

# Immunohistochemical Localization of Short Chain Cartilage Collagen (Type X) in Avian Tissues

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**ABSTRACT** Monoclonal antibodies were produced against the recently described short chain cartilage collagen (type X collagen), and one (AC9) was extensively characterized and used for immunohistochemical localization studies on chick tissues. By competition enzyme-linked immunosorbent assay, antibody AC9 was observed to bind to an epitope within the helical domain of type X collagen and did not react with the other collagen types tested, including the minor cartilage collagens 1 $\alpha$ , 2 $\alpha$ , 3 $\alpha$ , and HMW-LMW. Indirect immunofluorescence analyses with this antibody were performed on unfixed cryostat sections from various skeletal and nonskeletal tissues. Only those of skeletal origin showed detectable reactivity. Within the cartilage portion of the 13-d-old embryonic tibiotarsus (a developing long bone) fluorescence was observed only in that region of the diaphysis containing hypertrophic chondrocytes. None was detectable in adjacent regions or in the epiphysis. Slight fluorescence was also present within the surrounding sleeve of periosteal bone. Consistent with these results, the antibody did not react with the cartilages of the trachea and sclera, which do not undergo hypertrophy during the stages examined. It did, however, lightly react with the parietal bones of the head, which form by intramembranous ossification. These results are consistent with our earlier biochemical analyses, which showed type X collagen to be a product of that subpopulation of chondrocytes that have undergone hypertrophy. In addition, either it or an immunologically cross-reactive molecule is also present in bone, and exhibits a diminished fluorescent intensity as compared with hypertrophic cartilage.

During much of embryonic development, and throughout the juvenile growth period, elongation of skeletal elements occurs chiefly by endochondral bone formation in the epiphyseal growth region. This process involves precise temporal and spatial changes in the deposition of new extracellular materials, largely of a cartilagenous nature, as well as degradation and removal of pre-existing matrices. These changes in matrix materials reflect differences in the cell populations present. In the growth region, chondrocytes undergo changes in which they sequentially progress through phases of rapid proliferation, extensive matrix synthesis, hypertrophy, and ultimate removal. Although these changes occur in an uninterrupted continuum, it has been demonstrated using the 12-d-old embryonic chick tibiotarsus that the epiphyseal growth region can be reproducibly subdivided into four zones (1). The cell populations in each zone are highly enriched for chondrocytes

in one of the stages of this continuum, as determined by both biochemical and morphological criteria (2, 3).

Initially, the only collagenous molecule detected in mature cartilage was type II (4, 5). This was also found to be the case during embryonic development (6–8), although some results suggested that additional cartilage collagens might exist (9). Further biochemical studies definitively demonstrated the existence of at least three other genetically distinct types of collagen in cartilage (10–12).

Some of these quantitatively “minor cartilage collagens” have been localized within the cartilage matrix (13–15). Radiolabeling experiments performed on cell and organ cultures of chondrocytes obtained from the different zones of the epiphyseal growth region in the embryonic chick tibiotarsus suggested that at least one of these collagens was synthesized preferentially, if not exclusively, in the zone of hypertrophy.

This molecule, which is about half the size of a "typical" collagen molecule (12, 16, 17), has been termed short chain cartilage collagen (12). We would now like to designate it as collagen type X, in accordance with previously accepted nomenclature (18). A similar, if not identical, molecule has been identified by several other investigators (19–22).

In mass cultures of passaged chondrocytes from the more mature zones of the embryonic tibiotarsus, type X becomes the predominant collagenous molecule secreted into the culture medium (12). From this source, it can be obtained in relatively large quantities without the need for proteolytic extraction procedures. The molecule contains both a collagenase-sensitive, triple helical domain and a pepsin-sensitive non-helical one. The complete molecule, with both domains intact, is referred to as the 59K<sup>1</sup> form since its constituent chains have an  $M_r$  of 59,000, as determined by SDS PAGE. Limited pepsin digestion of the nature 59K form removes the nonhelical domain, generating a molecule with  $M_r$ -45,000 chains, termed the 45K form. Results in good agreement with these are obtained when rotary-shadowed preparations of the two forms of the molecule are examined by electron microscopy (17).

Although the helical domain of type X is unusually small, it is also unusually stable. The denaturation temperature of the helical structure in both forms of the molecule, as analyzed by circular dichroism (CD) spectropolarimetry at 222 nm, is ~47°C, a temperature that is considerably higher than that of most collagen molecules (23). This shows that type X can exist within the extracellular matrix as a triple helical molecule and provides additional evidence that it is not a breakdown product of a larger molecule.

In the present study, we report the production of a monoclonal antibody specific for a conformation-dependent epitope located within the helical domain of type X collagen. We also employ this antibody for immunohistochemical studies which suggest that type X collagen is a skeletal-specific molecule. In cartilage it is indeed restricted to the zone of hypertrophying chondrocytes as our previous biochemical analyses suggested. It is also present in lesser amounts in bone derived from both endochondral and intramembranous ossification. It is not, however, found in detectable amounts in any nonskeletal tissue examined. These results are consistent with and extend our previous observations on type X collagen.

## MATERIALS AND METHODS

**Collagen Production:** The 59K form of type X collagen was purified from the culture medium of chondrocytes that had been maintained in monolayer culture for 6 wk, as previously described (12). The collagen was precipitated from the medium by the addition of solid ammonium sulfate to 30% of saturation (176 mg/ml) in the presence of protease inhibitors (5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide). The precipitate was recovered by centrifugation (10,000 *g* for 30 min) and resolubilized in potassium phosphate buffer (0.13 M K<sub>2</sub>HPO<sub>4</sub>, 5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.6). The ammonium sulfate precipitation was repeated a second time. Contaminating type II collagen was removed by dialysis against 0.5 M acetic acid, 0.9 M NaCl, under which conditions it precipitates. Type X collagen was then recovered by precipitation by raising the NaCl concentration to 2.0 M. The 45K form of type X was generated by limited pepsin digestion of the 59K form, as previously described (12).

<sup>1</sup> *Abbreviations used in this paper:* CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay; HMW and LMW, high and low molecular weight collagen, respectively; 59K and 45K, the  $M_r$ -59,000 complete form and the  $M_r$ -45,000 helical form of type X, respectively.

**Hybridoma Production:** Female SJL/J mice (The Jackson Laboratory, Bar Harbor, ME) were each immunized subcutaneously with 200 µg of the 59K form of type X collagen in 200 µl of an emulsion containing complete Freund's adjuvant. Equal volumes of adjuvant and collagen solution in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>) were mixed in two connecting syringes. The mixture was then sonicated for 5 s. The mice were boosted 2 wk later with a second subcutaneous injection of 200 µg in incomplete adjuvant and then were rested for 6 mo. Prior to the fusion procedure, the mice received four daily intraperitoneal injections of 100 µg of type X in PBS without adjuvant. Their spleens were removed on the fifth day.

The procedure used for the production of hybridomas is described elsewhere (24) and is briefly summarized here. All cultures of hybridomas and the NS-1 myeloma cells used for hybridoma production were grown in Dulbecco's modified Eagle's medium (4.5 g/liter glucose [Gibco Laboratories, Grand Island, NY]) supplemented with 10% fetal calf serum (Sterile Systems, Logan, UT) and gentamycin (50 µg/ml). Splenocytes ( $3 \times 10^7$ ) were obtained from the immunized mice and fused with NS-1 myeloma cells ( $10^7$ ) in 37.5% polyethylene glycol. The cells were suspended in complete medium and plated in 96-well culture plates at a density of  $2.5 \times 10^4$  myeloma cells per well. The next day, hypoxanthine/aminopterin/thymidine medium was added to select for hybridomas.

Hybridomas were selected by enzyme-linked immunosorbent assay (ELISA) and cloned twice by limiting dilution. Selected clones were expanded; sodium azide was added to medium to 0.05% and the medium was stored at 4°C. Hybridoma cells were frozen in complete medium containing 10% DMSO.

**ELISA:** ELISAs were performed using a Hybridoma Screening Kit (Bethesda Research Laboratories, Gaithersburg, MD) and Immulon II microtiter plates (Dynatech Corp., Alexandria, VA). Wells were coated for 2 h with 100 µl of a 0.5 µg/ml solution of either the 45K or the 59K form of type X collagen in 0.1 M Na<sub>2</sub>CO<sub>3</sub>, 0.4 M NaCl, pH 8.5. Residual binding sites on the plate were blocked by a 2-h incubation with 110 µl of a 1% BSA solution in PBS, pH 7.8, followed by three more washes with PBS. All antibody dilutions, and collagen dilutions for inhibition studies, were made in PBS (pH 7.1) that contained 1% BSA. Incubations and washings during these assays were performed at room temperature unless stated otherwise. Hybridoma antibodies (100 µl/well) were incubated with type X-coated plates for 1 h at 25°C or overnight at 4°C. Subsequent steps involving incubation with the β-galactosidase-conjugated goat anti-mouse IgG and reaction with the *p*-nitrophenyl-β-D-galactoside substrate were performed according to the directions supplied with the screening kit. The absorbance of the wells at 414 nm was determined with an ELISA plate reader (Bio-Tek Instruments, Inc., Burlington, VT).

Competition ELISAs were performed with native collagen types I, II, III, IV, V, 1α, 2α, 3α, HMW-LMW, and the two forms of type X, as well as the thermally denatured forms of type X. Type I collagen was purified from lathyrus chick skin (5). Type II and 1α, 2α, 3α collagens were isolated from a pepsin extract of adult chicken sternal cartilage (9) and separated by sequential precipitation from 0.5 M acetic acid at 0.9 M NaCl and 1.2 M NaCl, respectively (11). The collagen types III, IV, and V, isolated from chicken gizzard, and HMW-LMW from sterna, were gifts of Richard Mayne (University of Alabama Medical School) (11). The assays were performed under nonequilibrium conditions (25). Aliquots of the collagen solutions were added to an equal volume of a 1:100 dilution of monoclonal antibody-containing culture medium and incubated in a microfuge tube overnight at 4°C. A 100-µl aliquot of the mixture was placed on type X-coated plates for 45 min. Subsequent steps were performed as described above.

Competition ELISAs were also performed on the two forms of type X heated to progressively higher temperatures. Aliquots of type X collagen (at 25 µg/ml) were heated to various temperatures for 30 min in a Lauda water bath (Brinkmann Instruments, Inc., Westbury, NY). Triton X-100 was included in the assay of the 59K form at a final concentration of 2% (see Results). Samples were cooled to 25°C and used in the competition ELISA experiments as described above.

The susceptibility of the type X collagen epitope to a purified bacterial collagenase Form III [Advanced Biofactures Corp., Lynbrook, NY] was tested in ELISA. Type X collagen was preincubated with 100 U of bacterial collagenase for 100 µg of collagen in 250 µl of 50 mM Tris, 0.2 M NaCl, 10 mM CaCl<sub>2</sub>, 5 mM *N*-ethylmaleimide, pH 7.5 buffer at 37°C for 18 h. The enzyme reaction was terminated by the addition of EDTA to 20 mM.

**Immunofluorescence Observations:** Various tissues from embryonic and adult chickens were removed, frozen in dichlorodifluoromethane at dry-ice temperature, and stored at -70°C until needed. Those that were ossified were demineralized for 3 d in 0.25 M EDTA at 4°C. Frozen tissues were embedded in Tissue-Tek II O.C.T. compound (Miles Laboratories, Inc., Elkhart, IN) and 8-µm sections were cut with a cryostat. Sections were placed on 12-spot, albuminized microscope slides (Shandon Scientific, Shandon Southern Instruments, Inc., Sewickley, PA). All cartilagenous tissue sections were pretreated for 1 h at 37°C with a 0.5% solution of testicular hyaluronidase

(Sigma Chemical Co., St. Louis, MO; Type 1-S, 500 U/mg) in PBS, washed with PBS, and dried. In addition to the anti-type X antibody, adjacent sections were reacted with monoclonal antibodies directed against either type I (26) or type II (27) collagen, which have been previously described. Tissue sections were incubated with monoclonal antibodies for 1 h and rinsed with PBS. The sections were then treated with rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Inc., Cochranville, PA) for 1 h, rinsed with PBS, and mounted in glycerol-PBS (95:5). Sections were examined using a Nikon Fluorophot equipped with an epi-illuminator and a 590-nm filter. Photographs were taken with Kodak Tri-X film rated at an ASA of 1600 and developed with Diafine (Acufine, Inc., Chicago, IL).

## RESULTS

### Monoclonal Antibody Production and Characterization

To produce the desired monoclonal antibodies, we fused splenocytes from mice hyperimmunized with the 59K form of type X collagen with NS-1 myeloma cells. All 400 culture wells showed positive hybridoma growth in hypoxanthine/aminopterin/thymidine selection medium, and these were screened for the production of potentially desirable monoclonal antibodies by ELISA using plates coated with type X collagen. 12 were positive and four of these were cloned by limiting dilution. In preliminary immunofluorescence histochemical assays, the clones derived from one original well, AC9, clearly produced a more intense fluorescent signal than those derived from any other original well. The monoclonal antibody produced by these clones was chosen for extensive characterization and subsequently for immunohistochemical localization studies.

Medium supernatants from AC9 cultures reacted with ELISA plates coated with either form of type X. They had high antibody titers and still gave a positive reaction when diluted >2,000-fold (data not shown). By competition ELISAs in which the plates were coated with the 45K form, the antibody reacted about equally well with both molecular forms of type X, but not with any other native collagen type tested (Fig. 1). Most notably it did not react with type II, nor with the minor cartilage collagens including  $1\alpha$ ,  $2\alpha$ ,  $3\alpha$  molecules (10) and HMW-LMW (11).

As can be seen in Fig. 1, the 59K form of type X was a

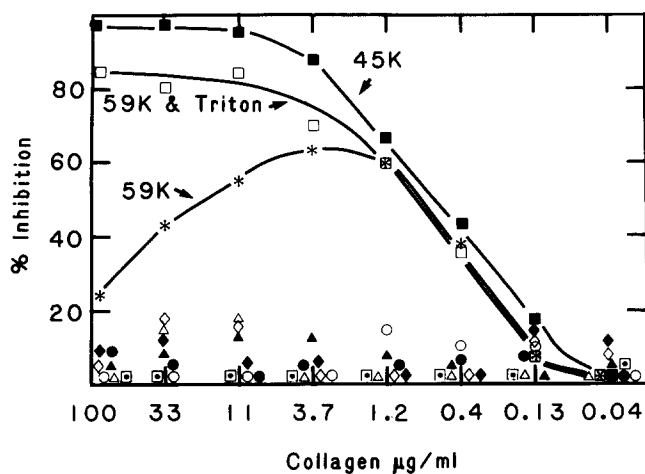


FIGURE 1 Competition ELISA using antibody AC9 and various concentrations of native collagens as inhibitors. ■, the 45K form of type X collagen; \*, the 59K form; □, the 59K form in the presence of 2% Triton X-100. Other collagen types including I (□), II (▲), III (○), IV (◇), V (△),  $1\alpha$ ,  $2\alpha$ ,  $3\alpha$  (●), and HMW-LMW (◆) showed no inhibition.

slightly less efficient inhibitor than was the 45K form. While this is undoubtedly due in part to the higher molecular weight of the 59K form, it may also reflect the tendency of this form of the molecule to aggregate when the nonhelical domain is present (17). In fact, in competition ELISA the 59K form of the molecule only showed a typical inhibition curve when it was reacted with the antibody in the presence of high concentrations of nonionic detergent (2% Triton X-100). If detergent was not present in the reaction mixture, an anomalous decrease in inhibition was observed at higher concentrations of inhibitor collagen. (In Fig. 1, compare the curve generated by 59K in the presence of Triton X-100, open squares, to that produced in its absence, asterisks.)

The binding of the antibody to both forms of the molecule suggested that it was directed against an epitope within their common helical domain, as is generally found to be the case when native collagens are used to immunize mice (28). In addition, bacterial collagenase digestion of type X collagen completely abolished the ability of the molecule to inhibit AC9 binding in ELISA (data not shown). We have previously demonstrated that a close correlation exists between the thermal denaturation temperature of the helix of a specific collagen type, as measured by CD spectropolarimetry, and the loss of monoclonal antibody binding, as determined by competition ELISA (29, 30). This provides strong additional evidence that the antibody is directed against the collagen, in addition to defining one of the characteristics of the epitope.

Samples of both the 45K and 59K forms of type X collagen were heated to progressively higher temperatures at neutral pH, allowed to cool to room temperature, and then assayed for antibody binding by competition ELISA (for details see the legend for Fig. 2 and Materials and Methods). As can be seen in Fig. 2, the 45K form shows a sharp loss of antibody binding with a denaturation temperature ( $T_m$ ) of 44°C. This is ~3° lower than the  $T_m$  determined by CD spectropolarimetry (23). Under identical conditions, the 59K form showed a different temperature response, and appeared to lose only about a third of its total inhibitory ability. This probably reflects the rapid renaturation of a large portion of this form of the molecule upon being cooled to room temperature. This

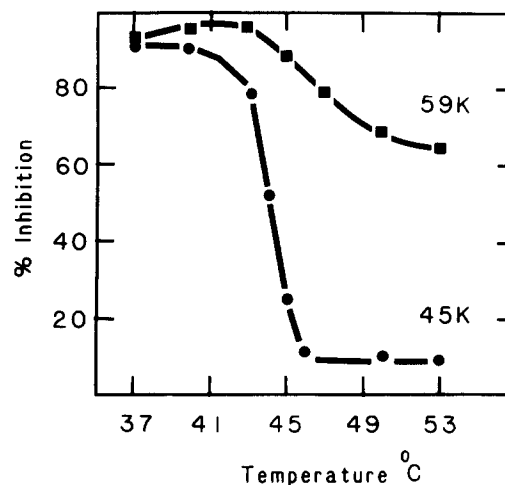


FIGURE 2 Thermal denaturation of the epitope recognized by the AC9 antibody, as measured by competition ELISA. The two forms of type X collagen were heated to various temperatures for 30 min and cooled to room temperature before incubation with the antibody. ●, the 45K form; ■, the 59K form. Triton X-100 (2%) was included in the assay of the 59K form of type X collagen.

result is fully consistent with our previous CD spectropolarimetric studies, and most probably is due to the presence of the nonhelical, globular domain which keeps the denatured chains in register (see Discussion).

### Immunohistochemical Analyses

For all immunohistochemical analyses, cartilage sections were predigested with testicular hyaluronidase to remove potentially masking proteoglycans before reacting with antibodies (7). Tissues containing bone were also decalcified with EDTA to remove hydroxyapatite, which can also mask the antigenic sites of type I collagen (8).

When the antitype X antibody was used to analyze unfixed cryostat sections of the distal half of the tibiotarsus from 13-d-old chick embryos (Fig. 3B), the overall distribution of this molecule was found to be much more restricted than was that of type II collagen (Fig. 3C), as evaluated by staining with a monoclonal antibody specific for that collagen type. The region of the limb in which the cartilage matrix begins to show a reaction for type X is slightly behind the front at which the chondrocytes have begun to hypertrophy (Fig. 3, arrowheads). This can be seen in Fig. 3 by comparing the location of the region of initiation of type X reactivity with the front of newest bone formation in the surrounding periosteal sleeve, which is identified in Fig. 3A by the arrowhead. The section in Fig. 3A has been reacted with a monoclonal antibody against type I collagen, which brightly stains the bone in a trabecular pattern. Numerous studies have shown that the region of chondrocytes which undergo hypertrophy generally corresponds to, and can be demarcated by, the sleeve of periosteal bone which surrounds it (31–33). Strong reactivity for type X extends uninterrupted to the edge of the marrow cavity (Fig. 3B, label *mc*), which is the terminal site where hypertrophied cells are removed.

Fig. 3B also indicates that type X collagen is present in the periosteal bone matrix, although the fluorescence in this tissue is greatly diminished compared with that in the hypertrophic cartilage. Note the light fluorescence in the periosteal layer of Fig. 3B (also described later), compared with its complete absence in the same region when reacted with the antitype II antibody (Fig. 3C), which for this tissue serves as a negative control.

The distribution of type X collagen in the extracellular matrices of endochondral bone is further illustrated in higher magnification fluorescence micrographs of the spongiosa of adult sternum shown in Fig. 4. The spongiosa characteristically is composed of spicules of bone matrix deposited on the surface of a core of hypertrophied cartilage (calcified) (34). Sections reacted with the antibody for type I collagen (Fig. 4A) show bright fluorescence in the bone matrix and lighter fluorescence in the cartilage matrix. That the cartilage matrix in this region contains both collagen types I and II is consistent with the previous observations of von der Mark and von der Mark (35). An adjacent tissue section reacted with antibody for type II collagen (Fig. 4C) shows fluorescence only in the cartilagenous core within the spicule. The antitype X antibody (Fig. 4B) shows a composite picture in which both cartilage and bone are reactive; the cartilagenous core is intensely fluorescent and the surrounding bone much less so.

These results raised two questions. Is type X present in cartilages that do not participate in endochondral bone formation and whose chondrocytes are therefore not destined to undergo hypertrophy? Is type X found in other bones that

form by intramembranous ossification and therefore have never been associated with a cartilagenous precursor?

To answer the first question, we examined the tracheal and scleral cartilages of 19-d embryos, neither of which participates in endochondral bone formation. Fig. 5 shows the results obtained with tracheal rings, which are cut in cross section. The cartilagenous matrix of these structures, which was sampled at several different sites along their circumference, was strongly reactive for type II collagen (Fig. 5B), and the surrounding, fibrous perichondrium was reactive for type I (Fig. 5A). Neither structure, however, showed detectable staining for type X collagen (Fig. 5C). Similar results were observed with the scleral cartilage of the eye (data not shown).

Membrane bones, however, did show reactivity for type X. Fig. 6 shows serial sections of a 19-d embryonic calvarium treated with antibody for type I (Fig. 6A), type X (Fig. 6B), and type II (Fig. 6C). The reactivity for type X is much less than for type I, and this intensity can vary somewhat in bone from different sources and ages of embryos. In all experiments we have tried, however, the fluorescence signal for type X is noticeably brighter than that obtained with the antitype II antibody which serves as a negative control. Similar patterns of reactivity and fluorescent intensities were also obtained when adult (8 wk) chicken calvaria were examined (data not shown). As additional controls (not shown), parallel sections of bone were reacted with other monoclonal antibodies whose specificities should have precluded their staining of this tissue, including one antibody specific for lens fiber cells and another directed against type IV collagen. These antibodies were all equally negative. Also, the reaction for type X could be completely eliminated by prereacting the antibody with an excess of 45K collagen. Another antitype X collagen monoclonal antibody, which we do not routinely employ since it appears to have a low affinity, reacted with bone, but less strongly than did AC9.

The tissue distribution of type X collagen appears to be exclusive to the skeletal system. Type X is detected near the hypertrophic cells of other cartilagenous tissues that undergo endochondral bone formation, including embryonic vertebrae and the cephalic aspect of the sternum (Schmid, T. M., E. Gibney, and T. F. Linsenmayer, manuscript in preparation). However, we have not found immunohistochemically detectable amounts in sections of nonskeletal tissues we have examined, including cornea, sclera, skin, large blood vessels, heart, skeletal muscle, brain, and as already described, the trachea.

### DISCUSSION

The data we have obtained with monoclonal antibody AC9 against type X collagen corroborate our earlier biochemical evidence suggesting that this molecule is a unique genetic type (12). The data are also consistent with our previous radiolabeling experiments (3, 16) which suggested that in cartilage the molecule is present only in the hypertrophic zone.

By a number of criteria, we have observed that monoclonal antibody AC9 is specific for type X collagen and that the epitope against which it is directed is conformation dependent and located within the helical domain. By competition ELISA it did not react with any other collagen type tested, which included other types currently known to exist in cartilage. The assignment of the epitope to the helical domain was made by several criteria, including: (a) its reactivity with both the 45K and 59K forms, whose structural common denominator

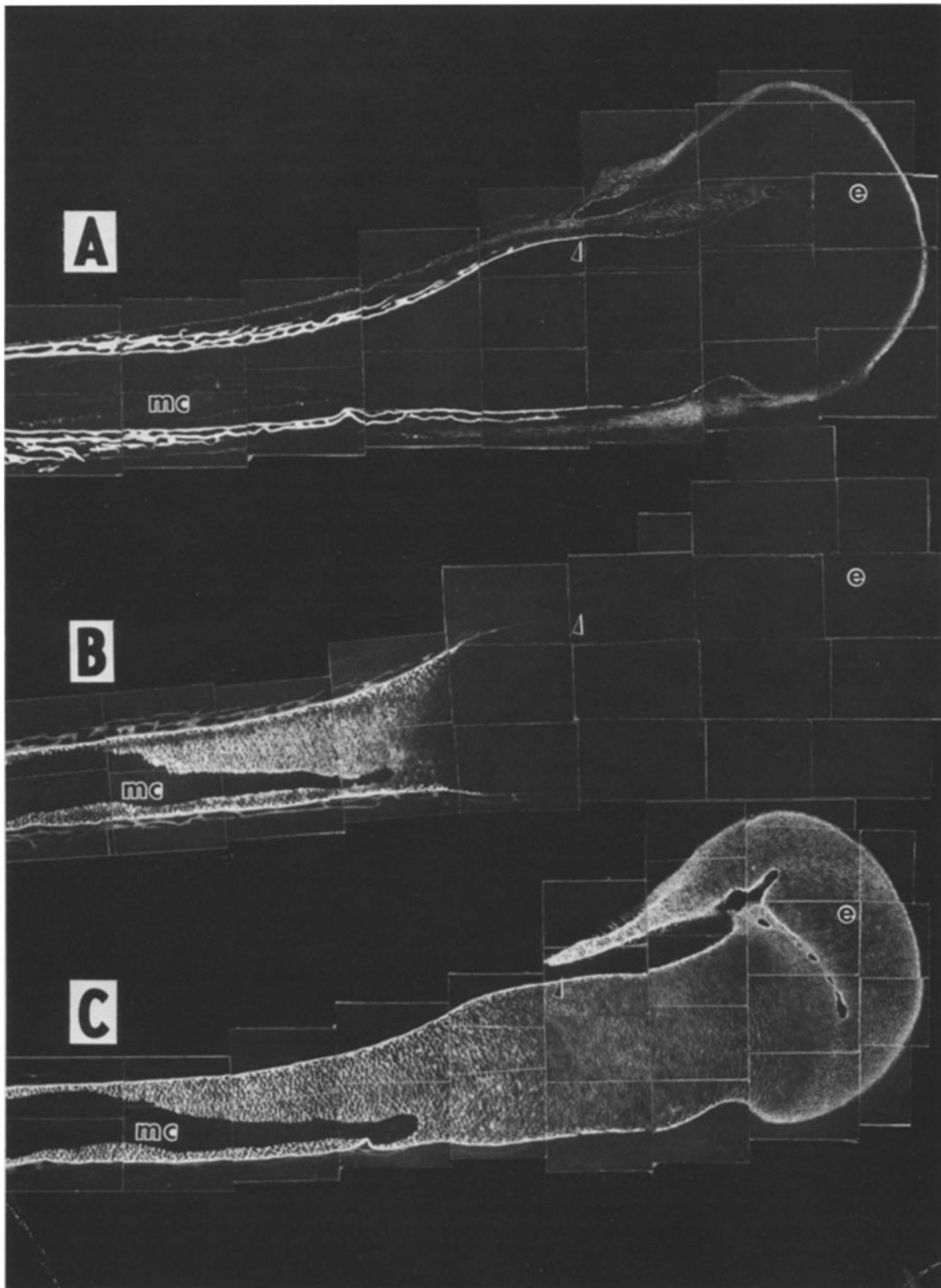


FIGURE 3 Photomicrographs of distal half of 13-d chick embryo tibiotarsus treated with monoclonal antibodies directed against collagen types I (A), X (B), and II (C). Arrowheads mark the distal boundary of hypertrophic chondrocytes. The marrow cavity (mc) and epiphysis (e) are labeled.  $\times 26$ .

is the helical domain; (b) the ability of highly purified bacterial collagenase to abolish reactivity; and (c) the correlation observed between antibody binding and the thermal denatura-

tion-renaturation properties of the different forms of the molecule.

The data obtained here in the thermal denaturation exper-

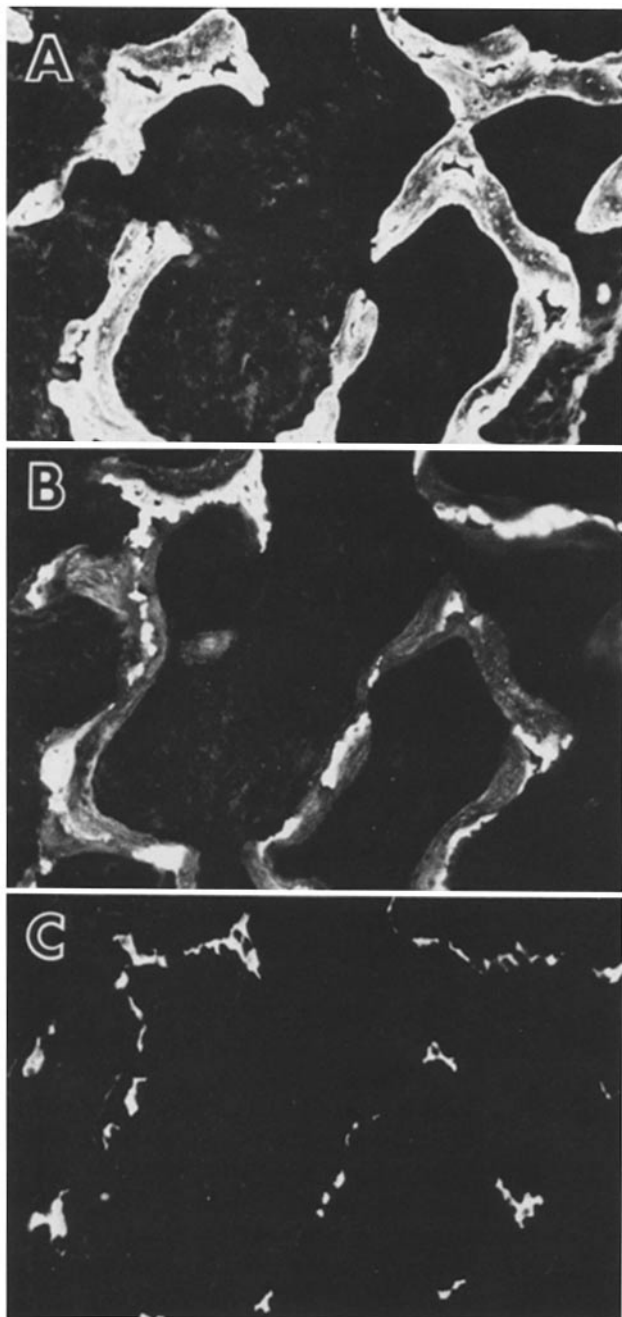


FIGURE 4 Photomicrographs of adult chicken sternum (7 wk) reacted with monoclonal antibodies directed against collagen types I (A), X (B), and II (C). Tissues were dissected from the calcified portion of the sternum and decalcified in EDTA for 1 wk.  $\times 100$ .

iments in all respects fit with those we previously obtained by monitoring the ellipticities of the molecules in the spectropolarimeter (23). The thermal denaturation curve of the 45K form as measured by competition ELISA generally paralleled that which we had previously obtained by CD spectropolarimetry. The  $T_m$  obtained from the thermal denaturation of the epitope (44°C) was  $\sim 3^\circ\text{C}$  lower than the  $T_m$  (47°C) of the entire molecule as measured by changes in its ellipticity. This difference is somewhat greater than we previously observed for similar correlations made for type V collagen and two different monoclonal antibodies specific for it (30). Possibly, the epitope against which AC9 is directed has a somewhat

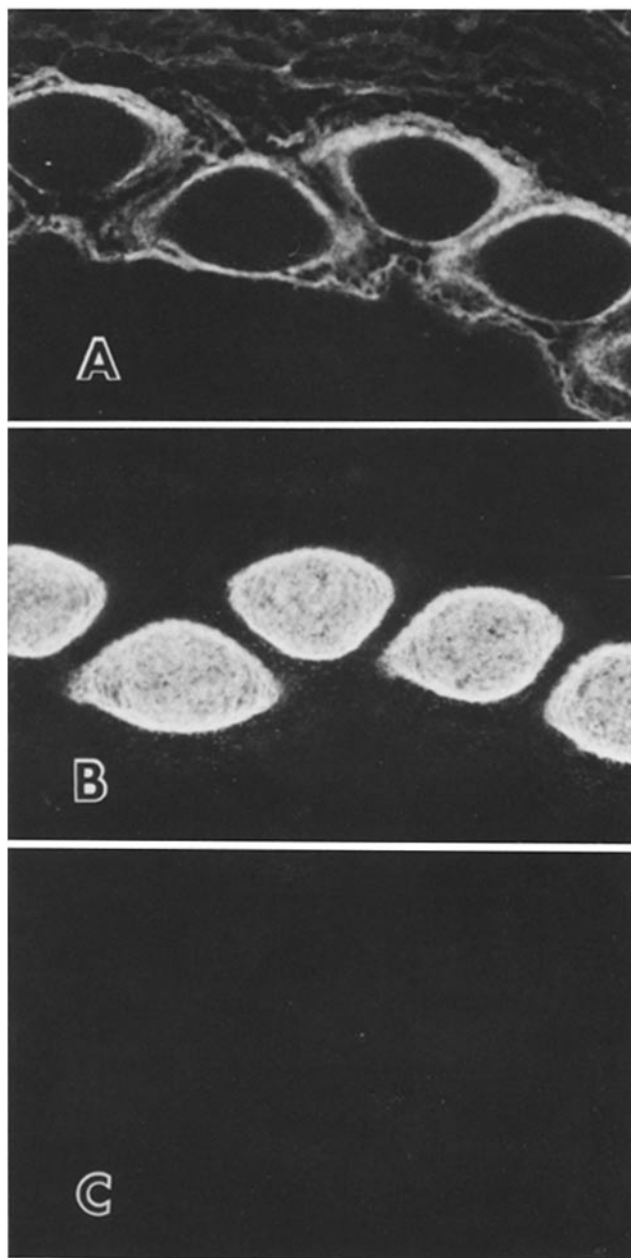


FIGURE 5 Photomicrographs of serial sections of 19-d embryonic chick trachea treated with monoclonal antibodies directed against collagen types I (A), II (B), and X (C).  $\times 100$ .

lower thermal stability than that of the overall helical structure of the type X molecule, which is the parameter that would be measured by CD spectropolarimetry. The 59K form exhibited a different denaturation profile. It appeared to lose only about one third of its total inhibitory ability, even at the highest temperatures examined. We are quite sure, however, that this result was due to rapid renaturation of a large portion of the denatured 59K molecules, since it is both qualitatively and quantitatively in good agreement with our previous CD analyses on this form of the molecule. In those experiments, we observed that when a sample of thermally denatured 59K molecules was cooled to room temperature, as was done in the ELISA experiments, about two thirds of the original helical signal returned within 40 min. Since the 45K form showed no detectable renaturation, as was also the case here, most likely renaturation in the 59K form is due to the presence



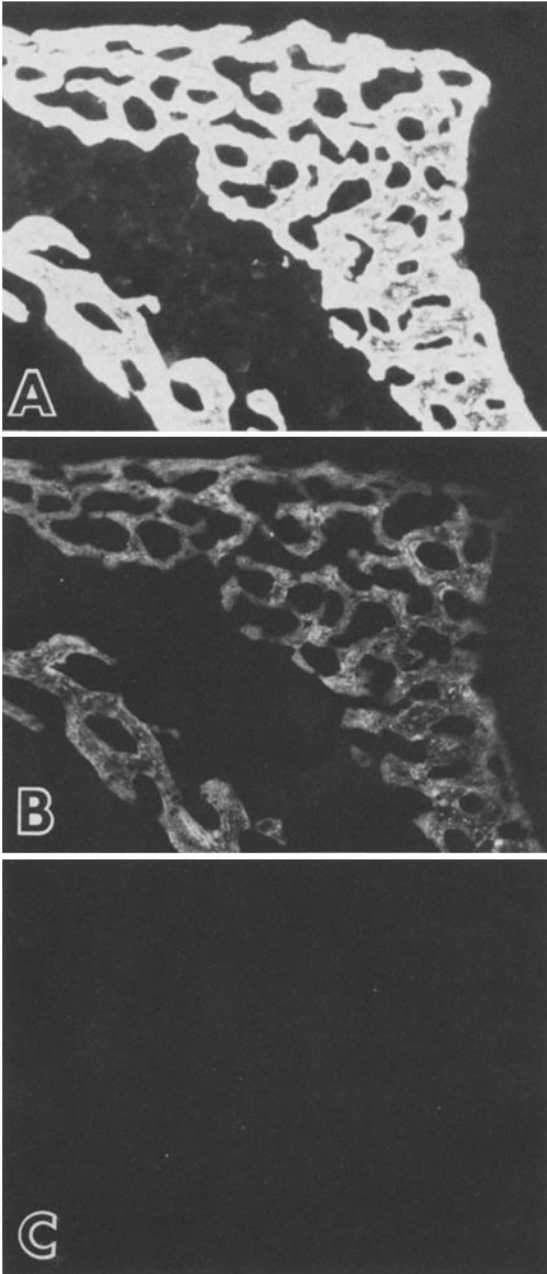


FIGURE 6 Photomicrographs of serial sections of 19-d embryonic chick calvaria treated with monoclonal antibodies directed against collagen types I (A), X (B), and II (C).  $\times 100$ .

of the nonhelical domain, which we know to be thermally stable (23). This probably keeps the chains of the denatured molecules in register, thus promoting rapid reformation of the helical structure upon cooling. Similar rapid renaturation has been reported for procollagen type I, which has a thermally stable nonhelical domain (36).

We have recently demonstrated (37) that anticollagen monoclonal antibodies that recognize conformation-dependent helical epitopes can serve as specific immunohistochemical probes for changes in this molecular conformation in tissues in situ. By using this approach, we were able to demonstrate that the  $T_m$  of the epitope of type IV collagen in basement membranes is 15–20°C higher in situ than that which is measured in solution (29). The conformation-dependent character of antibody AC9, as demonstrated here, should make it

a useful probe for similar studies on the in situ structure and assembly of type X collagen.

Our immunohistochemical analyses suggest that type X collagen is a skeletal-specific molecule and, as demonstrated here, may be present in bone as well as in hypertrophic cartilage. All of the controls, as described in Results, are consistent with this binding being specific. However, as judged by the fluorescent signal produced, it may be present in exceedingly small amounts. This conclusion must remain tentative since, in preliminary attempts, we have not been able to extract identifiable quantities of type X from calvaria. Thus, we can not say for certain that the epitope detected in bone belongs to the same type X molecule present in cartilage; it may be in another, closely related one.

In cartilage, the overall staining pattern obtained is consistent with the molecule being exclusively a product of hypertrophic cartilage. It is not found in cartilages that do not undergo hypertrophy. We have also observed that throughout embryonic development of the tibiotarsus, the temporal and spatial distribution of type X collagen is consistent with this conclusion (38). Thus the process of chondrocyte hypertrophy does not simply involve quantitative changes in the same battery of matrix molecules the cells had been synthesizing at an earlier phase in their life cycle, as might be the case if the cells were simply shutting down their biosynthetic machinery in preparation for their eventual removal. Instead, hypertrophy seems to be an active process, involving the *de novo* synthesis of at least one qualitatively different macromolecule.

Thus far we have not observed detectable amounts of type X collagen in any location outside of the skeletal system. But such a conclusion must always be considered as tentative until all possible adult and embryonic tissue sources have been examined, and at all stages of development. Type II collagen, for example, once thought to be found exclusively in cartilage, has been found in both the vitreous of the eye and the avian primary corneal stroma. In the latter structure, it is synthesized only in very young embryos (39). Also, masked antigenic sites are always a possibility (30).

We can only speculate as to what role(s) type X collagen performs in the skeletal system. Its high concentration in hypertrophic cartilage suggests that it might be involved in the processes of matrix erosion, vascularization, or calcification, all of which occur in this region. Its presence in bone was somewhat unexpected, since the extracellular matrices of bone and cartilage are dramatically different. They do, however, have one common feature in the presence of matrix vesicles (40, 41), which are thought to be the initial sites of hydroxyapatite formation (42, 43). Whether or not type X collagen is associated with these structures, or acts in concert with them in the process of calcification, is an interesting possibility which we are pursuing.

We thank Ms. Anita Mentzer and Ms. Eileen Gibney for excellent technical assistance, Dr. Jerome Gross for helpful discussions and suggestions throughout the course of this work, and Dr. Richard Mayne for providing some of the collagens used as standards.

This work was supported by National Institutes of Health grants AM-3564 and EY-05191. Thomas Schmid is a fellow of the Arthritis Foundation. This is publication No. 973 of the Robert W. Lovett Group for the Study of Diseases Causing Deformities.

Received for publication 6 June 1984, and in revised form 1 October 1984.

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