Macromolecular Organization of Chicken Type X Collagen In Vitro

Alvin P. L. Kwan,* Christine E. Cummings,‡ John A. Chapman,‡ and Michael E. Grant*

*Department of Biochemistry and Molecular Biology, and ‡Department of Medical Biophysics, University of Manchester, Manchester M13 9PT, United Kingdom

Abstract. The macromolecular structure of type X collagen in the matrices of primary cultures of chick hypertrophic chondrocytes was initially investigated using immunoelectron microscopy. Type X collagen was observed to assemble into a matlike structure within the matrix elaborated by hypertrophic chondrocytes. The process of self assembly was investigated at the molecular level using purified chick type X collagen and rotary-shadowing EM. It was shown that under neutral conditions at 34°C, individual type X collagen molecules associate rapidly into multimeric clusters via their carboxy-terminal globular domains forming structures with a central nodule of carboxy-terminal domains and the triple helices radiating outwards. Prolonged incubation resulted in the formation of a regular hexagonal lattice by lateral association of the juxtaposed triple-helical domains from adjacent multimeric clusters. This extended lattice may play an important role in modifying the cartilage matrix for subsequent events occurring in endochondral bone formation.

MOLECULAR heterogeneity of collagen in the cartilage extracellular matrix is now well documented (for reviews see Eyre et al., 1987; Grant et al., 1988; Mayne, 1989). Such heterogeneity in matrix macromolecular components enables cartilage to function not only as cushioning material to distribute load on bone articulating in cartilagenous and synovial joints but also to mediate the growth and development of the skeleton. The complexity in the organization of the cartilage extracellular matrix is best demonstrated in the epiphyseal growth plate where five genetically distinct collagen types, namely types II, VI, IX, X, and XI, are found to be components of the extracellular matrix (Grant et al., 1988). Within the epiphyseal growth plate, chondrocytes go through a series of well-defined differentiation stages (for review see Caplan, 1988) with concomitant changes in the synthesis and deposition of new extracellular matrix components. As a consequence, the epiphyseal growth plate can be divided into various histologically distinct zones (Stocum et al., 1979). The transition from cartilage to bone takes place in the hypertrophic zone where mineralization and vascularization of the cartilage matrix occurs before the invasion of osteogenic cells from the subchondral bone, a sequence of events collectively known as endochondral ossification.

Type X collagen is a low molecular weight collagen found exclusively in the matrix of the hypertrophic zone of the epiphyseal growth plate cartilage (Schmid and Linsenmayer, 1983, 1985; Kielty et al., 1985; Gibson and Flint, 1985; Kwan et al., 1986*a*). This molecule is comprised of three apparently identical polypeptides of M_r 59,000 known as the α1(X) chains. Biochemical analyses of chicken and bovine collagen X and nucleotide sequencing of the respective cDNAs have shown that the chicken α1(X) chain consists of three distinct domains: a pepsin-resistant collagenous domain of M_r 45,000; a 170-amino acid residues-long carboxy-terminal noncollagenous domain and a smaller NH_2-terminal domain (Kielty et al., 1985; Schmid et al., 1983; Ninomiya et al., 1986). The structure of the bovine α1(X) chain is similar to the avian polypeptide but contains a slightly longer triple-helical domain (Ayad et al., 1988; Thomas et al., 1991). Despite the detailed studies on the characterization and immunolocalization of collagen X (Kielty et al., 1985; Schmid et al., 1984; Kwan et al., 1986*a*, *b*; Ninomiya et al., 1986), little is known about its exact functional role(s) in the hypertrophic cartilage matrix. However the site of its synthesis and the timing of its appearance in the growth plate has led investigators to postulate that collagen X is involved in the processes of endochondral ossification. The synthesis of this molecule has also been demonstrated in the cartilagenous callus that forms during bone repair (Grant et al., 1987). A link between collagen X synthesis and cartilage mineralization has been demonstrated by studies in vivo with rachitic chickens (Kwan et al., 1989) and in vitro by hypertrophic chondrocytes in cultures (Thomas et al., 1990; Wu et al., 1990). A clearer picture of this relationship may emerge once the structural organization of collagen X in the extracellular matrix is known. In this paper, we present evidence for a unique supramolecular organization of type X collagen molecules.

Materials and Methods

Chondrocyte Cultures and Extraction of Matrix-associated Collagens

Chondrocytes were isolated from the hypertrophic regions of 17-d-old embryonic chick tibial epiphyseal cartilage by digesting the tissues for 3 h at 37°C with bacterial collagenase type 1A (10 mg/ml) (Sigma Chemical Co., Poole, England) and trypsin (0.3 mg/ml) in DME (Gibco, Paisley, Scot-

land). Cells were maintained in primary cultures in DME supplemented with 10% (vol/vol) heat-inactivated FBS (Advance Protein Products Ltd., Brockmoor, England) as described by Kielty et al. (1985).

Collagens were extracted from the cell layers of these primary cultures for qualitative examination of collagen types deposited in these matrices. Proteoglycans were first extracted from the cell layers with 4 M guanidinium hydrochloride in 0.5 M Tris/HCl buffer, pH 7.4 containing 25 mM EDTA, 25 mM ϵ-amino-*n*-caproic acid, 2 mM PMSF, and 10 mM *N*-ethylmaleimide (Sigma Chemical Co.) for 16 h at 4°C. After guanidinium chloride extraction, the cell layers were further extracted with 0.5 M acetic acid containing 1 mg/ml pepsin (Sigma Chemical Co.) for 24 h at 4°C. The pepsin extracts were dialyzed extensively against 0.5 M acetic acid and lyophilized. Collagen polypeptides in the pepsin extracts and in the final residue were analyzed by SDS-PAGE according to the methods described by Laemmli (1970).

Immunoelectron Localization of Type X Collagen in Chick Chondrocyte Cultures

Type X collagen deposited within the matrix elaborated by chick hypertrophic chondrocytes in culture was examined by immunoelectron microscopy using the preembedding techniques described by Bruns et al. (1986). Cell culture material was rinsed with PBS, pH 7.4, and incubated with mAb MC7 (Kwan et al., 1989) at 4°C for 8 h in PBS containing 0.1% (wt/vol) BSA, and 1% (vol/vol) normal goat serum (DAKOPATTS, High Wycombe, Bucks, England). The cell layer was then rinsed thoroughly with PBS at 4°C for 8 h on a rotary mixer before being incubated for 16 h at 4°C with 10-nm colloidal gold conjugated goat anti-mouse IgG (Janssen Life Sciences Products, Wantage, Oxon, England) which had been diluted five times with PBS containing 0.1% (wt/vol) BSA. The cell layer was washed extensively for 8 to 10 h and was transferred into 0.1 M sodium cacodylate buffer (Sigma Chemical Co.), pH 7.4. The washed cell layer was fixed in half-strength Karnovsky's fixative for 30 min at 4°C, rinsed with cacodylate buffer, and postfixed with 2% (vol/vol) osmium tetroxide (Emscope, Herts, England). Samples were dehydrated with increasing concentrations of ethanol and embedded in epoxy resin (Emscope). Ultrathin sections were cut with a Reichart Om U2 ultramicrotome (C. Reichert, Vienna, Austria) and collected on uncoated 200 mesh copper grids (TAAB Lab. Equipment Ltd., Reading, Berks, England). Sections were stained with aqueous 1% (wt/vol) uranyl acetate in cacodylate buffer and lead citrate before examination with an electron microscope (model 300; Philips Electronic Instruments Inc., Mahway, NJ) at 80 kV.

Aggregation Studies of Purified Chick Type X Collagen

Purification of Chick Type X Collagen. Chick cartilage collagens were isolated from culture media of the primary hypertrophic chondrocyte cultures by adding solid ammonium sulphate to 30% saturation. Different collagen types were separated by differential salt fractionation procedures and type X collagen was precipitated at 2 M NaCl concentration in 0.5 M acetic acid, desalted by dialysis against 0.5 M acetic acid and lyophilized (Kielty et al., 1985). In some experiments purified chick type X collagen was digested with pepsin (100 μg/ml) in 0.5 M acetic acid for 16 h at 4°C to obtain molecules devoid of the amino- and carboxy-terminal noncollagenous domains.

Turbidimetric Determination of Type X Collagen Aggregation in Solution. The formation of insoluble aggregates of type X collagen was measured as the increase in the turbidity of a type X collagen solution incubated at 34°C under the conditions used in the generation of banded collagen fibrils from type I collagen (Mould et al., 1985; Kadler et al., 1988). Lyophilized chick type X collagen, purified as mentioned above, was dissolved in 0.1 M acetic acid in a concentration of 1 mg/ml. The solution was dialyzed against 5 mM acetic acid for 16 h at 4°C to reduce the acidity of the solution. The dialyzed collagen was further diluted with distilled water to the desired concentrations (800 and 400 μg/ml). The diluted type X collagen solutions were prewarmed to 34°C by incubating for 15 min before mixing in a quartz microcuvette with an equal volume of 2× concentrated PBS which had also been preheated to 34°C. Changes in turbidity at 34°C of the neutralized type X collagen solutions were assayed by monitoring the absorbance change at 313 nm in a spectrophotometer (Ultraspec 4050; LKB Instruments Inc.) fitted with a temperature-controlled cuvette holder.

Rotary-shadowing EM. Diluted type X collagen solution in 5 mM acetic acid was further diluted with distilled water to ~4 μg/ml and mixed with an equal volume of 2× concentrated PBS as described in the above turbidity measurement experiment and was incubated at 34°C for up to 1 h. Aliquots of the mixture before incubation and at specific time points during the incubation period were taken and prepared for rotary-shadowing EM using the mica sandwich techniques described by Mould et al. (1985). A 5 μl droplet of the samples was sandwiched between two sheets of freshly cleaved mica (TAAB Lab. Equipment Ltd.) and the aggregates of collagen X were allowed to be absorbed onto the mica surfaces for 5 min. The mica sandwiches were washed in 0.2 M ammonium acetate, blotted dry with filter paper and then frozen in liquid nitrogen. The mica sandwiches were split open under liquid nitrogen, dried under vacuum, and after drying, the specimens were rotary shadowed with platinium wire on a tungsten filament at an angle of 4°, and then coated with carbon. The carbon replicas were floated off onto distilled water surface and picked up on uncoated 200 mesh copper grids. Specimens were examined in an electron microscope (1200EX; JEOL UK Ltd., London, England) at 120 kV. Length measurements of the type X aggregates were carried out on micrographs using a modified Microsemper software package (Synoptics, Cambridge, England) on an Olivetti M28/Matrox PIP1024 frame store system with TV input. Magnification was calibrated using a line-grating replica of 2,160 lines/mm (TAAB Lab. Equipment Ltd., Reading, Berks, England).

Results

Extraction of Extracellular Matrix Components from Chondrocyte Cultures

Collagens synthesized and deposited by embryonic chick tibial hypertrophic chondrocytes in primary cultures were extracted by 0.5 M acetic acid containing pepsin. Analyses of these pepsin extracts and the residues by SDS-PAGE have shown that the matrix elaborated by chondrocytes contains

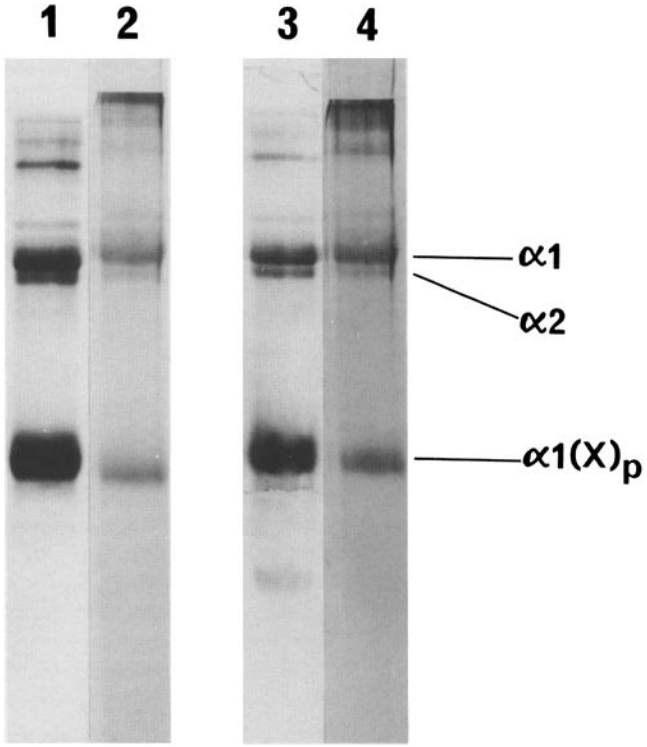

Figure 1. SDS-PAGE (8%) of collagenous polypeptides extracted from matrices elaborated by embryonic chick tibial hypertrophic chondrocytes in primary cultures. Samples were (lanes *1* and *3*) pepsin extracts from a 3-wk-old primary chondrocyte culture and (lanes *2* and *4*) collagenous polypeptides in the final residue after pepsin extraction. Polypeptides in lanes *3* and *4* were reduced by β-mercaptoethanol before electrophoresis. The label α1 was used to show the position of α1(I) and α1(II) chains of type I and II collagens, respectively. The positions of the α2(I) and α1(X)$_p$, M_r 45,000, are also shown.

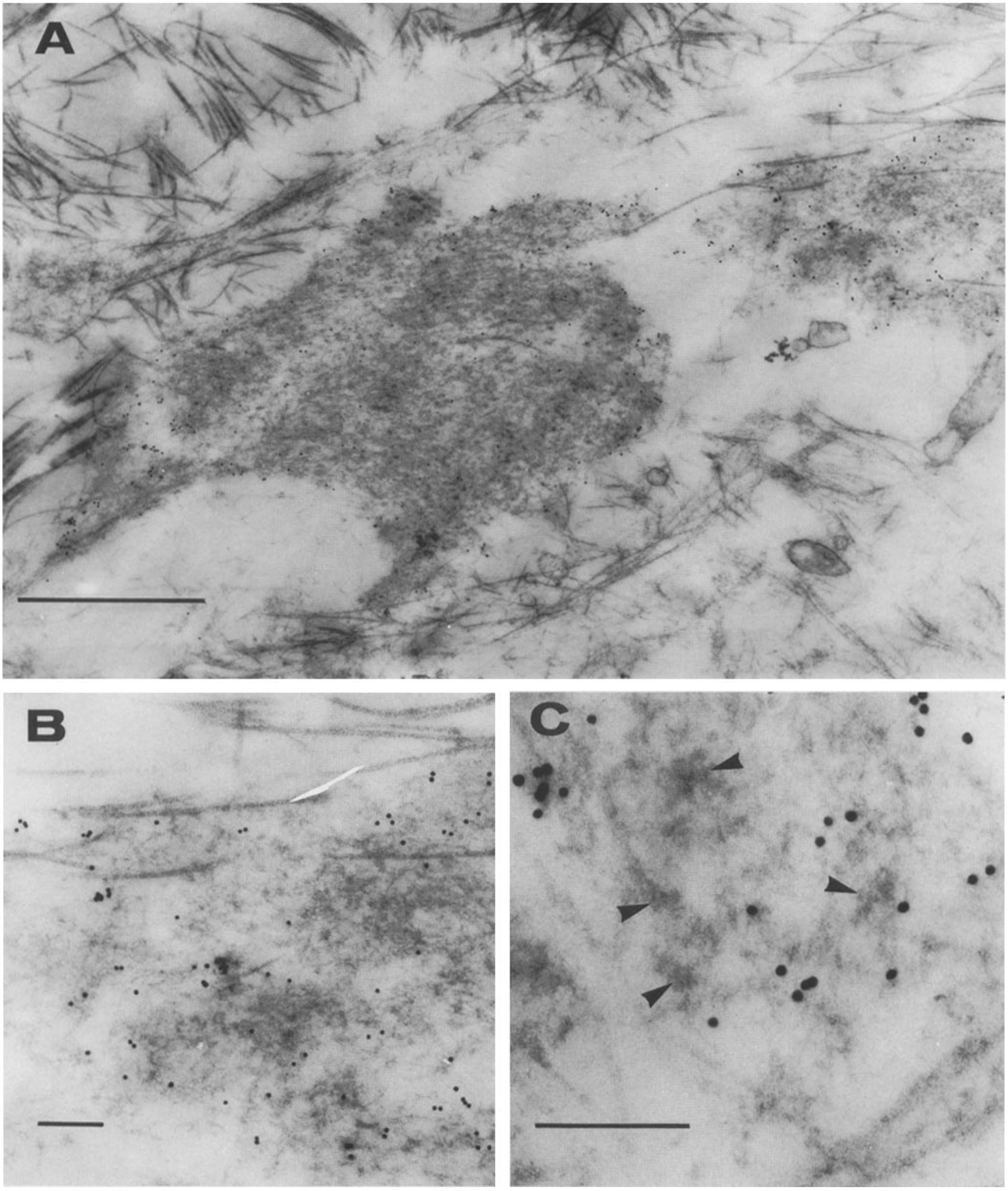

Figure 2. Immunoelectron microscopic localization of type X collagen in matrices elaborated by chick hypertrophic chondrocytes in culture. The cell matrices were incubated with mAb MC7 followed by incubation with 10-nm colloidal gold–conjugated anti–mouse IgG before processing for transmission EM. (*A*) Low magnification electron micrograph showing the association of colloidal gold particles with large matlike materials structurally distinct from the surrounding thin banded collagen fibrils. No colloidal gold particles were found to be associated with the banded collagen fibrils which are presumed to be fibrils of type II collagen. (*B* and *C*) Higher magnification electron micrographs showing some structural details of the collagen X mat which appears to be composed of irregularly spaced electron-dense nodules (*arrowheads*) interconnected by a fine filamentous network. Bars: (*A*) 1 μm; (*B* and *C*) 150 nm.

collagen types II and X (Fig. 1). Trace amounts of the α2(I) chain of type I collagen were also detected in these cell layers which may be attributed to the presence during the early phase of cell culture of dedifferentiated chondrocytes which are known to synthesize type I collagen (von der Mark and Conrad, 1979). Quantitation of the relative abundance of the different collagen types by laser densitometry has indicated that type X collagen represents ~60 to 70% of the total collagen deposited in the cell layer. These matrices therefore provide excellent in vitro models for the studies of the macromolecular organization of collagen type X in the extracellular space.

Immunoelectron Microscopic Localization of Type X Collagen

The ultrastructural organization of chick collagen X in the matrix was initially investigated by immunolocalization studies on matrices elaborated by cultured chick hypertrophic chondrocytes using mAb MC7 which recognizes an epitope within the triple helical domain (Kwan et al., 1989). The antibody-bound structures were identified by using 10-nm colloidal gold conjugated anti-mouse IgG (Fig. 2). The gold particles were localized within large feltlike structures which had no obvious banded fibrillar arrangements and was distinct from the surrounding thin cross-striated fibrils, presumed to be fibrils of type II collagen. These thin collagen fibrils have an average diameter of 10–20 nm (Fig. 2 *A*) which is the typical fibril size of type II collagen found within the cartilage matrix. At higher magnifications, the network formed by type X collagen was seen to consist of thin filaments joining to form an irregular lattice work (Fig. 2, *B* and *C*). No obvious association between the type X collagen network and the thin collagen fibrils was observed in any of the numerous sections studied.

Molecular Aggregation and Rotary-shadowing EM

To investigate, at the molecular level, the processes that govern the assembly of type X collagen monomers into macromolecular structures observed by immunoelectron microscopy, a series of aggregation studies was conducted in vitro and monitored using rotary-shadowing electronmicroscopic techniques. Under conditions when type I collagen monomers could be induced to form fibrils, solubilized purified chick type X collagens readily form insoluble aggregates as shown by the increase in the turbidity of the solution during the incubation period (Fig. 3). The change of turbidity can be divided into two phases: phase I corresponding to a rapid increase in absorbance which occurred within the first 2 min of incubation; phase II corresponding to a steady increase in turbidity at a much slower rate. Direct visualization of the aggregates formed in phase I by EM have shown that the rapid increase in turbidity is because of the formation of large insoluble aggregates formed by the association of type X monomers via their globular carboxy-terminal domains. Fig. 4 is a montage of electronmicrographs showing an array of aggregates of type X collagen ranging from dimers to multimeric clusters formed via aggregation of the carboxy-terminal domains. Each aggregate consists of a central nodule of carboxy-termini with the triple helical rods radiating outwards (Fig. 4, *A–C*). Further incubation resulted in the coalescing of a number of these clusters into a large regular latticework (Fig. 4 *G*). The increase in the size of this lattice

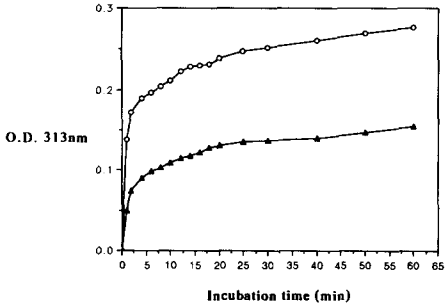

Figure 3. Turbidimetric behavior of purified chick type X collagen molecules in solution at 34°C. Type X collagen solutions in 5 mM acetic acid were neutralized with 2× concentrated phosphate-buffered saline to give final concentrations of 200 (▲) and 400 μg/ml (○) and incubated at 34°C. Changes in turbidity of the solutions were assayed by monitoring absorbance at 313 nm.

during the incubation period corresponds to the slow and steady increase of turbidity of the incubated type X collagen suspension. As shown in Fig. 5 the lattice consists of nodules, formed by the clustering of the carboxy-terminal globular domains, interconnected by a filamentous network formed by the triple-helical moieties of the collagen X molecules. The nodules within this lattice, in its fully extended state, were arranged in a hexagonal array. The average distance between two nodules within the hexagonal lattice was ~103 ± 12.8 nm (n = 148), a distance shorter than the measured length of 130 nm of the triple helical domain of a type X collagen molecule (Kielty et al., 1985). Fig. 6 shows a histogram representing the distribution of the internodular lengths which has the highest frequency between 100 and 105 nm. The short nodule to nodule distance may be in part because of the formation of super-helical structures amongst the adjacent helical domains. Lateral interactions between helical domains within individual multimeric clusters were frequently observed as the thickening of the triple helical moieties of the clusters (Fig. 4). This type of interaction appears to be independent of the noncollagenous domains as shown in Fig. 7 which showed that pepsin-treated type X collagen molecules, consisting only of the triple helical domain, could associate laterally to form molecules with a thicker molecular width/diameter. Partially associated molecules were also apparent in these preparations as shown in the figure as Y-shaped molecules. The electron microscopic data presented above imply that both parallel and anti-parallel lateral overlapping of the triple helices are required for the formation of the lattice work. The nature of these interactions is currently under intense investigation in this laboratory.

Discussion

To date, there is mounting evidence that type X collagen is an important matrix component which is expressed at sites of endochondral ossification during normal skeletal development (Schmid and Linsenmayer, 1985; Kwan et al., 1986) and also in pathological circumstances where new bone formation occurs, e.g., fracture repair or osteoarthritis (Grant et al., 1987; Kirsch et al., 1990; Thomas et al., 1991; Hoyland et al., unpublished work). However, the exact functional role of type X collagen in cartilage has remained undefined, in part because there is no clear information regarding the

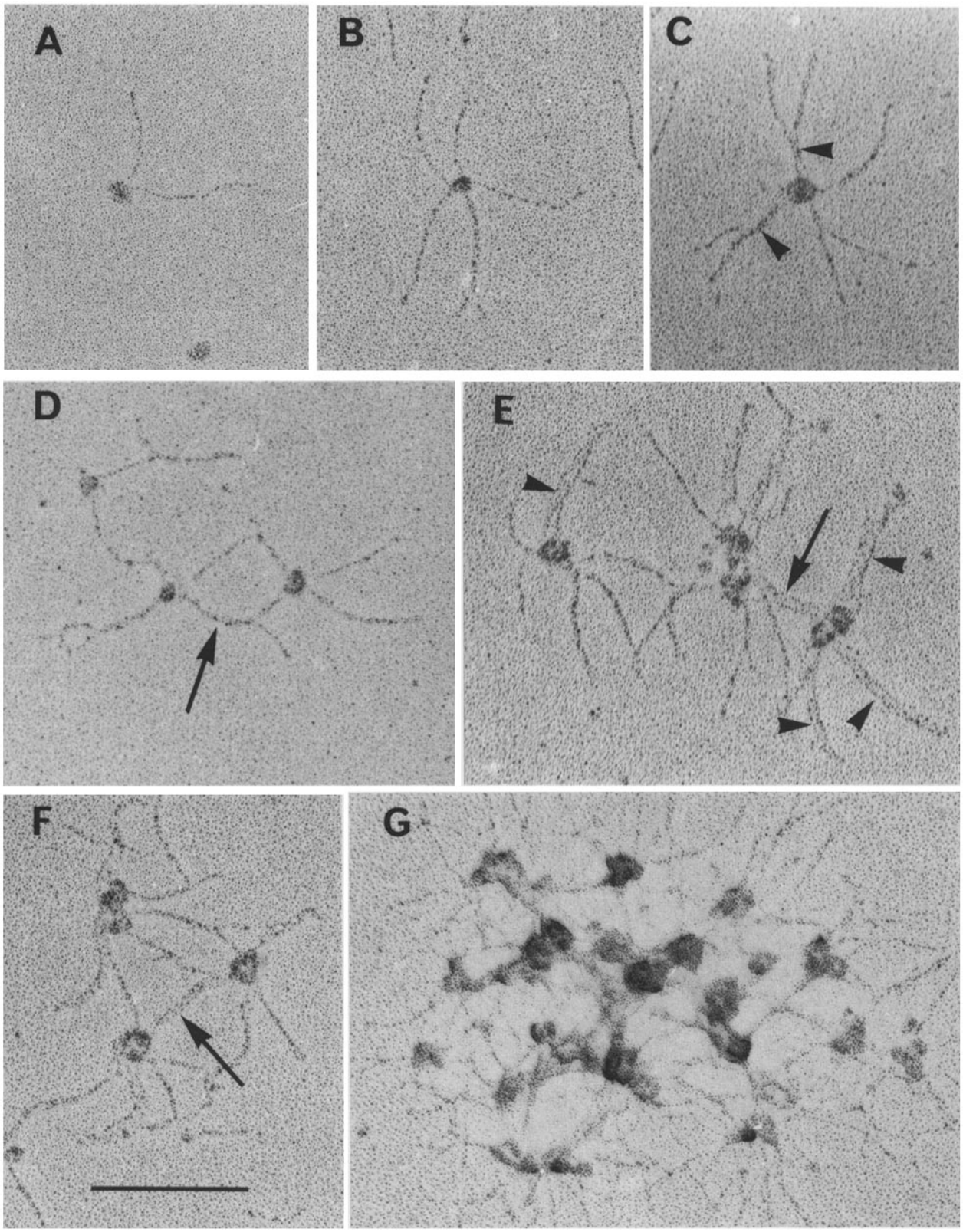

Figure 4. Electron micrographs of molecular aggregates of type X collagen formed in vitro by incubating purified chick type X collagen in PBS at 34°C. The aggregates were applied onto mica sheets and rotary shadowed with platinum before being examined in an electron microscope (1200EX; JEOL UK Ltd.). Dimers (*A*) and multimers (*B* and *C*) were formed by the association of the carboxy-terminal domains during the early phase of the incubation period. Association between adjacent multimers was mediated by interactions between juxtaposed triple helical domains (*arrows*) (*D*, *E*, and *F*). In register lateral overlapping of the triple helices within an individual multimeric cluster was also observed as much thicker helical arms (*arrowheads*; *C* and *E*). Large aggregates containing a number of multimers were found from samples taken 5 min after the initiation of aggregation (*G*). Bar, 150 nm.

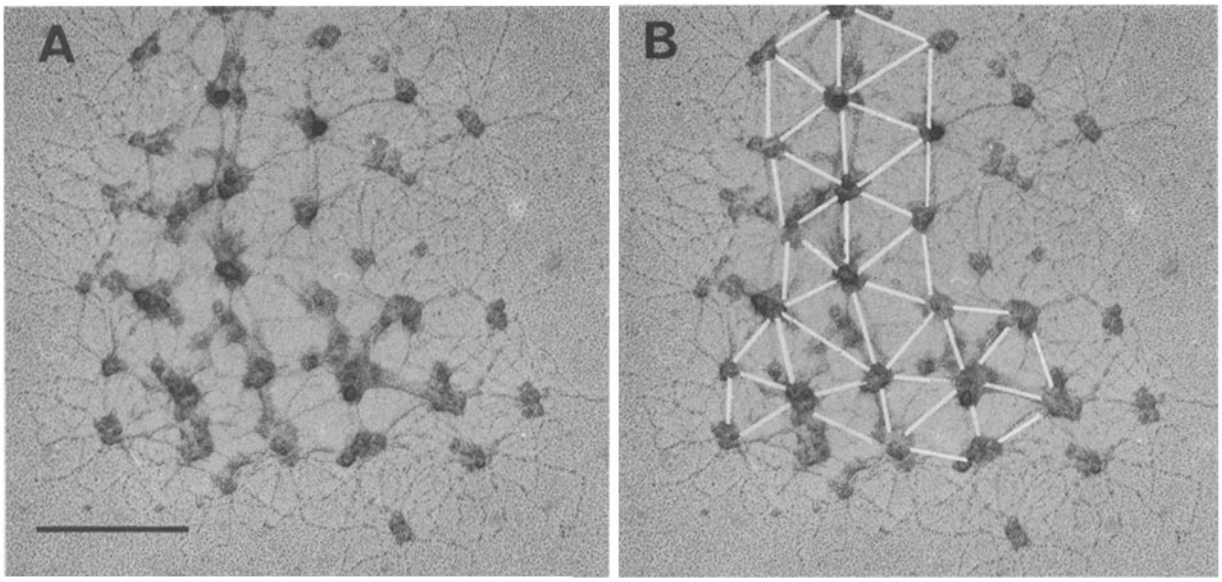

Figure 5. Rotary shadowing electron micrographs showing part of the extended network of type X collagen aggregates. This network contains regularly spaced nodules of aggregated carboxy-terminal noncollagenous domains interconnected with filamentous structures formed via interactions between adjacent triple helices (*A*). The nodules in this lattice were arranged into a regular hexagonal array. The hexagonal nature of the type X collagen lattice is highlighted in micrograph (*B*). Bar, 200 nm.

ultrastructural organization of this molecule and its interactions with other components in the extracellular matrix. In the present investigation, the supramolecular organization of type X collagen synthesized by chick hypertrophic chondrocytes in primary cultures was first studied by immunoelectron microscopy. The matrices elaborated by these chondrocytes over a 2–3-wk period in culture have been shown to be particularly rich in collagen X (Fig. 1). These cell systems offer certain advantages in immunohistochemical investigations of collagen X organization in that the cell layers (~0.1-mm thick) allow rapid penetration of antibodies without the need to resort to hyaluronidase/chondroitinase treatment as is required for such studies in intact cartilage. Using a mAb that recognizes the triple helical domain of chick type X collagen (Kwan et al., 1989), it was found that collagen X molecules in the cell layers do not form banded fibrils but associate to form a feltlike network (Fig. 2). These observations indicated that the structures formed by collagen X in vitro are comparable to those detected in vivo (Schmid and Linsenmayer, 1990). However, conventional EM of either cell culture product or intact tissues lacks the resolution to provide detailed structural information and the possible introduction of artefacts of fixation cannot be excluded. Accordingly we have considered it appropriate to adopt an alternative strategy to investigate the ultrastructural organization of type X collagen.

The nonfibrillar nature of type X collagen is clearly demonstrated by immunoelectron microscopy and the finding is consistent with gene cloning studies which suggested type X belongs to a family of collagens distinct from the fibrillar collagen family (for reviews see Ninomiya et al., 1990; and Kielty et al., 1991). Some clues concerning the extracellular

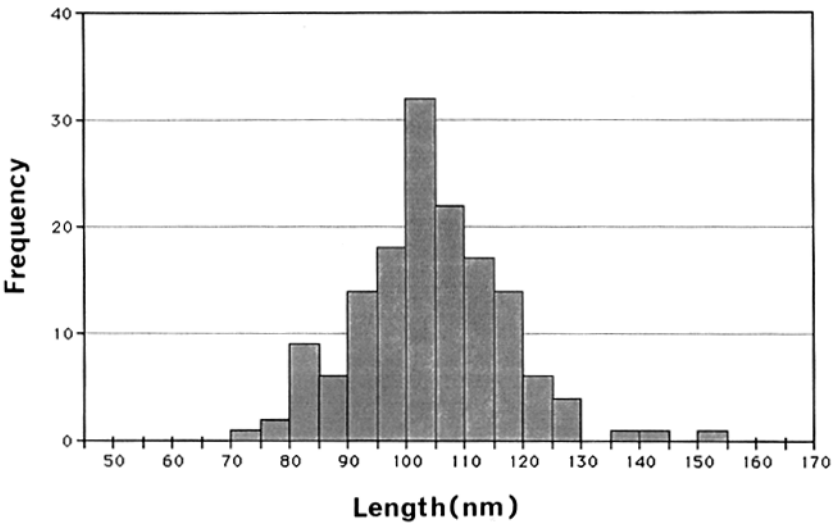

Figure 6. Distribution of the internodular lengths within the hexagonal type X collagen lattice formed by aggregation of type X collagen monomers. Length measurements were performed directly on micrographs taken at 25,000× magnification. A mean distance of 103 ± 12.8 nm was obtained from measuring the lengths of 148 internodular filaments.

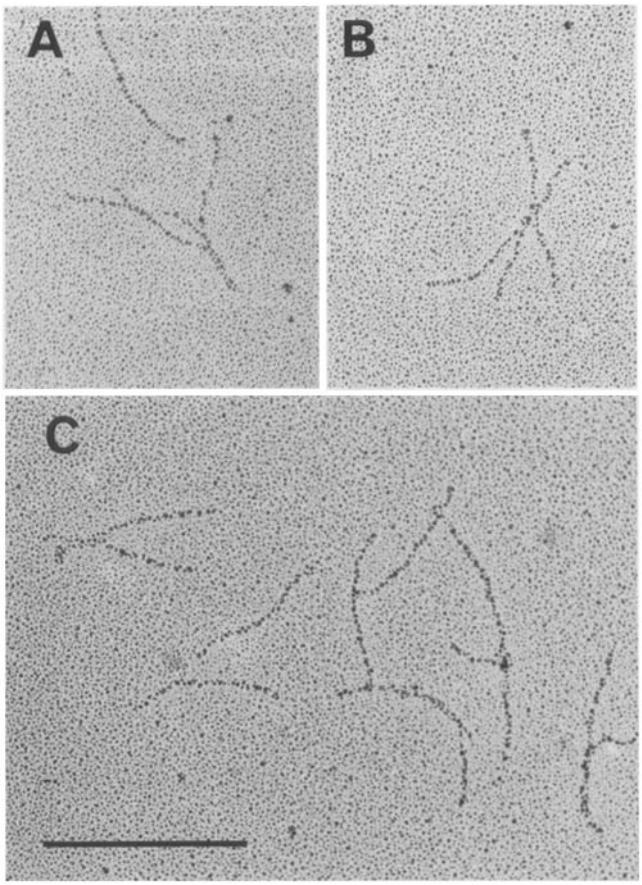

Figure 7. Rotary-shadowing electron micrographs of molecular aggregates of pepsin-treated chick type X collagen. (*A*, *B*, and *C*). Aggregates were formed by lateral association of the triple helical domains independent of the presence of the carboxy-terminal domains. Bar, 150 nm.

organization of type X collagen may be discerned from its biochemical properties, e.g., high thermal stability (Schmid and Linsenmayer, 1984) and hydrophobicity (Kwan et al., 1986*b*) but more particularly cDNA cloning has revealed its distinctive amino acid composition. Especially noteworthy is the sequence of the carboxy-terminal domain which contains high levels of aromatic amino acid residues, namely tyrosine, phenylalanine, and tryptophan residues and among which 13 out of the 15 tyrosine residues in the entire α1(X) chain are found within the carboxy-terminal domain (Ninomiya et al., 1986). Work in this laboratory and others has demonstrated remarkable homologies in the carboxy-terminal domains of chicken (Ninomiya et al., 1986), bovine (Thomas et al., 1991), human (J. T. Thomas, personal communication), and mouse (Kong, R., personal communication) α1(X) chains. In particular, the positions of the 13 tyrosyl residues and that of the putative *N*-linked oligosaccharide attachment site are found to be highly conserved (Thomas et al., 1991). Such observations suggest an important role of the conserved amino acid residues in the structure and function of type X collagen. However, the lack of homology with the carboxy-terminal propeptides of the fibrillar collagens may indicate a role for the carboxy-terminal domain of type X collagen additional to its involvement in alignment of the three α chains during helix formation. In the case of collagen type IV, the assembly of the basement membrane collagenous network involves important interactions via both the NH_2- and COOH-terminal domains (Yurchenco et al., 1984). Such interactions and the self-assembly process of type IV collagen have been studied by rotary shadowing EM (Yurchenco et al., 1984) and a similar approach has been adopted here to study the self-assembly of type X collagen.

These studies have demonstrated for the first time, the formation in vitro of large aggregates based on a regular hexagonal lattice (Fig. 5). The process of aggregation resembles aspects of the self assembly of type IV collagen from basement membranes (Yurchenco et al., 1984). Firstly, the purified type X collagen molecules undergo a rapid thermal gelation at neutral pH without an appreciable lag phase (Fig. 3); secondly, the aggregation appears to be initiated by associations of the large carboxy-terminal noncollagenous domains followed by the interactions of the juxtaposed triple helical domains to form an extensive latticework (Fig. 4). In control experiments using pepsin-treated type X collagen, the molecules lacking their carboxy-terminal domains tended to form thin filaments by lateral aggregation but no evidence was obtained for a network structure and hexagonal lattices were never observed. It was therefore concluded that the carboxy-terminal domain of type X collagen is of paramount importance in the initiation and maintenance of the ordered assembly of type X collagen. One might speculate on the potential role of the hydrophobic carboxy-terminal domain in promoting and stabilizing the hexagonal network structure seen in Fig. 5. The possibilities of the aggregates being further stabilized by disulphide links has arisen with the demonstration of a single cysteine residue in the carboxy-terminal domain of the α1(X) chain (Ninomiya et al., 1990). Indeed, disulphide-linked aggregates of M_r 120,000 have been isolated in long-term chondrocyte cultures (Kwan et al., 1986; Schmid et al., 1989). The marked insolubility of type X collagen in tissue suggests that nonreducible covalent cross-links also have a role to play in stabilization of this special matrix structure (Reginato et al., 1986).

In this study, a unique ordered structure of type X collagen has been described for the first time. However, it is not yet clear whether the same hexagonal arrangement of type X collagen is present in cartilage. The recent observation of irregular filamentous networks of type X collagen in ultrathin sections of embryonic chick cartilage provides the only data in support of a type X collagen network in vivo (Schmid and Linsenmayer, 1990). Further electron microscopic studies using tissue processing techniques such as slam freezing and high pressure freezing, which are known to preserve the ultrastructural integrity of cartilage, will be needed to ascertain whether the hexagonal lattice structure revealed by rotary-shadowing EM occurs in vivo. It is of great interest however, that EM has revealed an analogous hexagonal lattice of type VIII collagen in bovine corneal Descemet's membrane (Sawada, 1982). This structure is synthesized by corneal endothelial cells and cDNA cloning of the rabbit α1 (VIII) chain has shown a high degree of similarity in the primary structures of type VIII and type X collagens. Comparison of the sequences in the carboxy-terminal noncollagenous domain of rabbit type VIII collagen (Yamaguchi et al., 1989) with those of chicken and bovine α1(X) chains (Thomas et al., 1991) showed 58 and 55% homologies at the amino acid levels, respectively, indicating type VIII and X collagens may belong

to a family of related extracellular proteins forming similar supramolecular structures in the extracellular matrix. However, the functions of these collagenous networks must differ in that the two collagen types appear to have markedly different locations in tissues (for review see Ninomiya et al., 1990).

The formation of a regular network in the matrix of hypertrophic cartilage may represent part of the modification processes of the extracellular environment before the later events in endochondral ossification, namely mineral deposition, vascular invasion from the metaphyseal vessels, and the degradation of calcified cartilage. An apparent correlation between type X collagen deposition and cartilage matrix mineralization in vivo has been demonstrated in a comparative study of type X collagen occurrence in normal and rachitic chicken growth plates (Kwan et al., 1989). Matrix vesicles are believed to have a role in the initiation of matrix mineralization and potential interactions between these membraneous vesicles and type X collagen have been proposed by Wu et al. (1989). In addition, ultrastructural studies using freeze-substitution techniques have shown that matrix vesicles in rat hypertrophic cartilage are associated with a filamentous network (Arsenault et al., 1988; Takagi et al., 1989). The molecular constituent of this filamentous network is yet unknown but the possibility that it is related to type X collagen cannot be excluded. In this context, the formation of a hexagonal lattice in the extracellular matrix may be an important prerequisite of matrix mineralization in that the type X collagen network could provide a framework for matrix and matrix vesicle interactions or the network may direct the propagation of the mineral. The remarkable similarities in structural organizations between type VIII, a product of endothelial cells, and type X collagens also provoke speculation that the collagen X lattice may influence the migration of invading endothelial cells during angiogenesis within the cartilage matrix. The determination of the links between the type X collagen network and the processes of endochondral ossification will require further studies in vivo and matrix reconstruction experiments in vitro.

The financial support of the Medical Research Council is gratefully acknowledged.

Received for publication 21 January 1991 and in revised form 19 April 1991.

References

Arsenault, A. L., F. P. Ottensmeyer, and B. Heath. 1988. An electron microscopic and spectroscopic study of murine epiphyseal cartilage: analysis of fine structure and matrix vesicles preserved by slam freezing and freeze substitution. *J. Ultrastruct. Mol. Res.* 98:32–47.

Ayad, S., A. P. L. Kwan, and M. E. Grant. 1987. Partial characterization of type X collagen from bovine growth plate. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 220:181–186.

Bruns, R. R., W. Press, E. Engvall, R. Timpl, and J. Gross. 1986. Type VI collagen in extracellular, 100-nm periodic filaments and fibrils: identification by immunoelectron microscopy. *J. Cell Biol.* 103:393–404.

Caplan, A. I. 1988. Bone development. *Ciba Found. Symp.* 136:3–21.

Eyre, D. R. 1987. A growing family of collagens in articular cartilage: identification of 5 genetically distinct types. *J. Rheumatol.* 14:25–27.

Gibson, G. J., and M. H. Flint. 1985. Type X collagen synthesis by chick sternal cartilage and its relationship to endochondral development. *J. Cell Biol.* 101:277–284.

Grant, W. T., G. J. Wang, and G. Balian. 1987. Type X collagen synthesis during endochondral ossification in fracture repair. *J. Biol. Chem.* 262:9844–9849.

Grant, M. E., S. Ayad, A. P. L. Kwan, G. P. Bates, J. T. Thomas, and J. McClure. 1988. The structure and synthesis of cartilage collagens. *In* The Control of Tissue Damage. A. M. Glauert, editor. Elsevier Science Publishing Co., Inc., Amsterdam, Netherlands. 3–28.

Kadler, K. E., Y. Hojima, and D. J. Prockop. 1988. Assembly of type I collagen fibrils *de novo*. *J. Biol. Chem.* 263:10517–10523.

Kielty, C. M., A. P. L. Kwan, D. F. Holmes, S. L. Schor, and M. E. Grant. 1985. Type X collagen, a product of hypertrophic chondrocytes. *Biochem. J.* 27:545–554.

Kielty, C. M., I. Hopkinson, and M. E. Grant. 1991. Collagen. *In* Extracellular Matrix and Inheritable Disorders of Connective Tissue. P. M. Royce and B. Steinmann, editors. Alan R. Liss, Inc., New York. In press.

Kirsch, T., and K. von der Mark. 1990. Isolation of bovine type X collagen and immunolocalization in growth-plate cartilage. *Biochem. J.* 265:453–459.

Kwan, A. P. L., A. J. Freemont, and M. E. Grant. 1986*a*. Immunoperoxidase localization of type X collagen in chick tibiae. *Biosci. Rep.* 6:155–162.

Kwan, A. P. L., C. H. J. Sear, and M. E. Grant. 1986*b*. Identification of disulphide-bonded type X procollagen polypeptides in embryonic chick chondrocyte cultures. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 206:267–272.

Kwan, A. P. L., I. R. Dickson, A. J. Freemont, and M. E. Grant. 1989. Comparative studies of type X collagen expression in normal and rachitic chicken epiphyseal cartilage. *J. Cell Biol.* 109:1849–1856.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Mayne, R. 1989. Cartilage collagens: what is their function, and are they involved in articular disease? *Arthritis and Rheumatism.* 32:241–246.

Mould, A. P., D. F. Holmes, K. E. Kadler, and J. A. Chapman. 1985. Mica sandwich technique for preparing macromolecules for rotary shadowing. *J. Ultrastruct. Res.* 91:66–76.

Ninomiya, Y., M. Gordon, M. van der Rest, T. Schmid, T. F. Linsenmayer, and B. R. Olsen. 1986. The developmentally regulated type X collagen gene contains a long open reading frame without introns. *J. Biol. Chem.* 261:5041–5050.

Ninomiya, Y., P. Castagnola, D. Gercke, M. K. Gordon, O. Jacenko, P. Lu-Valle, M. McCarthy, Y. Muragaki, I. Nishimura, S. Oh, N. Rosenblum, N. Sato, S. Sugrue, R. Taylor, G. Vasios, N. Yamaguchi, and B. R. Olsen. 1990. The molecular biology of collagens with short triple-helical domains. *In* Extracellular Matrix Genes. L. Sandell and C. D. Boyd, editors. Academic Press, New York. 79–125.

Reginato, A. M., J. W. Lash, and S. A. Jimenez. 1986. Biosynthetic expression of type X collagen in embryonic chick sternum cartilage during development. *J. Biol. Chem.* 261:2897–2904.

Sawada, H. 1982. The fine structure of the bovine Descemet's membrane with special reference to biochemical nature. *Cell Tissue Res.* 226:241–255.

Sawada, H., H. Konomi, and K. Hirosawa. 1990. Characterization of the collagen in the hexagonal lattice of Descemet's membrane: its relation to type VIII collagen. *J. Cell Biol.* 110:219–227.

Schmid, T. M., and T. F. Linsenmayer. 1983. A short chain (pro)collagen from aged endochondral chondrocytes. *J. Biol. Chem.* 258:9504–9509.

Schmid, T. M., and T. F. Linsenmayer. 1984. Denaturation-renaturation properties of two molecular forms of short-chain cartilage collagen. *Biochemistry.* 23:533–558.

Schmid, T. M., and T. F. Linsenmayer. 1985. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. *J. Cell Biol.* 100:598–605.

Schmid, T. M., and T. F. Linsenmayer. 1989. Chains of matrix-derived type x collagen: size and aggregation properties. *Connective Tissue Research.* 20:215–222.

Schmid, T. M., and T. F. Linsenmayer. 1990. Immunoelectron microscopy of type X collagen: supramolecular forms within embryonic chick cartilage. *Develop. Biol.* 138:53–62.

Stocum, D. L., R. M. Davis, M. Leger, and H. E. Conrad. 1979. Development of the tibiotarsus in the chick embryo: biochemical activity of histologically distinct zones. *J. Embryol. Expl. Morphol.* 54:155–170.

Takagi, M., T. Sasaki, A. Kagami, and K. Komiyama. 1989. Ultrastructural demonstration of increased sulfated proteoglycans and calcium associated with chondrocyte cytoplasmic processes and matrix vesicles in rat growth plate cartilage. *J. Histochem. Cytochem.* 37:1025–1033.

Thomas, J. T., R. P. Boot-Handford, and M. E. Grant. 1990. Modulation of type X collagen gene expression by calcium β-glycerophosphate and levamisole: implications for a possible role for type X collagen in endochondral bone formation. *J. Cell Sci.* 95:639–648.

Thomas, J. T., A. P. L. Kwan, M. E. Grant, and R. P. Boot-Handford. 1991. Isolation of cDNAs encoding the complete sequence of bovine type X collagen: evidence for the condensed nature of mammalian type X collagen genes. *Biochem. J.* 273:141–148.

Von der Mark, K., and G. Conrad. 1979. Cartilage cell differentiation. *Clin. Orthopaedics Rel. Res.* 139:185–205.

Yamaguchi, N., P. D. Benya, M. van der Rest, and Y. Ninomiya. 1989. The cloning and sequencing of a1(VIII) collagen cDNAs demonstrate that type VIII collagen is a short chain collagen and contains triple-helical and carboxyl-terminal non-triple-helical domains similar to those of type X collagen. *J. Biol. Chem.* 264:16022–16029.

Yurchenco, P. D., and H. Furthmayr. 1984. Self-assembly of basement membrane collagen. *Biochemistry.* 23:1839–1850.

Wu, L. N. Y., G. R. Sauer, B. R. Genge, and R. E. Wuthier. 1989. Induction of mineral deposition by primary cultures of chicken growth plate chondrocytes in ascorbate-containing media: evidence of an association between matrix vesicles and collagen. *J. Biol. Chem.* 264:21346–21355.