, F. Esch, S. Shimasaki, and J. Termine. 1987. The M_r 24,000 phosphoprotein from developing bone is the NH_2-terminal propeptide of the α1 chain of type I collagen. *J. Biol. Chem.* 262:13457–13463.

Fleischmajer, R., R. Timpl, L. Tuderman, L. Raisher, M. Wiestner, J. S. Perlish, and P. N. Graves. 1981. Ultrastructural identification of extension aminopropeptide of type I and III collagens in human skin. *Proc. Natl. Acad. Sci. USA.* 78:7360–7364.

Fleischmajer, R., B. R. Olsen, R. Timpl, J. S. Perlish, and O. Lovelace. 1983. Collagen fibril formation during embryogenesis. *Proc. Natl. Acad. Sci. USA.* 80:3354–3358.

Fleischmajer, R., J. S. Perlish, and R. Timpl. 1985. Collagen fibrillogenesis in human skin. *Anal. NY Acad. Sci.* 460:246–257.

Fleischmajer, R., J. S. Perlish, R. E. Burgeson, F. Shaikh-Bahai, and R. Timpl. 1990. Type I and type III collagen interactions during fibrillogenesis. *Ann. NY Acad. Sci.* 580:161–175.

Goldring, M. B., L. J. Sandell, M. L. Stephenson, and S. M. Krane. 1986. Immune interferon suppresses levels of procollagen mRNA and type II collagen synthesis in cultured human articular and costal chondrocytes. *J. Biol. Chem.* 261:9049–9056.

Hayashi, M., Y. Ninomiya, J. Parsons, K. Hayashi, B. R. Olsen, and R. L. Trelstad. 1986. Differential localization of mRNAs of collagen types I and II in chick fibroblasts, chondrocytes, and corneal cells by in situ hybridization using cDNA probes. *J. Cell Biol.* 102:2302–2309.

Horlein, D., J. McPherson, S. H. Goh, P. Bornstein. 1981. Regulation of protein synthesis: translational control by procollagen-derived fragments. *Proc. Natl. Acad. Sci. USA.* 78:6163–6167.

Kohno, K., G. R. Martin, and Y. Yamada. 1984. Isolation and characterization of a cDNA clone for the amino-terminal portion of the pro-α1(II) chain of cartilage collagen. *J. Biol. Chem.* 259:13668–13673.

Kosher, R. A., and M. Solursh. 1989. Widespread distribution of type II collagen during embryonic chick development. *Dev. Biol.* 131:558–566.

Kosher, R. A., W. M. Kulyk, and S. W. Gay. 1986. Collagen gene expression during limb cartilage. *J. Cell Biol.* 102:1151–1156.

Kravis, D., and W. B. Upholt. 1985. Quantitation of type II procollagen mRNA levels during chick limb cartilage differentiation. *Dev. Biol.* 108:164–172.

Lewis, M. E., T. G. Sherman, and S. J. Watson. 1985. *In situ* hybridization histochemistry with synthetic oligonucleotides: strategies and methods. *Peptides.* 2:75–87.

Linsenmayer, T. F., and C. D. Little. 1978. Embryonic neural retina collagen: *in vitro* synthesis of high molecular weight forms of type II plus a new genetic type. *Proc. Natl. Acad. Sci. USA.* 75:3235–3239.

Linsenmayer, T. F., G. N. Smith, and E. D. Hay. 1977. Synthesis of two collagen types by embryonic chick corneal epithelium *in vitro*. *Proc. Natl. Acad. Sci. USA.* 74:39–43.

Moore, K. L. 1988. *The Developing Human.* M. Wonsiewicz, editor. W. B. Saunders Co., Philadelphia. 334–348.

Morris, N. P., and H. P. Bachinger. 1987. Type II collagen is a heterotrimer with the composition (1α, 2α, 3α) retaining non-triple-helical domain. *J. Biol. Chem.* 262:11345–11350.

Morris, N. P., D. R. Keene, R. W. Glanville, H. Bentz, and R. E. Burgeson. 1983. Isolation and partial characterization of a new human collagen with an extended triple-helical structural domain. *J. Biol. Chem.* 261:5638–5644.

Muragaki, Y., I. Nishimura, A. Henney, Y. Ninomiya, and B. R. Olsen. 1990. The α1(IX) collagen gene gives rise to two different transcripts in both mouse embryonic and human fetal RNA. *Proc. Natl. Acad. Sci. USA.* 87:2400–2404.

Newsome, D. A., T. F. Linsenmayer, and R. L. Trelstad. 1976. Vitreous body collagen. Evidence for a dual origin from the neural retina and hyalocytes. *J. Cell Biol.* 71:59–67.

Nishimura, I., Y. Muragaki, and B. R. Olsen. 1989. Tissue-specific forms of type IX collagen-proteoglycan arise from the use of two widely separated promoters. *J. Biol. Chem.* 264:20033–20041.

O'Rahilly, R., and E. Gardner. 1978. The Embryology of Movable Joints. Leon Sokoloff, editor. *In* The Joints and Synovial Fluid, Vol. 1. Academic Press, Inc., New York. 49–103.

O'Rahilly, R., and D. M. Meyer. 1979. The timing and sequence of events in the development of the human vertebral column during the embryonic period proper. *Anat. Embryol.* 157:167–176.

Paglia, L. M., M. Wiestner, M. Duchene, L. A. Ouelette, D. Horlein, G. R. Martin, and P. K. Muller. 1981. Effects of procollagen peptides on the translation of type II collagen messenger ribonucleic acid and on collagen biosynthesis in chondrocytes. *Biochemistry.* 20:3523–3527.

Prieto, A. L., F. S. Jones, B. A. Cunningham, K. L. Crossin, and G. M. Edelman. 1990. Localization during development of alternatively spliced forms of cytotactin mRNA by in situ hybridization. *J. Cell Biol.* 111:685–698.

Ryan, M. C., and L. J. Sandell. 1990. Differential expression of a cysteine-rich domain in the amino-terminal propeptide of type II (cartilage) procollagen by alternative splicing of mRNA. *J. Biol. Chem.* 265:10334–10339.

Ryan, M. C., M. Sieraski, and L. J. Sandell. 1990. The human type II procollagen gene: identification of an additional protein-coding domain and location of potential regulatory sequences in the promoter and first intron. *Genomics.* 8:41–48.

Sandell, L. J., and C. D. Boyd. 1990. Conserved and divergent sequence and functional elements within collagen genes. *In Extracellular Matrix Genes.* L. J. Sandell, and C. D. Boyd, editors. Academic Press, Inc, New York. 1–56.

Sandell, L. J., Y. Yamada, A. Dorfman, and W. B. Upholt. 1983. Isolation and characterization of genomic DNA coding for type II collagen. *J. Biol. Chem.* 258:11617–11621.

Sandell, L. J., H. Prentice, D. Kravis, and W. Upholt. 1984. Structure and sequence of the 3'-end of the chicken type II procollagen gene: characterization of the region encoding the carboxy-telopeptide and propeptide. *J. Biol. Chem.* 259:7826–7834.

Shepard, T. 1975. Normal and abnormal growth patterns: growth and development of the human embryo and fetus. *In* Endocrine and Genetic Diseases of Childhood and Adolescence. L. I. Gardner, editor. W. B. Saunders Co. Philadelphia. 1–8.

Shurtz-Swirski, R., D. Lewinson, P. Shenzer, and M. Silbermann. 1989. Effects of different concentrations of serum on cartilage growth in an organ culture system. *In Vitro Cell. & Dev. Biol.* 25:995–999.

Sokolov, B. P., M. B. Sher, and V. N. Kalinin. 1989. Modified method for peptide mapping of collagen chain using cyanogen bromide-cleavage of protein within polyacrylamide gel. *Anal. Biochem.* 176:365–367.

Smith, G. N., T. F. Linsenmayer, and D. A. Newsome. 1976. Synthesis of type II collagen *in vitro* by embryonic chick neural retina tissue. *Proc. Natl. Acad. Sci. USA.* 73:4420–4428.

Stirpe, N. S., K. T. Dickerson, and P. F. Goetinck. 1990. The chick embryonic mesonephros synthesizes link protein, an extracellular matrix molecule usually found in cartilage. *Dev. Biol.* 137:419–424.

Su, M. W., V. Benson-Chanda, H. Vissing, and F. Ramirez. 1989. Organization of the exons coding for pro αl(II) collagen N-propeptide confirms a distinct evolutionary history of this domain of the fibrillar collagen genes. *Genomics.* 4:438–441.

Thom, J. R., and N. P. Morris. 1991. Biosynthesis and proteolytic processing of type XI collagen in embryonic chick sterna. *J. Biol. Chem.* 266:7262–7269.

Thorogood, P., J. Bee, and K. von der Mark. 1986. Transient expression of collagen type II at epitheliomesenchymal interfaces during morphogenesis of the cartilagenous neurocranium. *Dev. Biol.* 116:497–509.

Upholt, W. B., and L. J. Sandell. 1986. Exon/intron organization of the chicken type II procollagen gene: intron size distribution suggests a minimal intron size. *Proc. Natl. Acad. Sci. USA.* 83:2325–2329.

von der Mark, K., H. von der Mark, R. Timpl, and R. L. Trelstad. 1977. Immunofluorescent localization of collagen types I, II, and III in the embryonic chick eye. *Dev. Biol.* 59:75–85.

von der Mark, H., K. von der Mark, and S. Gay. 1976. Study of differential collagen synthesis during development of the chick embryo by immunofluorescence. I. Preparation of collagen type I and type II antibodies and their application to early stages of chick embryo. *Dev. Biol.* 48:237–249.

Vuorio, E., L. J. Sandell, D. Kravis, V. C. Sheffield, T. Vuorio, A. Dorfman, and W. B. Upholt. 1982. Construction and partial characterization of two recombinant cDNA clones for procollagen from chicken cartilage. *Nucleic Acids Res.* 10:1175–1192.

Wiestner, M., T. Kreig, D. Horlein, R. W. Glanville, R. Fietsek, and P. K. Muller. 1979. Inhibiting effect of procollagen peptides on collagen biosynthesis in fibroblast cultures. *J. Biol. Chem.* 254:7016–7023.

Wu, C. H., C. B. Donovan, and G. Y. Wu. 1986. Evidence for pretranslational regulation of collagen synthesis by procollagen propeptides. *J. Biol. Chem.* 261:10482–10484.

Wu, C. H., C. M. Walton, and G. Y. Wu. 1991. Propeptide-mediated regulation of procollagen synthesis in IMR-90 human lung fibroblast cell cultures. *J. Biol. Chem.* 266:2983–2987.