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Long-range movement and fibril association of type X collagen within embryonic cartilage matrix

(extracellular matrix/immunohistochemistry/matrix assembly)

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ABSTRACT **A recent immunoelectron microscopic study of type X collagen in developing cartilage gave results that could be explained by movement of the molecule from one region of the cartilage matrix to another, there becoming associated with preexisting collagen fibrils. In the present study, to test the feasibility of this model we incubated pieces of nonhypertrophic, embryonic chicken sternal cartilage (which has no endogenous type X collagen) in medium with type X collagen and then used immunofluorescence and immunoelectron microscopy to evaluate movement of the molecule through the matrix. The results show that type X collagen molecules can indeed pass through embryonic sternal cartilage matrix and subsequently become fibril-associated.**

In dense connective tissues, such as cartilage, diffusion of nutrients and small macromolecules is thought to be important for normal physiological processes. Movement of large macromolecules, however, is generally thought not to occur to any appreciable extent. Studies on native cartilage, usually articular, have generally agreed that the size of the diffusing molecule and its net charge are important parameters in determining the ability of a molecule to diffuse through the matrix. The upper size limit is generally estimated to be about that of hemoglobin, ≈60 kDa (1, 2).

The development and growth of extracellular matrix-rich tissues require the assembly and remodeling of supramolecular structures, such as collagen fibrils. For many of these structures, the subunits utilized have extremely large Stokes' radii. If severe constraints on macromolecular movement apply, then matrix growth and remodeling would necessarily be confined largely to pericellular regions. Conversely, if a structural macromolecule could move extensively through extracellular matrix, it would be possible for a cell to exert long-range influences on assembly and remodeling.

In a recent immunoelectron microscopic study of developing cartilage, we (3) obtained results that could be explained by the movement through cartilage matrix of type X collagen, a hypertrophic cartilage-specific molecule. In this model, some of the newly secreted type X collagen moves away from the synthesizing chondrocyte and out into the matrix. There, it becomes associated with preexisting type II collagen fibrils, possibly altering their properties such as ability to undergo calcification and/or removal (4–6). However, the available data on the size limitation for diffusion through cartilage questioned the likelihood of this. By rotary shadowing analyses (7), type X collagen is only half the length (138 nm) of a "typical" fibrillar collagen. It is nevertheless a large molecule (177 kDa) that, due to its rod-like shape, behaves on gel filtration chromatography like a globular molecule of much higher molecular mass (8).

To test the feasibility of our model, in the present study we incubated pieces of nonhypertrophic embryonic sternal cartilage, which has no endogenous type X collagen, in medium containing type X collagen. Then, by immunofluorescence microscopy and by radiolabeling, we evaluated movement of the molecule into the matrix. Immunoelectron microscopy was also performed. The results show an extensive movement of type X collagen molecules through sternal cartilage matrix and their subsequent association with fibrils containing type II collagen.

METHODS

Sterna were removed from embryonic chickens (usually 15 days of incubation) and dissected into Hanks' saline. The perichondrium was stripped off, and pieces from the caudal one-half to one-third of the sternum (the "permanent" cartilaginous region, which does not synthesize type X collagen) were used.

For the experimental incubations, the medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and exogenous collagen. The concentration of type X collagen routinely employed was 150 μg/ml. Fifteen micrograms per milliliter gave essentially the same result, but with a decrease in the intensity of the fluorescence signal. Sternal pieces (usually three or four) were added to the medium (either 10 ml in a 15-ml snap-top polystyrene tube or 1 ml in a 1.5-ml microcentrifuge tube). The tubes were then incubated for various times at 37°C on a Nutator rocking table (VWR Scientific) to ensure gentle mixing.

The exogenous type X collagen employed was isolated as described (8) from the medium of aged cell cultures of hypertrophic tibiotarsal chicken chondrocytes. The preparations, as analyzed by SDS/PAGE, consisted mostly of the intact (59-kDa) form of type X collagen, with the remainder being other cartilage collagens. This degree of purification was deemed sufficient for these studies. Inherent in the experimental design, the immunofluorescence assay (see below) using anti-type X collagen monoclonal antibody X-AC9 (9) specifically detected the added type X collagen.

Type I collagen was purified from lathyritic chicken skin, and type I procollagen was isolated from monolayer cultures of 18-day chicken embryo fibroblasts (10). Immunofluorescence detection of these collagens was with the anti-type I collagen monoclonal antibodies I-BA1 and I-DD4, which are directed against helical epitopes at opposite ends of the type I molecule (ref. 11 and unpublished results).

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For immunofluorescence histochemical analyses, the incubation medium was removed, and the sternal pieces were quickly rinsed in phosphate-buffered saline and fixed in 4% paraformaldehyde for 20 min. Further processing for cryostat sectioning and for the indirect immunofluorescence procedure with anti-collagen monoclonal antibodies was as described (12). To ensure that we were evaluating penetration of the type X collagen deep into the matrix from the surface, the sternal pieces were sectioned perpendicular to their long axis, and sections near the cut ends were not used.

Immunoelectron microscopy employing ultrathin cryostat sections was performed as recently described (13). In brief, the incubated pieces of cartilage were fixed in paraformaldehyde/lysine/periodate fixative, infiltrated with sucrose at 4°C, mounted on stubs, and frozen. Sections (100 nm) were cut with a Reichert FC4 ultracryotome, placed on Formvar-coated grids, and reacted with antibodies complexed to colloidal gold particles or with uncomplexed primary antibodies, which were then identified with a second antibody–gold complex. Sections were viewed in a Phillips CM10 electron microscope.

Radiolabeled type X collagen was purified from hypertrophic chondrocytes incubated in the presence of [^{3}H]-proline. For experimental incubations, 2000 dpm of radiolabeled type X collagen was added along with 150 μg of unlabeled collagen per ml. Then, at various time points, labeled sternal pieces (carefully size-matched) were removed from the incubation medium. Some were subsequently incubated for a chase period in type X-free medium. The labeled tissues were quickly rinsed to remove surface-associated label. Then the sternal pieces were solubilized in Beckman tissue solubilizer 450 for scintillation counting in Ready organic liquid scintillation cocktail (Beckman).

In situ hybridizations for mRNA encoding type X collagen were performed according to Hayashi *et al.* (14), with a *Pst* I–*Pvu* II fragment of pYN3116 (15) as a cDNA probe.

RESULTS

The experimental system consisted of pieces of nonhypertrophic sternal cartilage, usually from 15-day embryos, gently shaken in medium to which exogenous type X collagen had been added. The presence of type X collagen in the tissue was assayed by immunofluorescence histochemistry with a monoclonal antibody specific for the molecule.

After a 2.5-hr incubation in type X collagen-containing medium (Fig. 1*A*), the molecule had penetrated most parts of the 15-day embryonic sternum. Sterna incubated in control medium without added type X collagen (Fig. 1*B*) showed no fluorescence for type X collagen. For comparison, type I procollagen did not penetrate the cartilage matrix by 2.5 hr, or even after an overnight incubation. The only detectable reactivity for type I collagen and procollagen was along the surface of the cartilage, reminiscent of a perichondrium (compare that for type I procollagen in Fig. 1*C* to the control in Fig. 1*D*). Similar results of type X collagen penetration were obtained with different ages of sterna (13–19 days of development) and different concentrations of type X collagen ranging from 150 μg/ml down to 15 μg/ml. At incubation concentrations lower than 15 μg/ml the immunofluorescence

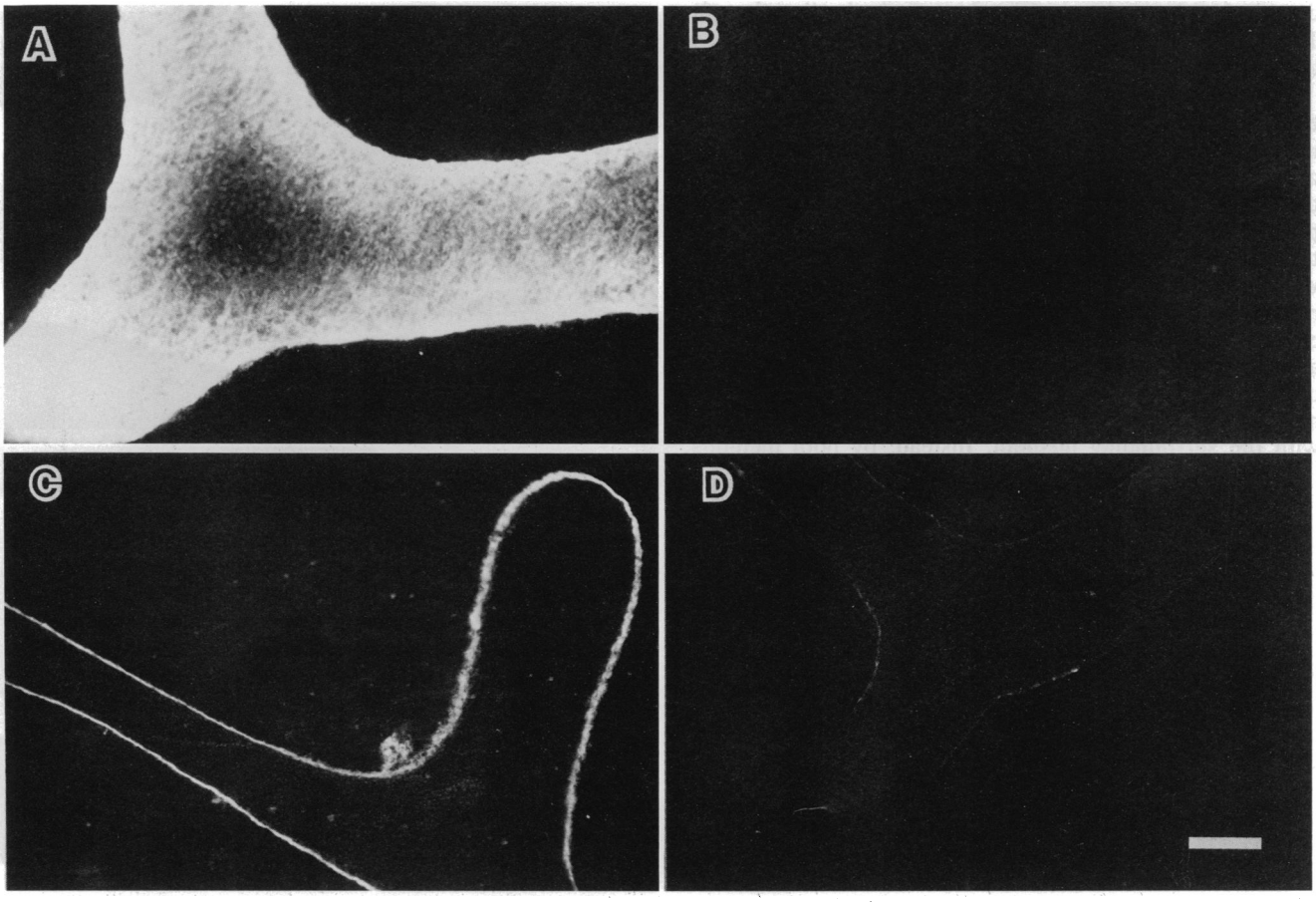

FIG. 1. Fluorescence micrographs of sections of sterna incubated for 2.5 hr in medium containing type X collagen (*A*) or type I procollagen (*C*) or in control medium without exogenous collagens (*B* and *D*). Sections were reacted with the monoclonal antibody for type X collagen (*A* and *B*) or with the monoclonal for type I collagen (*C* and *D*). In *D* the thin line of fluorescence most likely represents the small amount of type I collagen normally present in the subperiosteal region. (Bar = 77 μm.)

signal produced was barely detectable, precluding evaluation of the results.

The degree to which the type X collagen penetrated the cartilage matrix was examined in a time course from 15 min to 2.5 hr (Fig. 2). By 15 min (the earliest time evaluated, Fig. 2*A*), penetration had already occurred to a depth of several cell diameters. This then progressed through 30 min (Fig. 2*B*) and 1 hr (Fig. 2*C*), until, by 2.5 hr (Fig. 2*D*), most of the sternum contained type X.

This net inward progression of type X collagen-specific immunoreactivity, and the short duration over which it occurred, suggested that the observed signal was derived from the exogenous type X in the medium. The possibility existed, however, that the incubation conditions had induced a rapid *de novo* synthesis of type X collagen by the sternal chondrocytes themselves. Analyses by *in situ* hybridization eliminated this. Even after an overnight incubation in type X-containing medium, the cells contained no detectable mRNA for type X collagen (data not shown). In fact, there appeared to be no direct involvement of the cells themselves. Movement occurred under conditions in which metabolism is severely reduced (at 4°C) or completely abolished (in freeze–thawed tissue) (data not shown).

The type X collagen molecule contains a COOH-terminal globular domain that bears some resemblance to similar domains in the procollagen forms of fibrillar collagens. It has been suggested that such domains can determine certain physical properties of a collagen molecule, such as solubility and fibril-forming capacity (see, for example, ref. 16). To ascertain whether the globular domain of type X collagen is required for movement, we tested molecules in which this domain had been removed by limited pepsin digestion (8). The movement of such molecules was indistinguishable from that of the intact collagen (data not shown).

We asked whether free diffusion (i.e., that occurring equally well in all directions) was the only process involved in the net inward movement. To test this we incubated pieces of cartilage in type X collagen-containing medium for 1 hr and then transferred them to medium without type X collagen for periods of "chase." Analyses were performed both by isotopic studies (Fig. 3), in which ^{3}H-labeled type X collagen was added to the incubation medium, and by immunofluorescence histochemistry (Fig. 4). If diffusion occurred equally well in both inward and outward directions without any retention in the tissue, one would expect a large decrease of type X content in the tissue incubated in the unsupplemented medium, due to outward movement and equilibration with the surrounding medium. Instead, by both methods,

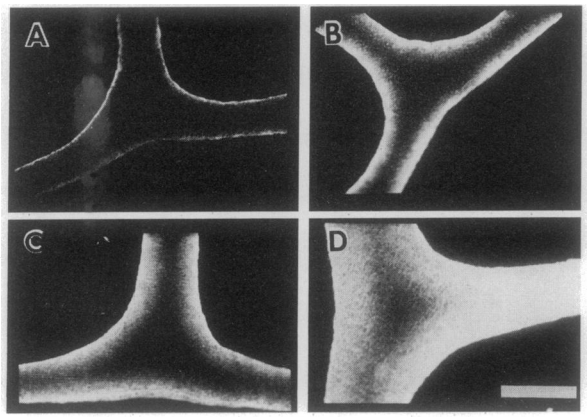

FIG. 2. Fluorescence micrographs of sections of sterna incubated in type X collagen-containing medium for 15 min (*A*), 30 min (*B*), 1 hr (*C*), and 2.5 hr (*D*). All sections were reacted with the monoclonal antibody for type X collagen. (Bar = 200 μm.)

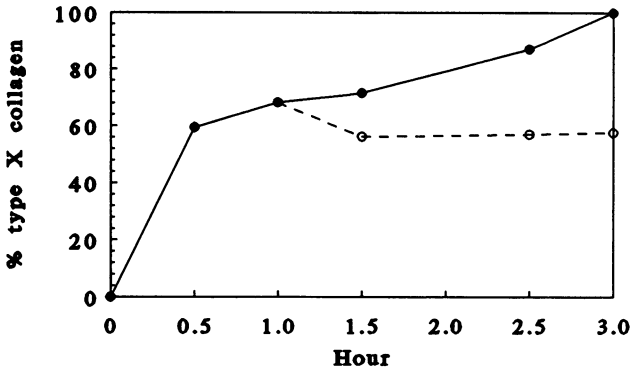

FIG. 3. Time-dependent uptake of ^{3}H-labeled type X collagen by sternal pieces. Filled circles show continuous uptake of radiolabel. Open circles show chases in unlabeled medium, following 1 hr of uptake. The 100% value is the uptake after 3 hr of continuous radiolabeling.

during the chase period there was, at most, a slight overall decrease observed in the tissue-associated type X collagen.

In the isotopic studies, the 3-hr time point of infiltration was used as the standard maximum because immunofluorescence microscopy had demonstrated that type X completely penetrated the tissue by that time. After 1 hr in medium containing ^{3}H-labeled type X, the tissue became infiltrated with type X to a level 68% of the maximum value; during the chase period more than 80% of this label remained associated with the tissue (Fig. 3).

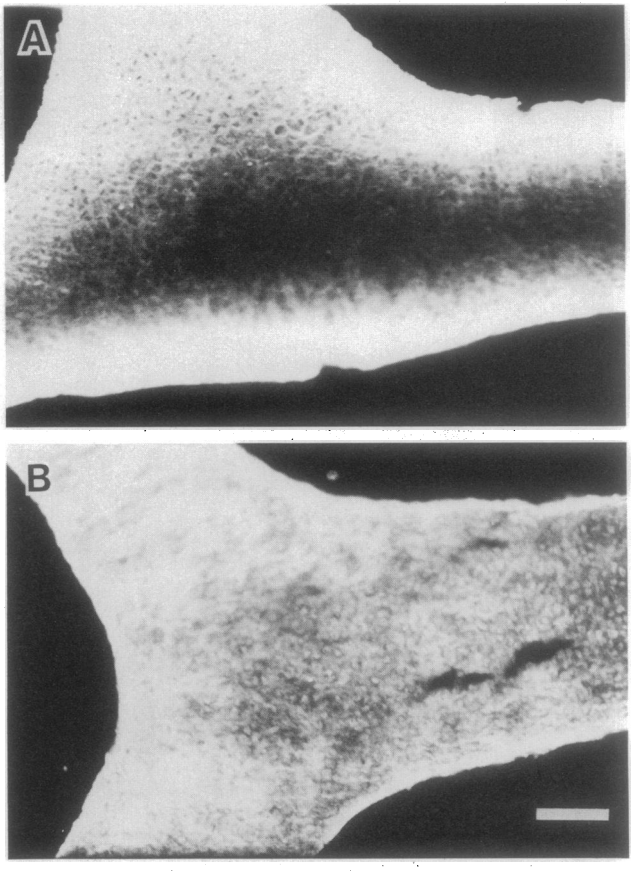

FIG. 4. Fluorescence micrographs of sterna incubated in type X collagen-containing medium for 1 hr (*A*) and 1 hr followed by a 2-hr chase incubation in unsupplemented medium (*B*). Sections were reacted with the monoclonal antibody for collagen type X. (Bar = 77 μm.)

Immunofluorescence analyses confirmed the retention of the infiltrated type X (compare Fig. 4 *A* and *B*) and also revealed that the collagen continued to move inward during the chase period.

The net inward movement of the type X collagen, and its subsequent retention within the matrix during the chase, could have resulted from a sequestration of the molecules, possibly by their association with the preexisting type II collagen-containing fibrils. Immunoelectron microscopic observations with anti-type X collagen–colloidal gold complexes supported this contention (Fig. 5). Most of the type X collagen (decorated with large gold–antibody complexes) that had moved into the cartilage matrix had become associated with fibrils. Very little was found in the interfibrillar space. The fibrils also reacted for type II collagen (small gold–antibody complexes). But, as we have observed (ref. 3 and unpublished observations), the labeling for type II collagen is at a low level, possibly due to the blocking of type II collagen epitopes by the coating of collagen types X and IX.

DISCUSSION

We have observed in this *in vitro* system an extensive movement of exogenous type X collagen molecules through embryonic chicken sternal cartilage matrix. This could mimic the *in vivo* behavior we proposed for newly secreted type X collagen (3). The data also support the contention that subsequent to its diffusion out into the matrix, type X collagen becomes associated with preexisting fibrils.

The motive force for the movement may simply be diffusion. The process of movement of type X collagen in the matrix does not seem to depend on active cellular metabolism. We have observed a similar extensive movement of type X collagen through cartilage at 4°C and in freeze–thawed tissue. Others (17) have observed that diffusion constants for molecules are similar whether in living cartilage or in cartilage poisoned with azide. The directionality of diffusion could be provided by sequestration of the inwardly moving molecules, effected by their association with the type II collagen fibrils. Once sequestered, such molecules would no longer be able to diffuse out into the surrounding medium, resulting in the observed net influx.

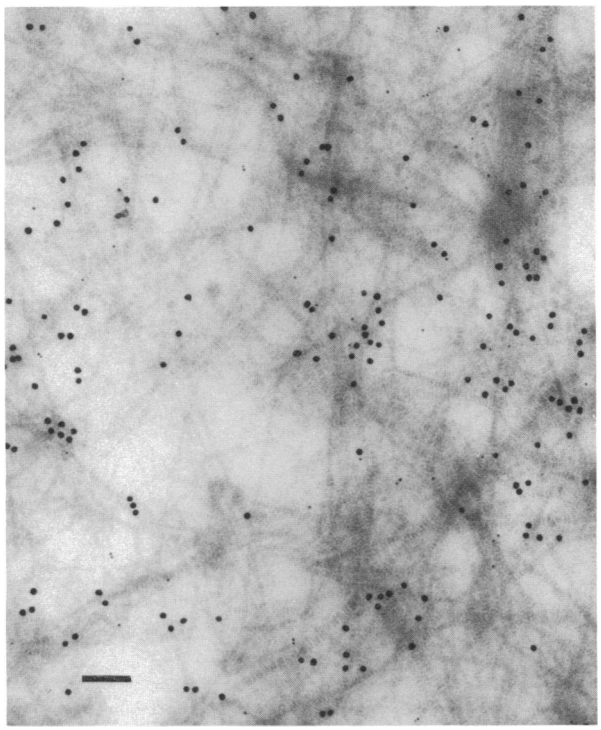

FIG. 5. Double-label immunoelectron micrograph of sternal cartilage that had been incubated in type X collagen-containing medium for 3 hr. Anti-type X collagen monoclonal antibody, 15-nm gold; anti-type II collagen monoclonal antibody, 5-nm colloidal gold. (Bar = 100 nm.)

The movement observed for the type X molecules probably depends on both the properties of the molecule and the matrix itself. In this study we employed the rapidly developing cartilage of the embryonic chicken sternum, which allowed extensive movement of type X collagen. But, when we similarly examined type X collagen penetration into mature human costal cartilage, we found it to be slight (unpublished results). So the source of the cartilage and/or its state of development appears to be a critical factor. As for the molecular properties that relate to the observed movement, the results suggest some selectivity for type X collagen, a rod-shaped molecule. Penetration of cartilage matrix was not observed with the other rod-like molecules tested, type I collagen and type I procollagen (which should not form fibrils under the conditions used) and the truncated vertebrate collagenase-digestion products of type I collagen (provided by Jerome Gross, Cutaneous Biology Research Center, Massachussetts General Hospital East) (unpublished observations). Laurent and coworkers (for reviews, see refs. 18 and 19) have shown that, in general, molecular diameter is an important parameter for the movement of asymmetric molecules through matrix. It can also be argued that rod-like molecules, due to their shape, may be able to make an ordered, end-on passage. Our results suggest that properties other than molecular shape and size may also be important determinants of a molecule's behavior. These properties are probably specific for the molecule and remain to be elucidated.

Whatever the mechanism, the ability of type X collagen molecules to move extensively through cartilage matrix and become associated with fibrils lends credence to the possibility that at least one function of type X collagen in the process of endochondral bone formation is to modify preexisting cartilage-collagen fibrils. It may also explain why small amounts of type X collagen have sometimes been detected in several tissues other than hypertrophic cartilage, such as developing bone (9) and notochord (20), and raises the general proposition that during development, cells can modify matrices at a distance.

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1. Maroudas, A. (1976) *J. Anat.* **122,** 335–347.
2. Maroudas, A. (1970) *Biophysical J.* **10,** 365–379.
3. Schmid, T. M. & Linsenmayer, T. F. (1990) *Dev. Biol.* **138,** 53–62.
4. Linsenmayer, T. F., Fitch, J. M., Schmid, T. M., Birk, D.E., Bruns, R. R. & Mayne, R. (1989) in *Collagen*, eds. Olsen, B. R. & Nimni, M. E. (CRC, Boca Raton, FL), pp. 141–170.
5. Schmid, T. & Linsenmayer, T. (1987) in *Structure and Function of Collagen Types*, eds. Mayne, R. & Burgeson, R. E. (Academic, New York), pp. 223–259.
6. Poole, A. R. & Pidoux, I. (1989) *J. Cell Biol.* **109,** 2547–2554.
7. Schmid, T. M., Mayne, R., Jeffrey, J. J. & Linsenmayer, T. F. (1986) *J. Biol. Chem.* **261,** 4184–4189.
8. Schmid, T. M. & Linsenmayer, T. F. (1983) *J. Biol. Chem.* **258,** 9504–9509.
9. Schmid, T. M. & Linsenmayer, T. F. (1985) *J. Cell Biol.* **100,** 598–605.
10. Pesciotta, D. M., Curran, S. & Olsen, B. R. (1982) in *Immunochemistry of the Extracellular Matrix*, ed. Furthmayr, H. (CRC, Boca Raton, FL), Vol. 1, pp. 91–110.
11. Linsenmayer, T. F., Gibney, E. & Fitch, J. M. (1986) *J. Cell Biol.* **103,** 1587–1593.

12. Fitch, J. M., Gibney, E., Sanderson, R. D., Mayne, R. & Linsenmayer, T. F. (1982) *J. Cell Biol.* **95,** 641–647.
13. Fitch, J. M., Birk, D. E., Linsenmayer, C. & Linsenmayer, T. F. (1990) *J. Cell Biol.* **110,** 1457–1468.
14. Hayashi, M., Ninomiya, Y., Parsons, J., Hayashi, K., Olsen, B. R. & Trelstad, R. L. (1986) *J. Cell Biol.* **102,** 2302–2309.
15. Ninomiya, Y., Gordon, M., Van der Rest, M., Schmid, T., Linsenmayer, T. F. & Olsen, B. R. (1986) *J. Biol. Chem.* **261,** 5041–5050.
16. Gross, J. & Bruns, R. R. (1984) in *The Role of Extracellular Matrix in Development*, ed. Trelstad, R. L. (Liss, New York), pp. 479–512.
17. Cumming, G. J., Handley, C. J. & Preston, B. N. (1979) *Biochem. J.* **181,** 257–266.
18. Preston, B. N., Laurent, T. C., Comper, W. D. & Checkley, G. J. (1980) *Nature (London)* **287,** 499–503.
19. Comper, W. D. & Laurent, T. C. (1978) *Physiol. Rev.* **58,** 255–315.
20. Linsenmayer, T. F., Gibney, E. & Schmid, T. M. (1986) *Dev. Biol.* **113,** 467–473.