Isolation of bovine type X collagen and immunolocalization in growth-plate cartilage

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Type X collagen was extracted with 1 M-NaCl and 10 mM-dithiothreitol at neutral pH from fetal-bovine growth cartilage and purified to homogeneity by using f.p.l.c. gel filtration on a Superose 12 column, followed by ion-exchange chromatography on a Mono Q column. The purified protein migrates in SDS/polyacrylamide gels with an apparent M_r of 58000 under reducing conditions and as a high- M_r oligomer in its unreduced form. The amino acid composition is similar to the published composition of chick type X collagen. Pepsin digestion at 4 °C decreases the M_r of the monomer to 43000; purified bacterial collagenase digests most of the molecule, leaving a non-collagenous domain of apparent M_r 15000, which probably represents the C-terminal globular domain. The IgG fraction from a rabbit antiserum raised against purified bovine type X collagen was specific for this collagen by the criteria of e.l.i.s.a. and immunoblotting after immunoabsorption with collagen types I, II, IX and XI. Immunofluorescence localization of type X collagen in sections of fetal-bovine and human cartilage was possible after acetone fixation of sections and hyaluronidase treatment. Type X collagen was restricted to the zone of hypertrophic and calcified cartilage inside the bone spicules of the growth plate.

INTRODUCTION

During long-bone development, chondrocytes differentiate along a series of discrete developmental stages which can be defined not only by morphological criteria, but also by their pattern of collagen types synthesized at each stage (for review, see von der Mark, 1980, 1986). Chondrocyte precursor cells in the undifferentiated limb mesenchyme synthesize type I collagen, this changing to type-II-collagen synthesis with the onset of chondrogenesis (Linsenmayer *et al.*, 1973; von der Mark *et al.*, 1976). The discovery of type X collagen in the culture medium of chondrocytes (Gibson *et al.*, 1982; Capasso *et al.*, 1982) and in the zone of growth cartilage (Schmid & Conrad, 1982) opened the possibility for unequivocal identification of hypertrophic chondrocytes *in situ* and *in vitro* with biochemical, recombinant DNA or immunological tools (Gibson *et al.*, 1983; Schmid & Linsenmayer, 1983, 1985a,b; Capasso *et al.*, 1984; for review see Schmid & Linsenmayer, 1987). Thus it has been possible to monitor precisely the differentiation in cell culture of embryonic-type-II-collagen-producing chick chondrocytes ('stage I chondrocytes'; Castagnola *et al.*, 1986) to type-X-collagen-producing hypertrophic chondrocytes ('stage II chondrocytes') using SDS/polyacrylamide-gel electrophoresis (Gibson *et al.*, 1983, 1984; Castagnola *et al.*, 1986, 1987) or Northern hybridization (Castagnola *et al.*, 1988).

With monoclonal antibodies (Schmid & Linsenmayer, 1985a,b; Summers *et al.*, 1988) and cDNA probes (Ninomiya *et al.*, 1986) type X collagen was located in hypertrophic cartilage of growth plates, in the calcifying cartilage of sternal and rib cartilage, but also in the developing notochord (Linsenmayer *et al.*, 1986) and in bone trabeculae of chick calvaria (Schmid & Linsen-

mayer, 1985a). Less information is available on the distribution of type X collagen in mammalian cartilage. Endochondral ossification in mammalian bone has rather different anatomical structures; however, the available chick type-X-collagen antibodies and probes fail to cross-react with the corresponding mammalian collagen or RNA respectively. Thus the preparation of antibodies and cDNA probes recognizing mammalian type X collagen has been gaining considerable interest, particularly in view of several genetic disorders that may involve defects in chondrocyte hypertrophy, e.g. thanatophoric dysplasia or other forms of chondrodysplasia and dwarfism.

Bovine type X collagen has been identified in organ culture of hypertrophic cartilage after radioactive labelling as a disulphide-linked high- M_r component that, after reduction, gives rise to α -chains of M_r 63000 (Grant *et al.*, 1985). By comparison, chicken type X collagen has been isolated from culture medium both as a non-disulphide-linked monomer with α -chains of M_r 64000 (Capasso *et al.*, 1982, 1984; Gibson *et al.*, 1982; Schmid & Linsenmayer, 1983) and as a disulphide-linked dimer of M_r 120000 (Kwan *et al.*, 1986). Analysis of the chicken type-X-collagen gene structure indicated the presence of cysteine residues in the C-terminal region of the primary transcript, which, however, are apparently lost during processing of the molecule (Ninomiya *et al.*, 1986). Similar to chicken type X collagen, bovine type X is converted into a 46000- M_r peptide after limited proteolysis with pepsin (Grant *et al.*, 1985). Ayad *et al.* (1987) reported the extraction of both 59000- and 49000- M_r forms of type X collagen from bovine growth plate by guanidine, suggesting processing of the 59000- M_r molecule *in situ*.

Up until now purification of a mammalian type X

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collagen to homogeneity such as to permit amino acid sequence analysis or the preparation of antibodies has not been achieved. Here we report on the extraction of type X collagen from fetal-bovine growth-plate cartilage with 1 M-NaCl and reducing agents, and describe chromatographic procedures for the purification of the molecule. With the purified protein we were able to raise in rabbits specific antibodies that could be used to localize type X collagen in the extracellular matrix of bovine growth-plate cartilage.

MATERIALS AND METHODS

Extraction of type X collagen

Epiphyseal cartilage from the long bones of calf fetuses (30–60 cm in length) was carefully cleaned from adhering connective tissue and homogenized in an ice bath in 1 M-NaCl/0.05 M-Tris/HCl, pH 7.4, containing proteinase inhibitors (1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride and 1 mM-*N*-ethylmaleimide), 10 mM-dithiothreitol and 0.1 mM- β -aminopropionitrile using an Ultra-Turrax homogenizer (Jahnke and Kunkel, Staufen, Germany). The homogenate was centrifuged for 1 h at 4 °C at 40000 g, and the supernatant was saturated to 33 % with $(\text{NH}_4)_2\text{SO}_4$. The precipitate containing type X collagen was spun down as described above. Alternatively, the 1 M-NaCl extract was dialysed directly against DEAE-cellulose starting buffer (0.2 M-NaCl/0.05 M-Tris/HCl, pH 7.4).

Chromatographic procedures

The 1 M-NaCl extract was applied to a 40 ml column of DEAE-cellulose (Whatman DE 52). Collagens were eluted at 4 °C from the column with the same buffer, followed by the elution of proteoglycans with 1 M-NaCl/0.05 M-Tris/HCl, pH 7.4.

For gel filtration, the DEAE-cellulose-purified collagens were concentrated by ultrafiltration, denatured at 50 °C and applied in 2 ml aliquots to a preparative Superose 12 (Pharmacia, Uppsala, Sweden; particle size 10–40 μm) column, using an f.p.l.c. unit (Pharmacia). The column was run at 32 °C and eluted with 0.2 M-NaCl/0.05 M-Tris/HCl, pH 7.4, at 1 ml/min under a constant pressure of 500 kPa.

Fractions from the Superose column were dialysed against 50 mM-Tris/HCl, pH 7.5, and applied to a Mono Q column (Pharmacia) equilibrated in the same buffer. The column was eluted under a pressure of 2 MPa using a linear gradient between 0 and 0.5 M-NaCl over 50 ml.

SDS/polyacrylamide-gel electrophoresis

Samples were dissolved in 3 % SDS/sample buffer (Laemmli, 1970), with or without dithiothreitol, denatured at 95 °C for 5 min and analysed by electrophoresis in 10 % polyacrylamide mini-slab gels. Gels were stained with Coomassie Blue or silver as described by Oakley *et al.* (1980).

Enzymic digestions

Collagen samples were digested with pepsin (0.2 mg/ml; twice-crystallized; Serva, Heidelberg, Germany) in 0.5 M-acetic acid for 24 h at 4 °C, then neutralized, dialysed and freeze-dried. For collagenase digestions, 10 μg samples were taken up in 0.1 M-NaCl/5 mM- CaCl_2 /

50 mM-Tris/HCl (pH 7.5)/0.1 mM-*N*-ethylmaleimide, heat-denatured for 30 min at 50 °C and incubated with 145 munits of proteinase-free purified collagenase (Advance Biofacture, Lynbrook, NJ, U.S.A.) for 4 h at 37 °C.

Amino acid analysis

Collagen samples were hydrolysed in 6 M-HCl at 110 °C under N_2 , evaporated to dryness and dissolved in 100 μl of 0.2 M- NaHCO_3 , pH 9.0. A 100 μl portion of dimethylaminobenzenesulphonyl chloride (dabsyl chloride) (2 mg/ml in acetone) was added and the reaction mixture was heated to 70 °C for 15 min, dried under vacuum and dissolved in 0.5 ml of 70 % ethanol. The derivatized amino acids were separated by h.p.l.c. on a Lichrospher 100 CDH-18 column (Merck, Darmstadt, Germany) and the Beckman h.p.l.c. system Gold. Elution was achieved with a gradient between 0.045 M-sodium acetate, pH 4.13, and acetonitrile/tetrahydrofuran (4:1, v/v).

Antibody preparation

Rabbits were immunized subcutaneously with 0.2 mg of purified bovine type X collagen coupled to haemocyanin, dissolved in phosphate-buffered saline (0.137 M-NaCl/2.7 mM-KCl/1.45 mM- KH_2PO_4 /8.0 mM- Na_2HPO_4 , pH 7.2) and emulsified in complete Freund's adjuvant. After two booster injections with the same amount of type X collagen in incomplete Freund's adjuvant, blood was collected from the ear vein, and the antiserum was absorbed with bovine collagen types II, IX and XI, human collagen type I and fibronectin, each coupled to Sepharose CL6B (Pharmacia). From the absorbed serum the IgG fraction was obtained by f.p.l.c. on Protein A-Superose (Pharmacia). Antibody titres were tested by using an e.l.i.s.a. (Rennard *et al.*, 1980) with 3,3',5,5'-tetramethylbenzidine as colour substrate. Microtitre dishes were coated with 50 μg of collagen/well.

Immunoblotting

Samples were blotted on to nitrocellulose filters (Bio-Rad, Richmond, CA, U.S.A.) after electrophoresis on 10 % (w/v) polyacrylamide gels as described by Towbin *et al.* (1979). After blocking with low-fat milk protein, blotted proteins were immunostained with rabbit anti-(collagen type X) IgG fraction (0.1 $\mu\text{g}/\text{ml}$) using peroxidase-conjugated goat anti-rabbit Ig (Bio Yeda, Rehovot, Israel) in a 1:1000 dilution as second antibody and α -chloronaphthol as colour substrate.

Immunofluorescence staining

Frozen sections of fetal-bovine epiphyseal cartilage were collected on polylysine-coated glass slides, fixed in acetone and digested with testicular hyaluronidase (2 mg/ml; Serva, Heidelberg, Germany) in phosphate-buffered saline, pH 5.0. Fluorescence staining with rabbit anti-(collagen type X) IgG (10 $\mu\text{g}/\text{ml}$), followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Behringwerke, Marburg,) was performed as described by von der Mark *et al.* (1976). Sections were viewed under a Leitz Diaplan microscope equipped for epifluorescence.

Fig. 1. F.p.l.c. molecular-sieve chromatography on Superose 12 (Pharmacia) of bovine collagens extracted from fetal growth plates with 1 M-NaCl under mild reducing conditions

After removing proteoglycans by DEAE-cellulose chromatography (see the Materials and methods section), the extract was dialysed into 0.2 M-NaCl/0.05 M-Tris/HCl, pH 7.4, denatured at 50 °C and applied to a preparative column of Superose 12 (1.5 cm × 40 cm) equilibrated in the above buffer. Elution was carried out at 32 °C. Fraction A contained type X collagen.

RESULTS

Isolation and purification of type X collagen

Sequential extractions of fetal-bovine growth-plate cartilage with (1) 1 M-NaCl at neutral pH, (2) 4 M-guanidinium chloride, (3) acetic acid and (4) pepsin, and analysis of the extracts on SDS/polyacrylamide gels under reducing conditions revealed a collagenase-susceptible 58 000- M_r molecule predominantly in the 1 M-NaCl extract (results not shown). The yield of this

Fig. 2. F.p.l.c. anion-exchange chromatography of type X collagen on Mono Q

Fraction A (Fig. 1) was dialysed into 0.05 M-Tris/HCl and applied to a Mono Q column equilibrated in the same buffer. The column was eluted with a linear NaCl gradient of 0–0.5 M over 50 ml.

Fig. 3. SDS/polyacrylamide-gel electrophoresis and immunoblotting of purified bovine type X collagen

Bovine type X collagen purified by Mono Q chromatography (see Fig. 2) was applied to SDS/10%-(w/v)-polyacrylamide gels without reduction (a) and after reduction with dithiothreitol (DTT) (b). The M_r of the non-reduced form is above 300 000. The gel was stained with Coomassie Blue. (c) Immunoblotting of lane b with a rabbit antiserum raised against the purified bovine type X collagen. The arrow on the left indicates the type X collagen oligomer. The arrowhead on the right indicates the type X collagen monomer.

molecule, which was later identified as type X collagen (see below), was considerably enhanced in the presence of 10 mM-dithiothreitol, suggesting increased solubilization of type X collagen by the cleavage of intermolecular cystine bonds without reducing intramolecular bonds.

The native type X collagen was separated from type II collagen and other neutral-salt-soluble cartilage-matrix components by f.p.l.c. gel filtration on a Superose 12 column at 32 °C (Fig. 1). The fraction eluted in the void volume was rechromatographed on Mono Q ion-exchange columns (Fig. 2); at 0.4 M-NaCl, type X collagen was eluted in a single peak which was analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 3). Without reduction, type X collagen hardly penetrated into the gel, indicating an M_r considerably higher than 180 000, which would correspond to $[\alpha 1(X)_3]$. After reduction, the rechromatographed material appeared as a single band of M_r 58 000 ± 4000.

Characterization as type X collagen

The amino acid composition of the purified material correlated well with the published analyses of chicken type X collagen obtained by protein hydrolysis (Quarto *et al.*, 1985) or deduced from the cDNA sequence (Ninomiya *et al.*, 1986) (Table 1). The collagenous nature of the protein was confirmed by its susceptibility to

Table 1. Amino acid composition of bovine and chick type X collagen

Amino acid	Species... Form...	Composition mol/100 mol		
		Bovine 59000 M_r	Chick	
			45000 M_r *	59000 M_r †
Cys		1.8	0	0.5
4-Hyp		10.3	12.6	8.0
Ser		2.5	2.0	4.4
Arg		2.0	2.9	3.0
Asx		5.2	3.9	4.1
Glx		7.8	8.5	8.3
Thr		0.7	1.3	2.0
Gly		33.0	33.3	25.7
Ala		8.6	5.8	4.5
His		1.1	0.8	1.2
Pro		9.7	11.7	11.8
Met		1.6	1.6	2.6
Val		3.3	2.4	4.0
Phe		2.7	1.2	2.3
Ile/Leu		5.8	7.3	8.0
Hyl		2.7	3.0	3.0
Lys		2.8	2.0	2.5

* From Quarto *et al.* (1985).

† From Ninomiya *et al.* (1986) and Lu Valle *et al.* (1988).

purified bacterial collagenase, which degraded the 58000- M_r component completely, leaving smaller fragments of M_r 15000 (Fig. 4). These components probably represent the non-collagenous domains of type X collagen (Ninomiya *et al.*, 1986).

Digestion of the native molecule with pepsin decreased the apparent M_r of the 50000- M_r α chain to 43000 on reducing SDS/polyacrylamide gels (Fig. 5), which is consistent with the result obtained after pepsin treatment of chick type X collagen and previous reports on bovine type X collagen (Grant *et al.*, 1985; Ayad *et al.*, 1987). Without reduction, bovine type X collagen migrated as a high- M_r component on top of SDS/polyacrylamide gels also after pepsin treatment, indicating that pepsin did not remove the cysteine bridges from the molecule [see also Grant *et al.* (1985) and Ayad *et al.* (1987)].

Preparation of rabbit antibodies against type X collagen

Rabbits were immunized with purified bovine type X collagen coupled to haemocyanin, and the antisera were tested for titres against several collagens by e.l.i.s.a. After absorption of the antiserum with fibronectin and collagen types I, II, IX, and XI, the IgG fraction was isolated by chromatography on protein A-Superose. The IgG fraction was positive for type X collagen in the e.l.i.s.a. at concentrations of 0.1 $\mu\text{g/ml}$ (half-maximal absorbance), and negative for all other cartilage collagens tested, as well as for fibronectin (Fig. 6). In the immunoblot, the antibody specifically labelled the 59000- M_r band of bovine type X collagen (Fig. 3) at concentrations of 0.1 μg of IgG/ml.

Immunofluorescence localization of bovine type X collagen

Frozen sections of fetal-bovine cartilage required fixation with acetone and subsequent digestion with testicular

Fig. 4. Collagenase digestion of bovine type X collagen

Purified bovine type X collagen was denatured at 50 °C for 30 min and then incubated with proteinase-free purified bacterial collagenase at 37 °C for 4 h in 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.5, containing 5 mM-CaCl₂ and 0.1 mM-N-ethylmaleimide (a) or with buffer only (b). The SDS/18 % polyacrylamide gels were silver-stained. C, collagenase; the arrow shows uncleaved 58000- M_r -type X collagen; the small arrowhead shows the possible non-collagenous domain of type X collagen.

Fig. 5. Pepsin digestion of bovine type X collagen

(a) Pepsin digestion at 4 °C without reduction leaves the molecule as a high- M_r component (arrowhead 1) of M_r similar to that of the non-pepsin-treated material (Fig. 3), indicating the presence of intramolecular disulphide bonds within the triple helix. After reduction with dithiothreitol (b), most of the pepsin-digested material migrated with an apparent M_r of 43000 (arrowhead 3). The 10 % polyacrylamide/SDS gel was Coomassie Blue-stained. (c) Indicates M_r markers. The arrowhead 2 indicates undigested type X monomers.

Fig. 6. E.I.s.a. of the IgG fraction from a rabbit anti-(bovine type X collagen) serum after absorption with types I, II, IX and XI collagen and fibronectin

Microtitre dishes were coated with 50 $\mu\text{g}/\text{ml}$ of pepsin-extracted collagen type I (human) ($\square$), types II ($\blacklozenge$), IX ($\blacklozenge$), X ($\diamond$) and XI ($\blacksquare$) (bovine) and fibronectin ($\square$) from human plasma. The concentration of the IgG solution was 50 $\mu\text{g}/\text{ml}$.

hyaluronidase to reveal type X collagen by immunofluorescence. The rabbit antibody located the protein exclusively in the zone of columnar hypertrophic cartilage beneath the cartilage–bone erosion zone (Fig. 7). It was also present in remnants of calcified cartilage within bone trabeculae of endochondral bone (Fig. 8). Zones of

resting cartilage and other tissues did not stain with anti-(collagen type X) antibody.

DISCUSSION

Although numerous studies on the biosynthesis, isolation, protein and gene structure and immunolocalization of chicken type X collagen have led to a complete characterization of this collagen type (for review, see Schmid & Linsenmayer, 1987), relatively little progress has been made in the isolation and characterization of mammalian collagen type X.

The existence of a collagen in mammalian growth-plate cartilage with properties similar to those of chicken type X collagen was first demonstrated in organ culture of fetal-bovine growth-plate cartilage after metabolic labelling with radioactive precursors (Grant *et al.*, 1985). From the zone of hypertrophic cartilage in the costochondral junction, a molecule of M_r 63 000 was extracted, which was converted into a 46 000- M_r form by mild pepsin digestion. In contrast with chick type X collagen, however, without reduction both forms migrated in SDS/PAGE as high- M_r components above β -chains of type II collagen, indicating the presence of intrachain and intermolecular cystine bridges (Grant *et al.*, 1985). Ayad *et al.* (1987) reported the extraction of the smaller, processed, 49 000- M_r form of type X collagen with 4 M-guanidinium chloride or with pepsin from fetal-bovine growth-plate cartilage, besides an unprocessed 59 000- M_r form. No significant amounts of bovine type X collagen

Fig. 7. (a) Immunofluorescence localization of type X collagen in the hypertrophic zone of a fetal-calf distal tibial growth plate and (b) phase-contrast picture of the same section (magnification $\times 480$)

Frozen sections were fixed with acetone, digested with testicular hyaluronidase and stained with 10 μg of absorbed rabbit anti-(bovine type X collagen) IgG/ml, followed by fluorescein isothiocyanate-labelled goat anti-rabbit IgG. Only the hypertrophic zone was positive. The zone of resting cartilage did not stain (not shown).

Fig. 8. Immunofluorescence localization of type X collagen in the spicules of calcified cartilage remaining in bone trabecules (zone of endochondral ossification)

The distal tibial epiphysis of a 25–30-week-old calf fetus is shown. Staining was done as in Fig. 7. Magnification $\times 400$.

were extractable with 1 M-NaCl (Ayad *et al.*, 1987), and from fetal-chick sternal cartilage, type X collagen was only extractable by pepsin digestion (Quarto *et al.*, 1985).

Here we show that substantial amounts of unprocessed native type X collagen could be extracted from fetal growth-plate cartilage with 1 M-NaCl at neutral pH under mild reducing conditions. Addition of 10 mM-dithiothreitol without denaturing agents was apparently sufficient to reduce intermolecular cystine bonds while leaving intramolecular disulphide bonds intact. Additional amounts of 59000- M_r type X collagen were obtained in subsequent guanidinium chloride extracts. We did not observe processed forms of M_r 43000–49000 (Ayad *et al.*, 1987), either in the 1 M-NaCl extract or in subsequent guanidinium chloride extracts.

The neutral-salt-soluble form of type X collagen was purified to homogeneity by using three conventional chromatographic procedures. The material was readily soluble and did not reveal any hydrophobic characteristics. A hydrophobic nature of type X collagen has been reported by Kwan *et al.* (1986) and Ayad *et al.* (1987) and would be consistent with a previously reported 29-amino-acid-long hydrophobic domain at the C-terminal end of the chicken type X collagen (Ninomiya *et al.*, 1986).

The identification of the purified 59000- M_r molecule as type X collagen is based on the following criteria: (i) like chicken type X collagen it is converted into a 43000- M_r form by limited digestion with pepsin; (ii) purified collagenase digests the molecule and leaves a 15000- M_r fragment intact, presumably the non-collagenous C-terminal domain; (iii) the amino acid composition is similar to that of chicken type X collagen, with the exception of two additional cysteine residues; (iv) antibodies to bovine type X collagen located the molecule in the zone of hypertrophic and calcified cartilage in the growth plate of fetal-bovine cartilage.

The processing of type X collagen is the subject of some controversy. In cell and organ culture studies on chicken growth-plate cartilage (Schmid & Conrad, 1982; Summers *et al.*, 1988), no processing of the 59000–63000-

M_r form to the 49000- M_r form was found; similarly, pulse-chase experiments using organ cultures of bovine rib cartilage did not show conversion of the synthesized 63000- M_r form of type X collagen (Grant *et al.*, 1985). Capasso *et al.* (1984), however, reported the conversion of the 64000- M_r form of type X collagen into a 30000- M_r fragment in pulse-chase-labelled chondrocytes, whereas Jimenez *et al.* (1986) found a 70000- M_r precursor form of type X collagen in long-term cultures of hypertrophic chicken chondrocytes. Ayad *et al.* (1987) reported the extraction of a smaller, processed, 49000- M_r form of type X collagen with 4 M-guanidinium chloride from fetal-bovine growth-plate cartilage, besides the unprocessed 59000- M_r form, and concluded that processing of type X collagen takes place in cartilage. We could not detect significant amounts of a 49000- M_r form of type X collagen in guanidinium chloride extracts of fetal-bovine growth cartilage.

In contrast with the developing chicken long bone, in which the zone of hypertrophic and calcifying cartilage stretches in an irregular manner from the mid diaphysis to the epiphysis (Schmid & Linsenmayer, 1985a,b), the corresponding region in mammalian long bones or ribs is confined to an anatomically rather narrow zone (Grant *et al.*, 1985). Immunofluorescence analysis of 20–30-week-old fetal-bovine epiphyseal cartilage with the affinity-purified antibody to type X collagen located this collagen specifically in that zone. Intense reactions were also observed in remnants of calcified cartilage in enchondral-bone trabecules. Preliminary studies have shown that the antibodies described can also be used for immunostaining of human tissues (T. Kirsch, K. von der Mark & G. Weseloh, unpublished work). Thus the availability of antibodies recognizing both bovine and human type X collagen offers new potential not only for studies on cartilage development, but also for studies on growth defects involving growth-plate cartilage.

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