Comparative Studies of Type X Collagen Expression in Normal and Rachitic Chicken Epiphyseal Cartilage

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Abstract. The levels of type X collagen in mineralizing normal chicken epiphyses and nonmineralizing rachitic chicken tibial epiphyses were measured and compared. Qualitative immunoperoxidase studies with anti-chick type X collagen monoclonal antibodies on sections from normal and rachitic cartilage demonstrated that the type X collagen levels in rachitic growth plates are reduced. Northern hybridization of mRNA and biosynthetic studies have confirmed that type X collagen synthesis in rickets is also decreased. In hypocalcemic rickets, the level of type X collagen mRNA is reduced by 80% whereas the level of type X collagen mRNA is only reduced by 50% in normocalcemic rickets. These observations provide additional evidence that type X collagen is involved in the process of cartilage mineralization and also suggest that the partial recovery of type X collagen synthesis in normocalcemic rickets may be related to the elevated plasma concentration of calcium. Calcium concentration may therefore play an important role in the control of type X collagen synthesis.

LONGITUDINAL growth of long bones is governed by activities of cells located within the epiphyseal cartilage. During development of the growth plate, chondrocytes in the epiphysis undergo a continuum of cell proliferation, maturation, and hypertrophy (Stocum et al., 1979). Finally, cells in the distal hypertrophic zone degenerate and the matrix becomes mineralized with the concurrent invasions of blood vessels and bone marrow. Chondrocytes within these different zones show varied cell morphologies and biochemical activities, as exemplified by the differences in the composition of their respective extracellular matrices. Biochemical analyses of cartilage matrices in recent years have revealed a high degree of collagen heterogeneity. To date, five genetically distinct collagen types have been shown to be associated with the epiphyseal cartilage, namely collagen types II, VI, IX, X, and XI (Grant et al., 1988).

Among these molecules, type X collagen is of particular interest since it is synthesised exclusively by hypertrophic chondrocytes in the part of the growth plate destined for matrix mineralization (Schmid and Linsenmayer, 1983, 1985; Kielty et al., 1984, 1985; Kwan et al., 1986*a*). Although this molecule has been characterized extensively in recent years (Kielty et al., 1985; Schmid et al., 1984; Kwan et al., 1986*b*; Ninomiya et al., 1986), the exact functional role of type X collagen remains to be defined. However, its temporal and spatial appearance within the cartilaginous matrix of growing epiphyses suggest its possible involvement in processes that lead to matrix mineralization during endochondral bone formation (Habuchi et al., 1985). Additional evidence for the association between type X collagen and endochondral ossification has been presented in a recent publication showing the synthesis of type X collagen in mineralizing fracture callus (Grant et al., 1987).

Vitamin D deficiency results in a failure of the mineralization process. In chickens with vitamin D–deficiency rickets, the epiphyseal growth plates of long bones are abnormally wide due mainly to an enlargement of the zone of chondrocyte proliferation and maturation. If the hypocalcemia that is also present in these rachitic chickens is normalized by increasing the calcium content in the diet, the width of this zone is similar to that found in vitamin D–replete chickens of similar age, but the zone of chondrocyte hypertrophy and degeneration is enlarged. It is within this latter zone that cartilage normally calcifies and is then replaced by mineralizing osteoid, but histological and microradiographic studies showed this process to be defective in both normocalcemic and hypocalcemic vitamin D–deficient chickens (Jande and Dickson, 1980). These animal models provided a convenient approach to investigate the relationship between vitamin D status and synthesis of type X collagen.

Materials and Methods

Production of Chickens with Hypocalcemic and Normocalcemic Rickets (HCR and NCR, Respectively)[1]

1-d-old Golden Comet cockerels (Sappa Ltd., Bury, St. Edmunds, UK)

1. *Abbreviations used in this paper*: HCR, hypocalcemic rickets; NCR, normocalcemic rickets.

were divided into three groups. One group was induced to develop HCR by maintenance on a rachitogenic diet (vitamin D–deficient diet containing 1.2% [wt/wt] Ca^{++} and 0.7% [wt/wt] phosphate). To produce NCR, birds were fed the rachitogenic diet for 3 wk, but after the first week their diet was supplemented with 1% (wt/wt) $CaCO_3$ for 7 d followed by 3% (wt/wt) $CaCO_3$ throughout the third week. The third group was given cholecalciferol (7.5 μg in 0.5 ml of arachis oil) orally twice each week in addition to the vitamin D–deficient diet and was defined as the control group. The birds were maintained for 3 wk and then anesthetized, bled by cardiac puncture, and killed by chloroform overdose. Serum levels of calcium, phosphorus, and alkaline phosphatase were analyzed using methods described elsewhere (Jande and Dickson, 1980).

Preparation of Monoclonal Antibodies to Chicken Type X Collagen

Chicken type X collagen was isolated and purified from chondrocyte culture media as previously described (Kielty et al., 1985). BALB/c mice (8–12 wk old) were each injected intraperitoneally with either 200 μl of native chick type X collagen in complete Freund's adjuvant (Sigma Chemical Co. Ltd., Poole, UK) or 200 μl of alum precipitate suspension containing 50 μg of type X collagen and 2×10^9 killed *Bortella pertussis* organisms (Burroughs Wellcome & Co., London, UK). The mice were allowed to rest for 3 wk after the initial immunization and were then reinjected intraperitoneally with 25 μg of type X collagen in incomplete Freund's adjuvant (200 μl). First, tail bleed and antibody titer determinations were performed 2 wk after the second injection. Mice showing positive responses to the immunization procedures were reinjected and rested for 4 wk before the final booster injection (25 μg of type X collagen in PBS) was administered. After 3 d, spleen cells were taken from these mice and mixed with myeloma x63-Ag8.653 in a spleen-to-myeloma ratio of 5:1 (Kearney et al., 1979). Fusion was achieved by adding to the mixed cell pellet 1 ml of 45% (wt/vol) polyethlene glycol (PEG 1500; BDH Chemicals Ltd., Poole, UK) in serum-free RPMI-1640 medium (Flow Laboratories, Rickmansworth, UK) containing 5% (vol/vol) DMSO. Hybridomas were selected by the hypoxanthine/aminopterin/thymidine selection method. Positive hybridomas were expanded, cloned, and subcloned by limiting dilution as described elsewhere (Galfre et al., 1977; Løvborg, 1982; Goding, 1980). Antibody specificities were assessed by ELISA with (*a*) purified or pepsinized chicken type X collagen; (*b*) chicken collagen types I, II, IX, and XI; and (*c*) bovine fibronectin. Mouse ascites fluid was prepared by intraperitoneal injection of 10^6 hybridoma cells into adjuvant-primed BALB/c mice. Antibodies were precipitated twice from the collected ascites fluid by ammonium sulphate precipitation at 50% saturation. Monoclonal antibodies purified from hybridoma cell lines MC7 and MB6 were used in immunolocalization studies.

Immunohistochemistry

The proximal tibial epiphyses were removed from freshly killed chickens and fixed in half-strength Karnovsky's fixative at 4°C for 16 h. Fixed tissues were dehydrated through a series of graded alcohol solutions and embedded in paraffin wax for sectioning. Dewaxed sections (5–7 μm thick) were treated with bovine testicular hyaluronidase (1 μg/ml in 0.05 M sodium acetate buffer, pH 5, with 25 mM NaCl) at room temperature for 60 min in a moist chamber. Treated sections were washed and incubated for 1 h with monoclonal antibodies MC7 and MB6, washed with PBS, and then stained with peroxidase-conjugated anti-mouse IgG for 60–90 min. Sections were immersed in 0.2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride containing 0.05% (vol/vol) H_2O_2 in PBS. Nuclei were counterstained with hematoxylin.

RNA Preparation

Total RNA was extracted from freshly dissected whole epiphyses or from different cartilage zones by the guanidinium isothiocyanate/cesium chloride method (Maniatis et al., 1982). Tissue fragments were homogenized with a homogenizer (Polytron Ultra-Turrax T25; IKA-Labortechnik, Janke & Kunkel GmbH & Co., Staufen, FRG) in 4 M guanidinium isothiocyanate (Fluka Chemicals Ltd., Glossop, UK) containing 5 mM sodium citrate, 0.1 M β-mercaptoethanol, and 0.5% Sarkosyl. Tissue debris were removed from the homogenate by centrifugation for 5 min at 1,000 *g*. Solid cesium chloride (1 g/2.5 ml) was added to the clarified homogenates which were then layered on top of a 2.5-ml CsCl cushion consisting of 5.7 M CsCl in 0.1 M EDTA. The RNA was pelleted by centrifugation for 12 h at 100,000 *g* (at 20°C) in a rotor (Ti50; Beckman Instruments, Inc., Fullerton, CA). The RNA pellet was resuspended in 10 mM Tris-HCl, pH 7.4, containing 5 mM EDTA and 1% SDS and digested with 50 μg of proteinase K (Gibco Laboratories, Paisley, Scotland) at 64°C for 1 h to remove any contaminating protein and proteoglycan. The digest was extracted once with a 1:1 mixture of phenol/chloroform. RNA was precipitated over 16 h after the addition to the aqueous phase of 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.2 vol of ethanol at −20°C. RNA was recovered by centrifugation, washed with 70% ethanol, and dried under vacuum before dissolving in diethyl-pyrocarbonate-treated water. Integrity of the isolated RNA was assessed by the presence of discrete 28s and 18s ribosomal RNA bands after electrophoresis in formaldehyde-containing agarose gels (Boot-Handford et al., 1987).

Northern Blot and Hybridization Analyses

Heat-denatured RNA (10 μg/sample) was electrophoresed on a 1% (wt/vol) agarose gel containing formaldehyde (2.2 M) followed by capillary blotting onto a nitrocellulose filter using a vacuum blotting apparatus (Vacugene; LKB Instruments, Inc., Gaithersburg, MD). The filter was air dried, baked under vacuum at 80°C for 2 h, and prehybridized for 6 h at 37°C in 50% formamide, 4× SSC buffer, 1 mg/ml sheared salmon sperm DNA, 1× Denhardt's solution (Boot-Handford et al., 1987), and 0.1% SDS. Hybridizations with heat-denatured ^{32}P-labeled chicken type X collagen cDNA (clone pYN 3116 kindly donated by Dr. B. R. Olsen, Harvard Medical School, Cambridge, MA) was carried out at 37°C for 16–24 h in prehybridization solution. ^{32}P-labeled cDNA probe was prepared by nick translating whole plasmids using the nick translation kit from Boehringer Mannheim Biochemicals (Lewes, UK).

Fluorograms were obtained by exposing the filters to x-ray film (XAR; Eastman Kodak Co., Rochester, NY) in the presence of intensifying screens at −80°C. The intensities of bands were assessed by absorbance per microgram of total RNA from densitometric scanning of the fluorograms.

Organ Cultures

Isolated epiphyseal cartilage was dissected into proliferative and hypertrophic zones. The distinct delineation of cartilage zones within the epiphyses of these 3-wk-old chickens allowed easy dissection without the need of visual aids. The regions corresponding to the border between proliferative and hypertrophic cartilage was discarded to avoid cross-contamination of cell populations. The cartilage zones were cultured in serum-free modified Biggers' medium (Dickson and Maher, 1985) containing 20 μCi/ml ^{3}H-proline, 150 μg/ml ascorbate, 100 μg/ml β-aminopropionitrile for either 2 or 12 h. At the end of incubation, labeled proteins from the culture medium were isolated by ammonium sulphate precipitation at 80% saturation. Labeled proteins from tissues were extracted with 50 mM Tris-HCl, pH 7.4, containing 4 M guanidinium chloride followed by extraction with 0.5 M acetic acid containing 1 mg/ml pepsin. Extracted polypeptides were

*Table I. Biochemical Analyses of Plasma Levels of Calcium, Phosphate, and Alkaline Phosphatase Activities of Chickens in Each Different Diet Group**

Diet group	Group size	Vitamin D status	Body weight	Calcium	Alkaline phosphatase	Inorganic phosphate
			g	*mg/100 ml*	*U/100 ml*	*mg/100 ml*
Control	17	+	264.8 ± 7	11.03 ± 0.13	10.42 ± 1.6	6.45 ± 0.44
HCR	17	−	155.5 ± 6.8	6.85 ± 0.44	142.3 ± 13.9	6.14 ± 0.57
NCR	18	−	218.8 ± 7.2	10.95 ± 0.48	75.04 ± 10.5	1.85 ± 0.21

* Values shown are expressed as mean ± SEM.

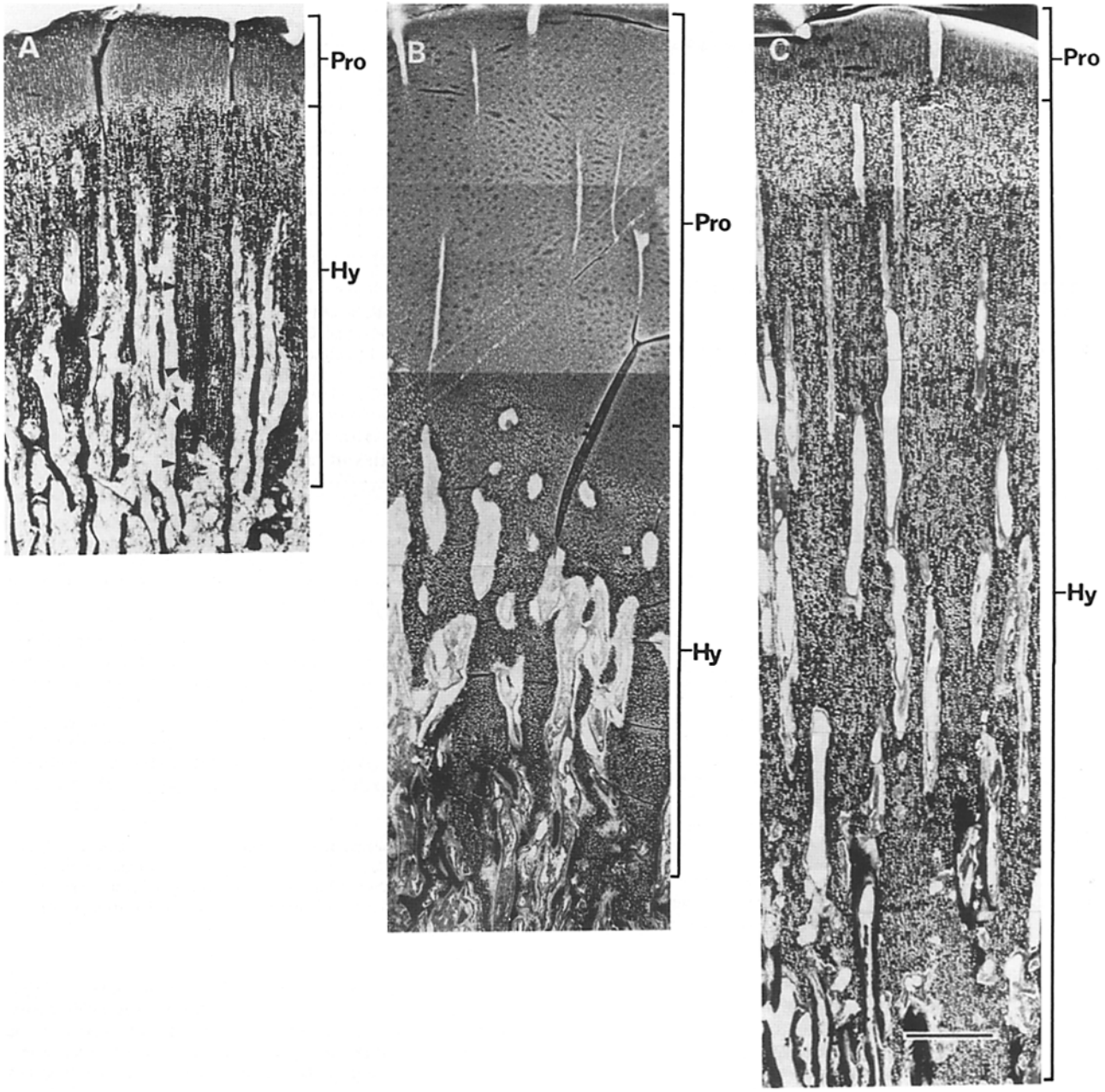

Figure 1. Montage of light micrographs of longitudinal sections through (*A*) normal (control), (*B*) HCR, and (*C*) NCR chicken distal tibial epiphyses. Tissues were fixed in half-strength Karnovsky fixative, embedded in paraffin, sectioned, and stained for mineral deposits with von Kossa stain. Note that the hypertrophic cartilage of the control epiphysis is extensively mineralized as shown by the heavy von Kossa staining (*arrowheads*) in the zones of hypertrophy and degeneration (*Hy*). The zones of chondrocyte proliferation and maturation are labeled (*Pro*). Nuclei were counterstained with hematoxylin. Cartilage matrix was stained with toluidine blue. Bar, 600 μm.

analyzed by SDS-PAGE (Laemmli, 1970) and fluorography as previously described (Kielty et al., 1985).

Results

Histology

Chickens raised on the vitamin D–deficient diet and given vitamin D_3 supplements (control group) showed no evidence of bony abnormalities as judged by growth plate histology (Fig. 1) and serum biochemistry (Table I). Those birds raised on the unsupplemented vitamin D–deficient diet exhibited classical symptoms of HCR (HCR group): subnormal body weight, elevated serum alkaline phosphatase, and low serum calcium. Chickens raised on the calcium-supplemented vitamin D–deficient diet (NCR group) had body weights and serum alkaline phosphatase levels that were intermediate between the other groups; serum calcium levels were similar to those in normal chickens, but the birds were markedly hypophosphatemic (Table I). The proximal tibiotarsal growth plates were abnormally wide in the HCR and NCR chickens. The extended width of the epiphyseal plate in HCR chickens was the result of the increase in the zone of proliferation and maturation which comprised ~50% of

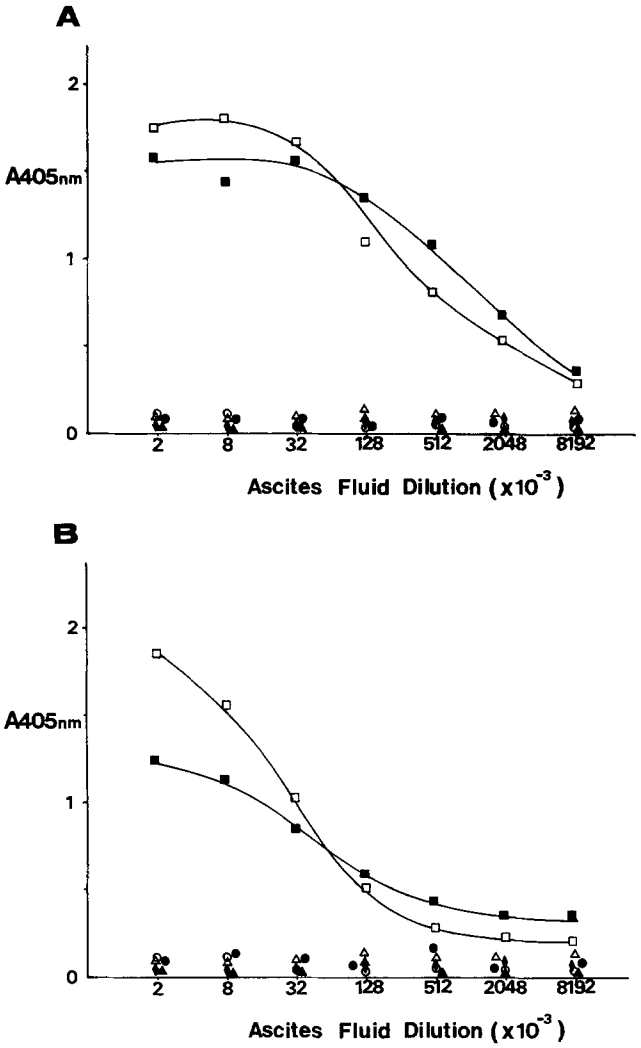

Figure 2. Determination of specificity of monoclonal antibodies derived from hybridoma lines MC7 and MB6. (*A*) ELISA of ascites fluid containing monoclonal antibody MC7 with coating of native type X collagen (■), pepsinised type X collagen (□), chick type I and II collagen mixture in a 1:1 ratio (●), type IX collagen (△), type XI collagen (▲), and fibronectin (♦). Concentrations of antigens are ~1 μg/well. The reactivity of normal mouse serum against native type X collagen is also indicated (○). (*B*) ELISA of ascites fluid containing monoclonal antibody MB6 with the same coatings.

the entire epiphyseal plate. In this zone, the cells exhibited an irregular distribution, which may be due to changes in the content and/or the composition of the extracellular matrix. In the NCR group, the width of the proliferative zone was not noticeably different from the control group, and the proliferating chondrocytes were arranged regularly in normal stacks. However, the zone of hypertrophy and degeneration was much wider than either the control or HCR group. In both NCR and HCR chickens, the cartilage matrix in the hypertrophic zone was poorly mineralized as shown by the low intensities of von Kossa staining (Fig. 1, *B* and *C*).

Immunolocalization of Type X Collagen

Changes in matrix composition with respect to the levels of type X collagen in rachitic cartilage were investigated by immunoperoxidase localization with monoclonal antibodies to chicken type X collagen. Monoclonal antibodies from hybridoma clones MC7 and MB6 were purified and screened by ELISA. These antibodies were shown to belong to the IgG_1 subtype, and their reactivities against type X procollagen and pepsinized type X collagen indicated that they both recognize epitopes within the triple-helical domain (Fig. 2). This observation has been confirmed by electronmicroscopic visualization of the antibody–antigen complexes after rotary shadowing (Kwan, A. P. L., manuscript in preparation). Fig. 3 shows micrographs of cartilage sections from normal and rachitic cartilage which have been treated with monoclonal antibody MC7. In normal, control chickens, intracellular type X collagen reactivity is observed from the beginning of the hypertrophic zone, and the intensity of the signal gradually increases upon progression to the lower hypertrophic zone. Matrix localization of type X collagen is observed only in the zone of ossifying cartilage (Fig. 3, *A* and *B*). Immunoperoxidase activities in rachitic epiphyses (HCR and NCR) were found to be very weak, and only faint cytosolic reactivity is detectable in the zone of hypertrophic chondrocytes. However, a slightly higher occurrence of cell-associated immunoperoxidase activity can be seen in sections of NCR chick epiphyseal plates (Fig. 3, *E* and *F*). Identical results were observed when monoclonal antibody MB6 was used (results not shown). These observations clearly demonstrate a change in the organization of the extracellular matrix in vitamin D–deficiency rickets. To assess whether the decreased levels of type X in rachitic epiphyseal plates could be attributed to decreased synthesis or the inability of rachitic cartilage matrix to accumulate this collagen, the following experiments were undertaken.

Analysis of Steady-State mRNA Levels

The steady-state levels of type X collagen mRNA from each group of chickens were assessed to confirm that the expression of type X collagen in vivo is affected by the lack of vitamin D in the diet. Northern analyses of chicken type X collagen mRNA from the whole epiphyses are shown in Fig. 4. These fluorograms indicate that the levels of the type X collagen mRNA in both HCR and NCR were diminished. Densitometric scanning of tracks of the fluorograms provided quantitation of the relative amounts of type X collagen mRNA in the epiphyses of these chickens. In three separate experiments, marked differences in the levels of type X collagen mRNA between control, HCR, and NCR chickens were consistently observed. In chickens with HCR, the steady-state level of type X collagen mRNA, expressed as units of mRNA per microgram of total RNA, extracted from the epiphyses is reduced to 18–20% of the value obtained from normal, control chickens, and the result corresponds well with the decrease in antigen–antibody reactivity on sections of rachitic epiphyses. The level of type X collagen mRNA in the NCR group is ~45% of the control level, which is consistent with the intermediate level of antibody reactivity on histological sections (Fig. 3).

Organ Culture Studies

Hypertrophic cartilage was cultured in the presence of [^{3}H]proline, and newly synthesized collagenous polypeptides from the culture media and tissue extracts (see Mate-

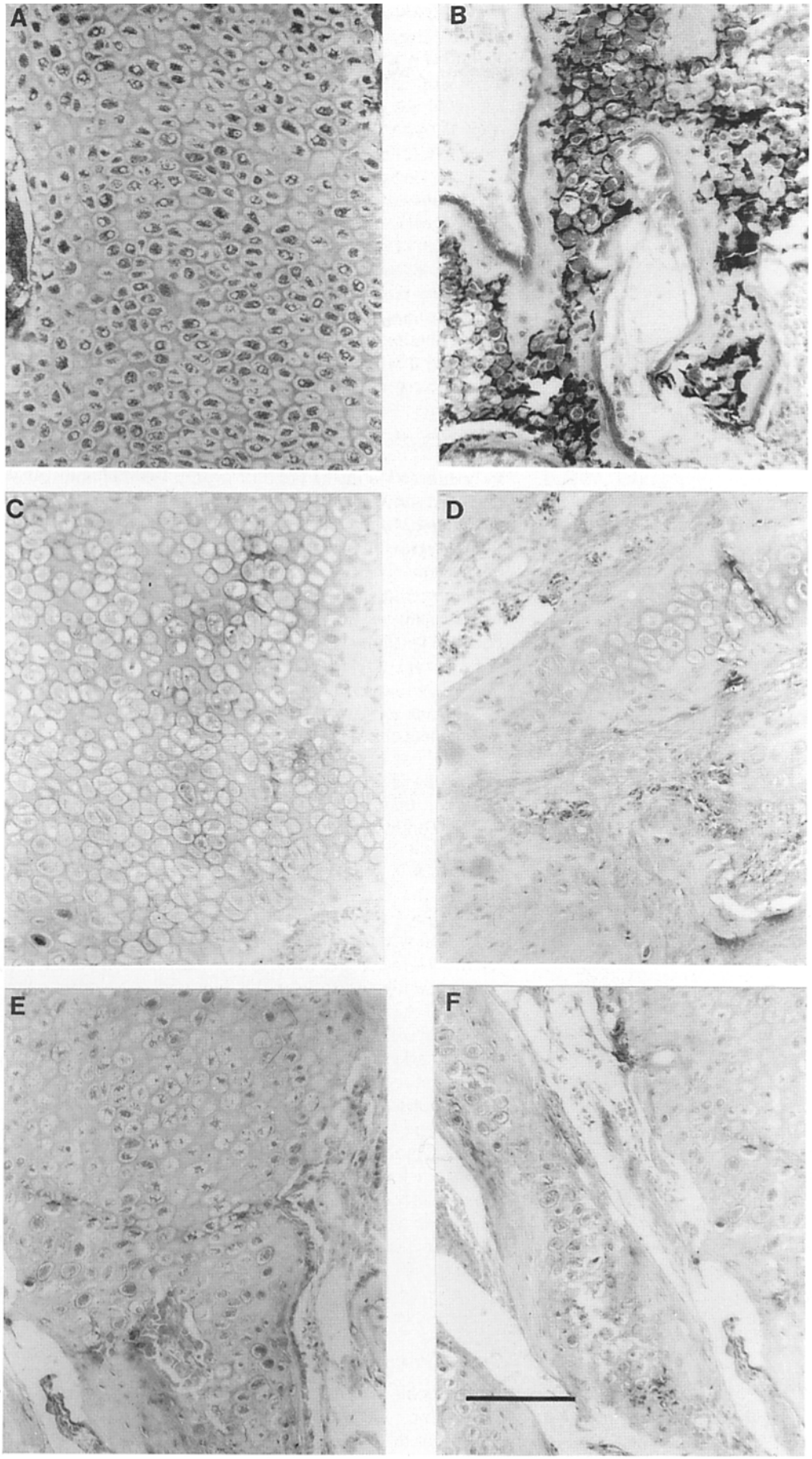

Figure 3. Immunoperoxidase localization of type X collagen in normal and rachitic chicken epiphyseal plates. Light micrographs of paraffin sections (7 μm) pretreated with bovine testicular hyaluronidase and stained with monoclonal antibodies MC7 followed by staining with peroxidase-conjugated anti-mouse IgG. (*A*) Photomicrograph of control epiphysis with peroxidase reactivity localized to the cytoplasm of the upper hypertrophic chondrocytes. (*B*) Strong immunoperoxidase reactivity is present in the lower hypertrophic zone of control epiphysis where mineralization has taken place. (*C* and *D*) Micrographs of sections from rachitic (HCR) epiphyses; only very weak peroxidase reactivity can be detected in the corresponding regions of rachitic epiphysis. (*E* and *F*) Micrographs of sections of NCR epiphysis showing intermediate peroxidase reactivity in these sections. Identical staining patterns were observed with monoclonal antibody MB6. Bar, 100 μm.

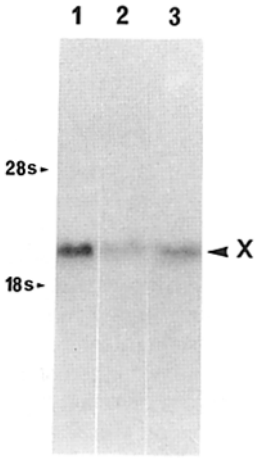

Figure 4. Northern analysis of total RNA from normal and rachitic chicken tibial ephiphyses. Total RNA (10 μg), extracted from pooled epiphyses, was electrophoresed in a formaldehyde-containing agarose gel, blotted, hybridized with ^{32}P-labeled chicken type X collagen cDNA pYN3116, washed, and exposed to x-ray film under the conditions described in Materials and Methods. Samples were (lane *1*) RNA extracted from control epiphyses; (lane 2) RNA extracted from HCR epiphyses; and (lane *3*) RNA from NCR epiphyses. The positions of the 28s and 18s ribosomal RNA bands are indicated.

rials and Methods) of the explants were analyzed by SDS-PAGE. The major [^{3}H]proline-labeled polypeptides isolated from various fractions of control, HCR, and NCR cultures were the α chains of collagen types II, I, and X (Fig. 5). In short-term organ cultures (incubation for 2 h), the percentages of the total extractable and nondiffusible radioactivity in the culture medium, 4 M guanidinium chloride, and pepsin extracts were ~15, 75, and 10%, respectively. Relative proportions of type X collagen polypeptides were assessed by densitometric scanning of the fluorograms after SDS-PAGE. In the cultures from control cartilages, type X collagen synthesis accounted for ~60% of the collagenous molecules recovered in the three extracts. In HCR and NCR cultures these figures were reduced to ~20 and 40%, respectively. Duplicate organ culture experiments yielded similar results, showing a marked reduction of type X collagen synthesis by rachitic cartilage: an observation that is consistent with the reduction of type X collagen immunoreactivity and reduced levels of type X collagen mRNA reported above.

However, when protocols involving prolonged incubation were adopted, considerable changes in the biosynthetic behavior of the normal and rachitic epiphyses were observed (Fig. 5). After 12 h of incubation, the distribution of extractable [^{3}H]proline counts in the culture medium, guanidinium chloride, and pepsin extracts were found to be ~40, 50, and 10%, respectively. In contrast to the 2-h incubations, where type X collagen synthesis was much lower than in the controls, after 12 h in culture, type X collagen synthesis was consistently higher in the rachitic cartilage cultures. After 12 h in culture, type X collagen accounted for ~45% of the collagenous molecules synthesized in controls, whereas the values obtained for HCR and NCR cultures had increased to ~60 and 70%, respectively.

Discussion

In this investigation of the relationship between mineralization and type X collagen expression in vivo, studies have been conducted with rachitic chickens fed on a vitamin D–deficient diet or the same diet supplemented with high levels of calcium. The findings reported here (*a*) of reduced immunohistochemical staining for type X collagen in rachitic hypertrophic cartilage, (*b*) of reduced synthesis of type X collagen by short-term cultures of explants from rachitic tissues, and (*c*) of reduced levels of extractable type X procollagen mRNA are all consistent with the hypothesis that the production of type X collagen is reduced in the vitamin D–deficient chickens. On the basis of the immunohistochemical studies, it was concluded that there was a decreased deposition of type X collagen in the matrix of rachitic birds. However, these results from immunoperoxidase localization studies contrast with the work of Reginato et al. (1988), who

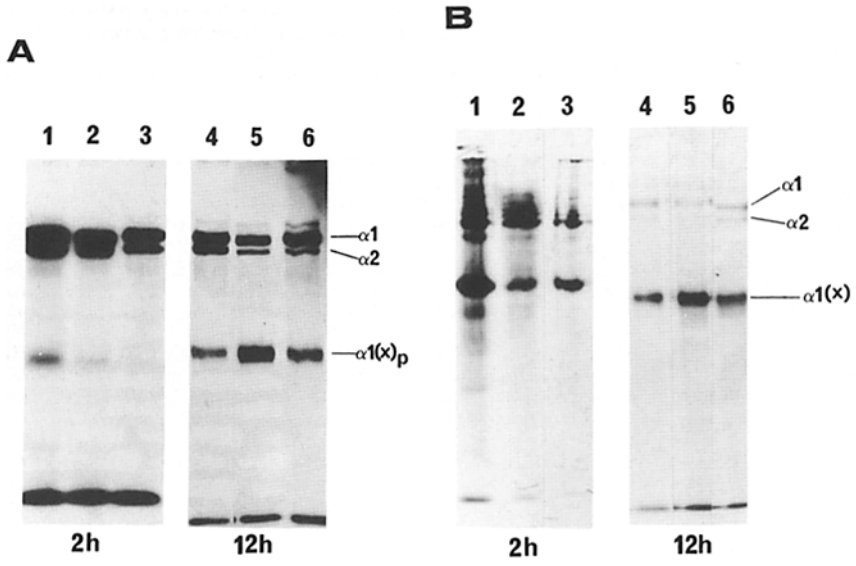

Figure 5. SDS-PAGE (8%) of proteins extracted from the short-term (2-h) and long-term (12-h) chicken epiphysis organ cultures. (*A*) Fluorogram of [^{3}H]proline-labeled collagenous polypeptides isolated from culture medium of 2- and 12-h (lanes *1–3* and *4–6*, respectively) organ cultures of hypertrophic cartilages dissected from control (lanes *1* and *4*), HCR (lanes *2* and *5*), and NCR (lanes *3* and *6*) chickens. All samples were subjected to limited pepsin digestion at 4°C for 16 h before electrophoresis. (*B*) Fluorogram of [^{3}H]proline-labeled collagenous polypeptides extracted from tissues in organ cultures by 4 M guanidinium chloride. Lanes *1* and *4* are of extracts from control hypertrophic cartilage in 2- and 12-h cultures, respectively. Lanes *2* and *5* show extracts from HCR hypertrophic cartilage from 2- and 12-h cultures, respectively. Lanes *3* and *6* show extracts from NCR hypertrophic cartilage from 2- and 12-h cultures, respectively. The label α1 was used to show the position of α1(I) and α1(II) chains of type I and type II collagens, respectively. The positions of the α2(I) and α1(X), 59,000 M_r, and α1(X)p, 45,000 M_r, are also shown.

report the increased presence of type X collagen in rachitic cartilage on the basis of type X collagen extractability from tissues. In analogous experiments in this laboratory, using 4 M guanidinium chloride and pepsin as extractants, no conclusive evidence was produced in support of the claim that type X collagen deposition increased in rachitic epiphyses (Kwan, A. P. L., and M. E. Grant, unpublished observations). An alternative approach was therefore adopted to investigate the capacity of normal and rachitic epiphyses to synthesize type X collagen. Decreased levels of type X collagen mRNA in HCR and NCR epiphyses were demonstrated by Northern hybridization techniques (Fig. 4), and these findings support the notion that type X collagen synthesis in rachitic cartilage is diminished. Although it can be argued that variations in the levels of type X collagen mRNA are the result of the differences in the dimensions of the proliferative and hypertrophic zones, two lines of evidence have indicated that these results reflect a genuine decrease in type X collagen gene expression in vitamin D–deficient chickens. First, the type X collagen mRNA level in epiphyses of NCR chickens showed a >50% reduction despite the highly elongated hypertrophic zone. Second, when mRNA isolated specifically from hypertrophic cartilage was analyzed, type X collagen mRNA content of HCR chickens was found to be reduced by 80% and a similar reduction in type X collagen expression in NCR hypertrophic cartilage was also observed (result not shown).

Organ culture experiments were conducted to study the biosynthetic behavior of normal and rachitic epiphyses in vitro. In the 2-h organ cultures, the amounts of type X collagen synthesized by HCR and NCR hypertrophic cartilage were shown to be lower than in the control tissues, an observation that is consistent with the results from the immunoperoxidase localization and mRNA experiments. However, markedly different results were obtained in the long-term (12-h) incubations, where the synthesis of type X collagen by rachitic cartilage increased dramatically during the prolonged incubation (Fig. 5). The differences in the results between the two organ culture systems can be explained if the biosynthesis of type X collagen is suppressed in vivo. The abnormally high level of type X collagen synthesis after the tissues had been in culture for >10–12 h may be caused by either the removal of such suppression or activation of type X collagen synthesis. Additional studies are being undertaken to investigate the nature of such suppression of type X collagen synthesis in rachitic cartilage and also to identify the possible factor(s) responsible for the induction of type X collagen synthesis.

Mechanisms by which vitamin D acts on skeletal tissues are not clearly understood, although collagen synthesis by cartilage and bone in explant cultures can be affected directly by vitamin D metabolites (Dickson and Maher, 1985). Since the primary function of vitamin D is the stimulation of the absorption of calcium through intestinal epithelial cells, the change in type X collagen synthesis in rickets may be a secondary effect related to calcium homeostasis, and changes in the local calcium concentration in the epiphyses may play an important role in the control of type X collagen gene expression. This effect of calcium on type X synthesis may also explain the higher levels of type X collagen synthesis in NCR chickens, which have been fed on a diet supplemented with very high levels of calcium carbonate. However, the intermediate levels of type X collagen in the NCR chicken epiphyses also suggest that calcium may be only one of the factors affecting type X synthesis since the levels of type X collagen in NCR chickens are still below control levels.

Type X collagen has been shown to be a product of hypertrophic chondrocytes, and studies conducted with chick embryo chondrocytes in culture have demonstrated that type X collagen synthesis is markedly influenced by a number of factors including matrix macromolecules (Gibson et al., 1982, 1983; Bates et al., 1987; Thomas and Grant, 1988), the levels of exogenous calcium and phosphate (Grant et al., 1988), and, particularly, calcium-β-glycerophosphate, a substrate used in the study of mineralization in vitro (Morris and Balian, 1985; Thomas, J. T., and M. E. Grant, unpublished observations). Although many studies have shown that type X collagen is synthesized when endochondral ossification occurs (Kwan et al., 1986*a*; Grant et al., 1987), no direct evidence for a relationship between type X collagen synthesis and matrix mineralization has been reported. Since type X collagen synthesis has been shown to precede matrix mineralization, it is therefore possible that the poor degree of mineralization in HCR and NCR epiphyses is the consequence of the low level of type X collagen, which has a putative role of providing a permissive matrix for deposition of minerals in calcifying cartilage (Schmid and Linsenmayer, 1985; Gibson and Flint, 1985). The findings in this report further substantiate the importance of type X collagen in matrix mineralization, and the control of type X gene expression may be an important key to the understanding of the process of cartilage mineralization.

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