

# Tenascin Is Associated with Chondrogenic and Osteogenic Differentiation In Vivo and Promotes Chondrogenesis In Vitro

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**Abstract.** The tissue distribution of the extracellular matrix glycoprotein, tenascin, during cartilage and bone development in rodents has been investigated by immunohistochemistry. Tenascin was present in condensing mesenchyme of cartilage anlagen, but not in the surrounding mesenchyme. In fully differentiated cartilages, tenascin was only present in the perichondrium. In bones that form by endochondral ossification, tenascin reappeared around the osteogenic cells invading the cartilage model. Tenascin was also present in the condensing mesenchyme of developing bones that form by intramembranous ossification and later was present around the spicules of forming bone.

Tenascin was absent from mature bone matrix but persisted on periosteal and endosteal surfaces.

Immunofluorescent staining of wing bud cultures from chick embryos showed large amounts of tenascin in the forming cartilage nodules. Cultures grown on a substrate of tenascin produced more cartilage nodules than cultures grown on tissue culture plastic. Tenascin in the culture medium inhibited the attachment of wing bud cells to fibronectin-coated substrates. We propose that tenascin plays an important role in chondrogenesis by modulating fibronectin-cell interactions and causing cell rounding and condensation.

**T**HE extracellular matrix (ECM)<sup>1</sup> is important for cartilage and bone differentiation and is the very basis of the function of the fully differentiated hard tissues. Cartilage differentiation begins with the rounding and condensation of mesenchymal cells, followed by differentiation which is characterized by the synthesis of cartilage-specific matrix components. Bone development can occur either by intramembranous or endochondral ossification. Intramembranous ossification takes place within a layer of mesenchyme, whereas endochondral ossification occurs within a cartilage model in which the chondrocytes hypertrophy and are then replaced by invading osteogenic cells.

Several immunohistochemical studies have been carried out which have demonstrated changes in ECM components during cartilage and bone differentiation. These ECM molecules can arbitrarily be divided into two overlapping groups: those which appear early in differentiation and may therefore promote this process, and those which are present in the differentiated tissue and have a predominantly structural role. Collagen type I, fibronectin, and a large chondroitin sulfate proteoglycan increase in density in the cartilage anlage of the embryonic chick limb bud at the time of mesenchymal condensation (11, 17). As cartilage differentiation proceeds, collagen type I and the large chondroitin sulfate proteoglycan are replaced by the cartilage-specific collagen type II and a cartilage-specific proteoglycan which persist throughout differentiation (11, 17, 31). Hyaluronic acid con-

centration remains constant in chondrogenic areas during condensation, but increases in peripheral nonchondrogenic mesenchyme (31). Collagen type I reappears as the cartilage model is destroyed during endochondral ossification (36). Collagen type X is present only in the hypertrophic cartilage which exists as an intermediate stage in endochondral ossification (29).

In vitro and in vivo studies have confirmed the importance of many of these ECM molecules in cartilage or bone differentiation. Chondrogenesis in vitro is stimulated by type I and type II collagen (18), chondroitin sulfate proteoglycan (20), hyaluronic acid (22), and by notochord ECM (19). Chondrogenesis in vitro is inhibited by fibronectin (27, 34, 38). Chondrogenesis and osteogenesis in vivo are stimulated by demineralized bone matrix (28).

Fully differentiated cartilage and bone each contain their own tissue-specific ECM components as well as ECM molecules found in other tissues. The particular combinations of ECM molecules are responsible for the special structural properties of the two tissues. The major components of cartilage matrix, hyaluronic acid-bound proteoglycans, and type II collagen, confer on cartilage its characteristic resilience. Bone matrix consists mainly of collagen type I and crystals of hydroxyapatite, the combination of which makes bone rigid. Cartilage and bone also contain minor tissue-specific collagen types, other proteins, and proteoglycans (discussed in references 5, 24, 26, and 35).

The ECM glycoprotein tenascin, which was originally described as myotendinous antigen and is probably identical to

1. *Abbreviation used in this paper:* ECM, extracellular matrix.

cytotactin (15), glioma mesenchymal extracellular matrix protein (GMEM) (4), and J1 (21), has a strikingly restricted tissue distribution during embryogenesis (7, 10). The perichondrium of vertebral and limb cartilage of chick fetuses before ossification is one of the tenascin-containing tissues (7). In a recent report it has been suggested that tenascin could be a structural component of differentiated cartilage (35). The present study was undertaken to obtain clues to the role of tenascin in cartilage and bone development and to determine whether it is present in the two differentiated tissues. The time of expression of tenascin during chondrogenesis and osteogenesis in the rat and mouse has been investigated by immunohistochemistry. The effect of exogenous tenascin on limb bud cultures undergoing chondrogenesis has also been investigated.

## Materials and Methods

### Protein Purification and Antisera

Tenascin was purified from the conditioned medium of primary chick embryo fibroblast cultures by affinity chromatography using a monoclonal antibody, as previously described (8). Fibronectin was purified from newborn calf serum using gelatin-agarose affinity chromatography as described (9). Rabbit antisera to tenascin and fibronectin were obtained previously (10, 13). Guinea pigs were immunized against tenascin purified from fibroblast-conditioned medium as described above. The guinea pig immunization schedules and processing of antisera were as previously described for rabbits (10).

### Frozen Sections and Immunofluorescence

Fetuses were obtained from outbred Sprague-Dawley rats and female BALB/c mice crossed with male CDI mice. Bronchi were dissected from adult Sprague-Dawley rats. Whole fetuses of various ages (vaginal plug = day 0) and bronchi were embedded in Tissue-Tek O.C.T. compound (Miles Laboratories, Inc., Naperville, IL), frozen in ethanol/dry ice, and stored at  $-70^{\circ}\text{C}$ . Sections (10–20- $\mu\text{m}$  thick) were cut at  $-20^{\circ}\text{C}$  and placed on gelatin-coated slides. Frozen sections through whole heads of 20-d-old rats were a kind gift from Dr. Tuomo Kantomaa (University of Oulu, Oulu, Japan). The 20- $\mu\text{m}$  sections were taken on a plastic tape and fixed in 96% methanol. All sections were stored in tight boxes at  $-20^{\circ}\text{C}$  until used.

Some sections were treated with bovine testicular hyaluronidase (Calbiochem, Lucerne, Switzerland; 0.1 mg/ml in PBS, 30 min,  $22^{\circ}\text{C}$ ) or collagenase (type I; Sigma Chemical Co., St. Louis, MO; 0.5 mg/ml in PBS, 20 min,  $37^{\circ}\text{C}$ ) and then washed in PBS before immunofluorescent staining. Sections were stained with anti-tenascin, anti-fibronectin, or preimmune serum by indirect immunofluorescence as previously described (10). For immunofluorescent staining anti-fibronectin was diluted 1:100 and anti-tenascin and preimmune sera were diluted 1:100 for rat tissues and 1:50 for mouse tissues. For immunoperoxidase staining the Vectastain ABC kit was used (Vector Laboratories, Inc., Burlingame, CA). The sections were incubated for 1 h at  $37^{\circ}\text{C}$  with anti-tenascin (diluted 1:200) or normal rabbit serum. After staining, the sections were mounted with Aquamount (Gurr, BDH Chemicals Ltd., Poole, UK). Some sections were stained with Alcian blue to stain glycosaminoglycans, and counterstained with hematoxylin and picro-ponceau and other sections were stained with hematoxylin and eosin by standard histological methods.

### Wing Bud Cultures

Wing bud cultures were prepared from 4-d-old chick embryos according to the method of Ahrens et al. (1) with minor modifications. Dissected wing buds were incubated for 10 min in a shaking waterbath in 0.1% trypsin (Gibco, Basel, Switzerland), 0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ), 10% FCS (Gibco) in calcium- and magnesium-free PBS. The resulting cell suspension was mechanically dissociated in DME/10% FCS until no clumps were visible. The cell suspension was adjusted to  $2 \times 10^6$  cells/ml. The cells were plated on 96-well cluster plates (Falcon Labware, Cockeysville, MD) in 100- $\mu\text{l}$  aliquots in DME/10% FCS. Twofold serial dilutions of the cells were prepared and each experiment was carried out at four cell densities, allowing us to select the optimal cell density for analysis. Cultures seeded too densely showed no difference in carti-

lage nodule formation between the different treatments and cultures seeded too sparsely contained insufficient cartilage nodules for analysis. The best results were usually obtained at a cell density of  $10^6$  or  $0.5 \times 10^6$  cells/ml. The wells were either used uncoated or after coating with a solution of fibronectin or tenascin at 50  $\mu\text{g}/\text{ml}$  in PBS for 15 min at room temperature. The wells were washed with medium before the cells were plated. In one experiment wells were coated with tenascin, fibronectin, or a solution of BSA (1% in PBS), then treated with bovine testicular hyaluronidase (Calbiochem; 0.1 mg/ml in PBS containing 1% BSA, 30 min,  $22^{\circ}\text{C}$ ) or 1% BSA in PBS and then washed with medium before the cells were plated. 3 or 4 d after plating, the cultures were fixed with 4% formaldehyde in PBS and stained with Alcian blue at pH 1 (1% in 0.1 M HCl; reference 23). The Alcian blue-stained nodules were counted and the results were analyzed using the *t* test.

### Attachment Assay

The dissociated wing bud cells were plated on the different substrates (described above) at  $2.5 \times 10^5$  cells/ml in DME/10% FCS using 50  $\mu\text{l}/\text{well}$ . 50  $\mu\text{l}$  of PBS or tenascin (50  $\mu\text{g}/\text{ml}$ ) in PBS were added immediately to the wells. After 16 h the cells were washed, fixed with 4% formaldehyde in PBS, stained with hematoxylin, and counted. Since the cells attached best to fibronectin, cell attachment on fibronectin was arbitrarily designated as 100% attachment.

### Immunofluorescence of Cell Cultures

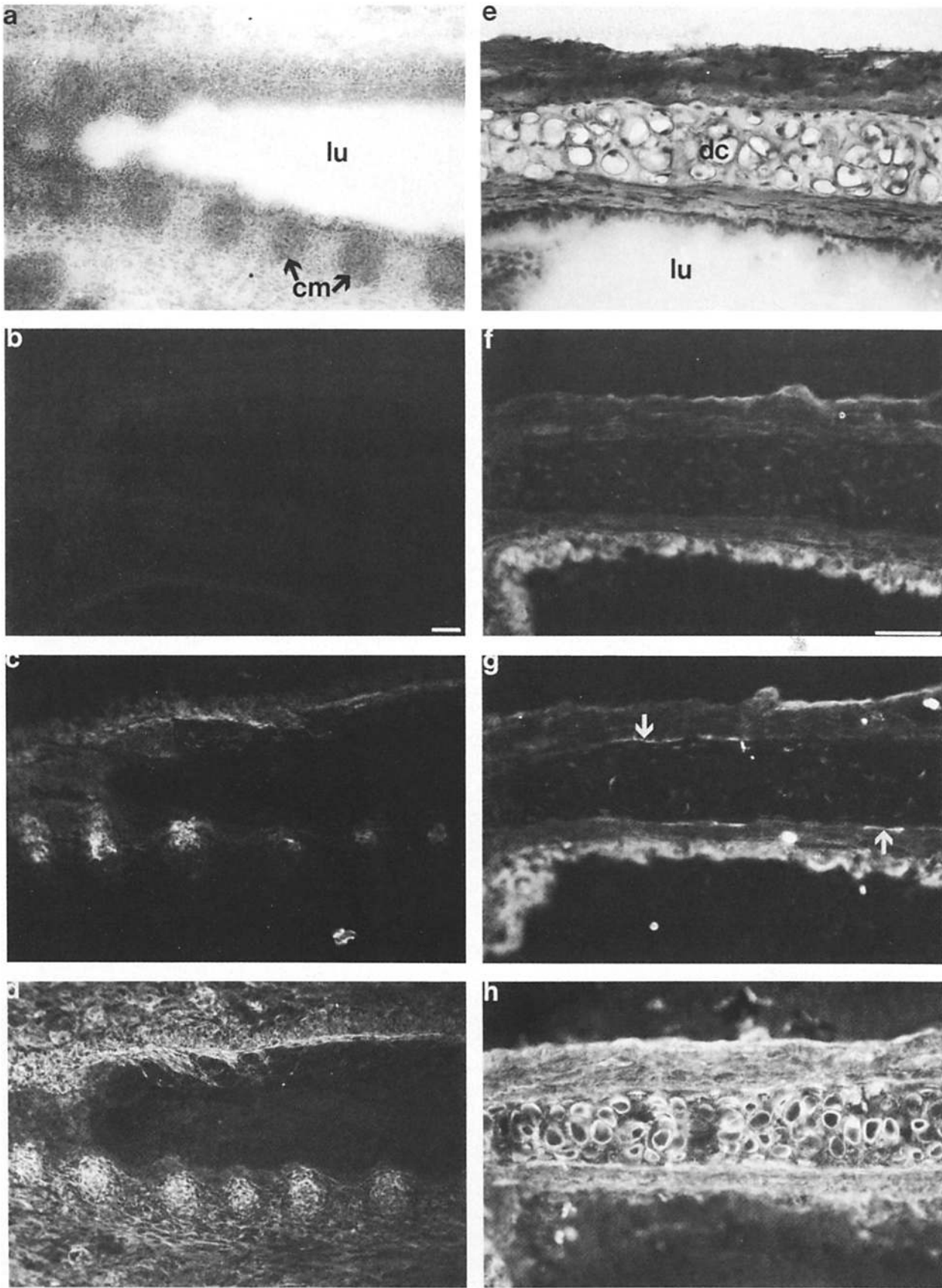
The cells were cultured on 8-chamber plastic slides (LabTek; Miles Laboratories, Inc.). Before staining they were washed with PBS, fixed with 4% formaldehyde in PBS for 20 min, treated with 0.2% Triton X-100 in PBS for 5 min, and washed three times for 5 min with PBS/0.1% BSA. They were stained with the antisera as described for the cryosections. The cultures that were double-stained for tenascin and fibronectin were treated first with rabbit anti-fibronectin, washed, then treated with guinea pig anti-tenascin. FITC goat anti-rabbit IgG (Miles-Yeda Ltd., Rehovot, Israel) and rhodamine-conjugated goat anti-guinea pig IgG (Cappel Laboratories, Malvern, PA; CooperBiomedical, Inc., Malvern, PA), each diluted 1:50 then mixed in equal volumes, were used to detect the anti-fibronectin and anti-tenascin antibodies, respectively.

## Results

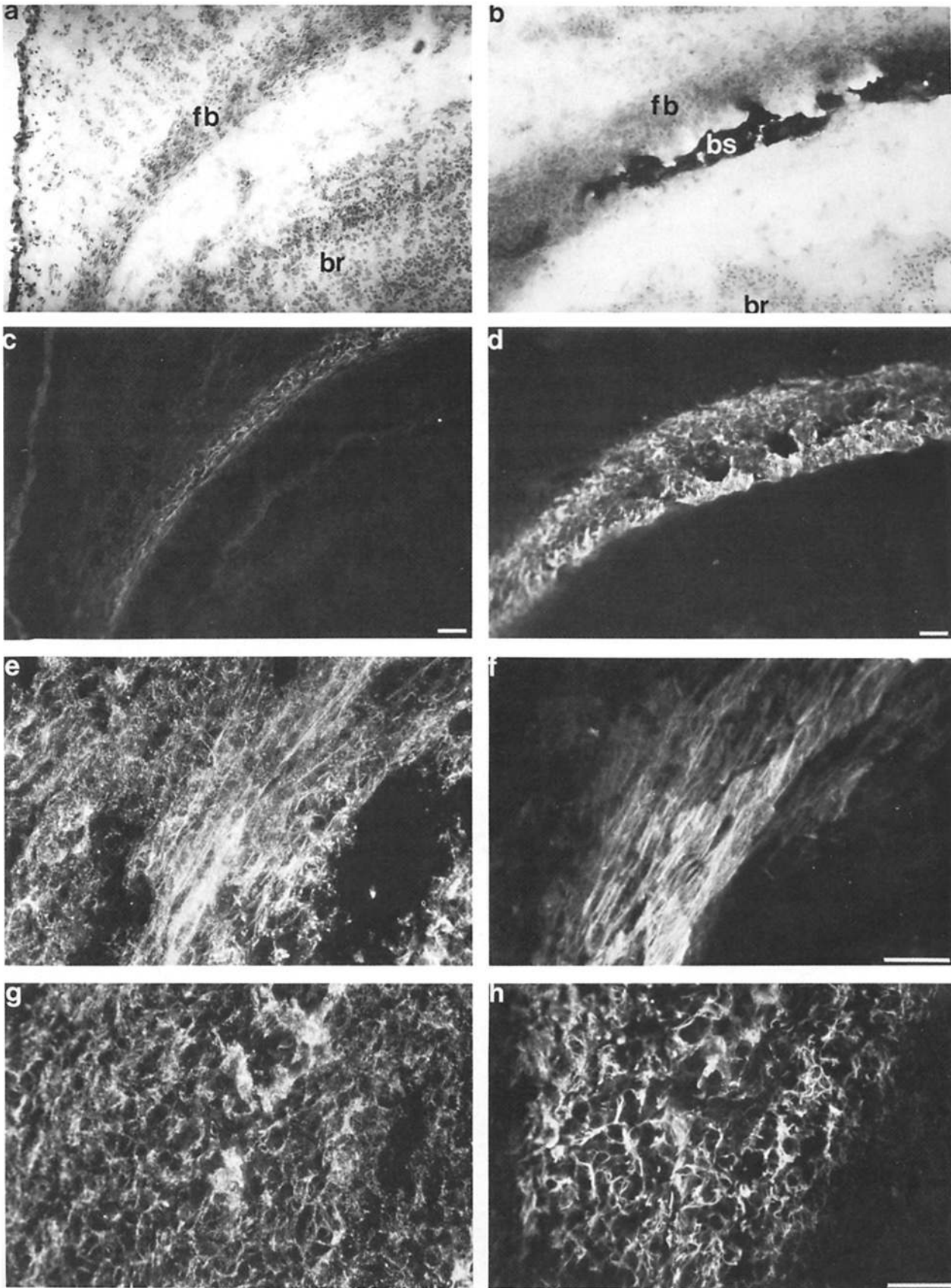
### Cartilage Development

Tenascin was detectable in the condensing mesenchyme of cartilage anlagen. Fig. 1, *a-d*, shows longitudinal sections through the trachea of a 17-d-old rat fetus. The condensing mesenchyme of the presumptive tracheal cartilage rings contained tenascin in a fibrillar distribution whereas the surrounding loose mesenchyme was negative for tenascin. Fibronectin, although present in a higher concentration in the cartilage anlagen, was additionally present throughout the surrounding mesenchyme. Tenascin was also selectively localized in the condensing mesenchyme of future intervertebral discs (Fig. 3 *b*).

As condensed cartilage mesenchyme begins to differentiate, cartilage-specific matrix is laid down and pushes the cells apart. In all the cartilages studied, including tracheal, sternal, and intervertebral disc cartilages, tenascin was progressively lost from the matrix with increasing chondrocyte differentiation. In the mature hyaline cartilage matrix of the adult rat bronchial rings no tenascin was detectable (Fig. 1, *e-h*). There was faint specific staining with anti-tenascin in the perichondrium. The differentiated fibrocartilage of intervertebral discs of 17-d-old rat fetuses also contained no tenascin; tenascin was, however, detectable in the matrix surrounding the condensing cells at the periphery of the discs (Fig. 3 *g*). In contrast, fibronectin was present in the differentiated hyaline cartilage of the bronchus (Fig. 1 *d*) and fibrocartilage of the intervertebral discs (Fig. 3 *h*).



**Figure 1.** Distribution of tenascin and fibronectin in fetal and adult rat cartilage. Serial longitudinal cryosections through 17-d-old fetal rat trachea (*a-d*) and adult rat bronchus (*e-h*) stained with Alcian blue and counterstained with hematoxylin and picro-ponceau (*a* and *e*), or by indirect immunofluorescence with preimmune (*b* and *f*), anti-tenascin (*c* and *g*), or anti-fibronectin (*d* and *h*) serum. *cm*, Condensing mesenchyme of presumptive cartilage rings; *dc*, differentiated cartilage; *lu*, lumen. The arrows in *g* indicate faint tenascin staining in the perichondrium. All other fluorescence in *g* is nonspecific as demonstrated in *f*. Bar, 50  $\mu$ m.



**Figure 2.** Distribution of tenascin and fibronectin in fetal membrane bones. Serial sagittal cryosections through the frontal bone of 15-d-old (*a* and *c*) and 17-d-old (*b* and *d*) rat fetuses, stained with Alcian blue counterstained with hematoxylin and picro-ponceau (*a* and *b*), or by indirect immunofluorescence with anti-tenascin (*c* and *d*). Higher magnification of serial sections of 15-d-old fetal rat frontal bone (*e* and *f*) and the membranous part of the mandible (*g* and *h*), stained with anti-fibronectin, (*e* and *g*) or anti-tenascin (*f* and *h*). *br*, brain; *bs*, bone spicules; *fb*, condensing mesenchyme of frontal bone. Bar, 50  $\mu$ m.

It has previously been demonstrated that treatment of cartilage sections with hyaluronidase unmasks fibronectin staining (37) and that tenascin staining in developing teeth is enhanced by hyaluronidase or collagenase pretreatment (Thesleff, I., E. Mackie, S. Vainio, and R. Chiquet-Ehrismann, manuscript submitted for publication). Thus, sections containing differentiated cartilage were treated with these enzymes before processing for indirect immunofluorescence. Neither hyaluronidase nor collagenase was able to unmask tenascin in mature cartilage matrix. In some partially differentiated cartilages, hyaluronidase treatment removed intracellular staining and unveiled pericellular staining with anti-tenascin, but staining was never unmasked in additional areas of tissue (not shown).

### **Bone Development**

Tenascin appeared with the mesenchymal condensation of forming bones which develop by intramembranous ossification. Tenascin was present in the newly condensed frontal bone of a 15-d-old rat fetus (Fig. 2, *a* and *c*). 2 d later the staining was more intense (Fig. 2, *b* and *d*). At this stage some bone spicules had been laid down and tenascin was present around these. Fig. 2, *e* and *f*, shows a higher magnification of the frontal bone of a 15-d-old rat fetus, stained with anti-fibronectin and anti-tenascin, respectively. Both fibronectin and tenascin were present in fibrils aligned in the plane of the future bone. The tenascin-containing fibrils, however, appeared to be thicker than the fibronectin-containing fibrils. Fig. 2, *g* and *h*, shows sections through the membranous portion of the mandible of a 15-d-old rat fetus. Once again tenascin-containing fibrils were thicker than fibronectin-containing fibrils, but in this case they were arranged without orientation.

Bones which form by endochondral ossification, including long bones, ribs, vertebrae, and some of the cranial bones, were examined in sections of whole rat and mouse fetuses. Tenascin appeared and disappeared during formation of the cartilage model, just as in the development of cartilage which remains as cartilage in the adult (described above). Strong staining with anti-tenascin was seen in the early condensations of rib and vertebral body anlagen in a 13-d-old rat fetus (Fig. 3, *a* and *b*). In the vertebral bodies of 17-d-old rat fetuses the well-differentiated cartilage matrix no longer contained tenascin, but fibronectin was still detectable (Fig. 3, *f-h*). By this stage the cartilage models of the ribs were being replaced by bone spicules (Fig. 3 *c*). Tenascin was absent from the hypertrophic cartilage but present around the invading osteogenic cells, around the bone spicules, and in the thick band of poorly differentiated presumptive osteoblasts surrounding the existing bone and cartilage (Fig. 3 *d*). Fibronectin was present throughout the cell layers and particularly abundant in the hypertrophic cartilage (Fig. 3 *e*).

The sequence of tenascin staining seen in ribs was also seen in developing limb long bones. In the femur of a 17-d-old mouse fetus tenascin was present in the perichondrium and in the matrix surrounding poorly differentiated chondrocytes at the growing ends of the bone. It was also present around the osteogenic cells replacing the cartilage model in the diaphysis of the bone (Fig. 4, *a-c*). Tenascin was not detectable in fully differentiated or hypertrophic cartilage.

Tenascin was undetectable in fully differentiated bone ma-

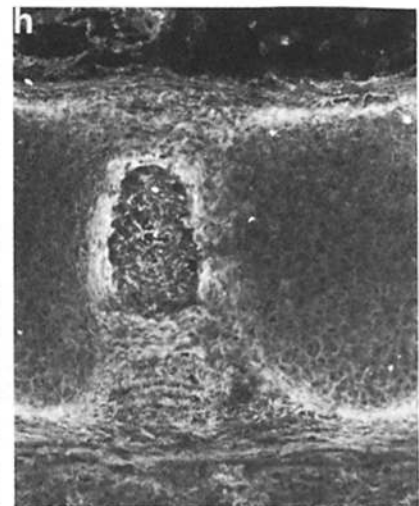
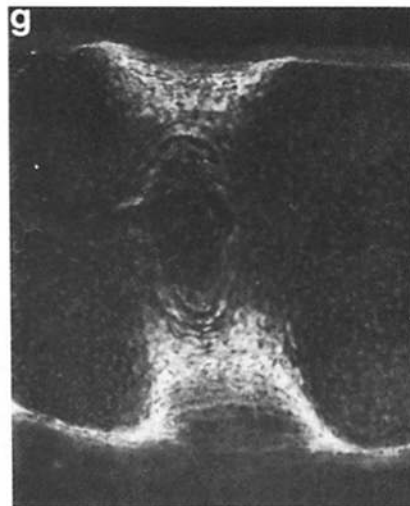
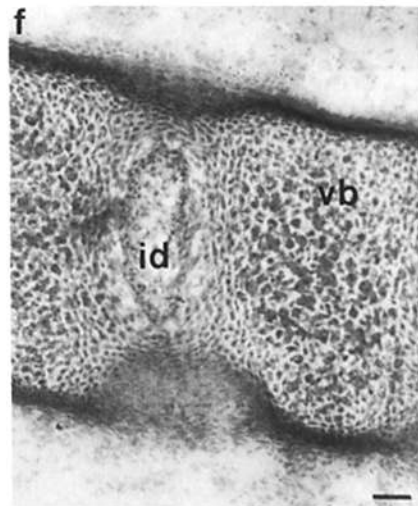
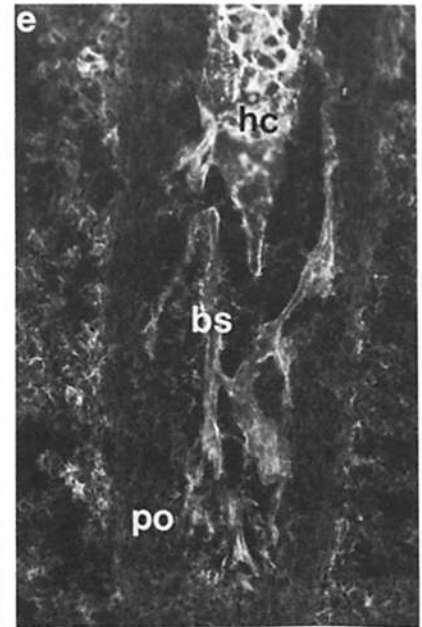
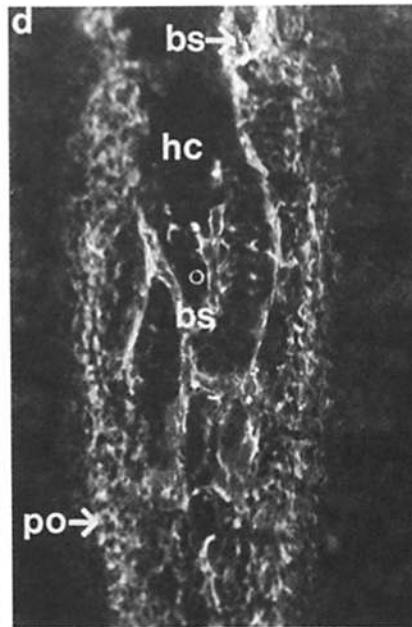
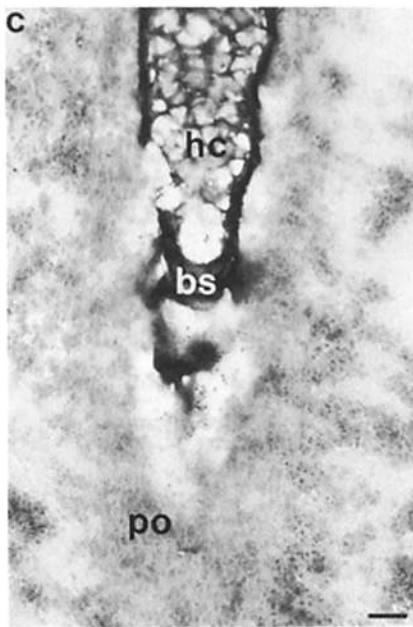
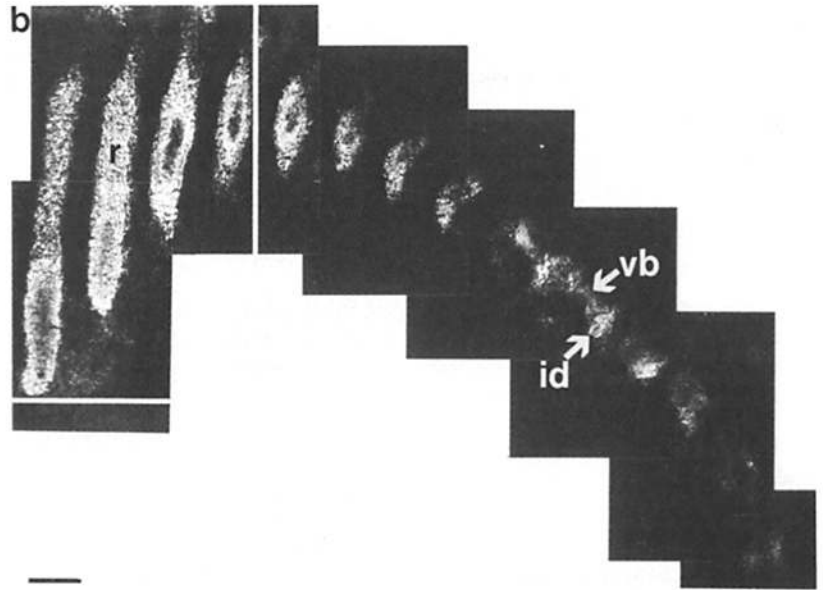
trix. The mandibular bone of a 20-d-old rat, which forms by intramembranous ossification, contained tenascin only on the periosteal and endosteal surfaces, as well as in bone marrow cavities (Fig. 4, *d* and *e*). The fully differentiated osteocytes embedded in the bone matrix were negative as was the mature bone matrix. Treatment of the sections with hyaluronidase or collagenase did not reveal other antigenic sites.

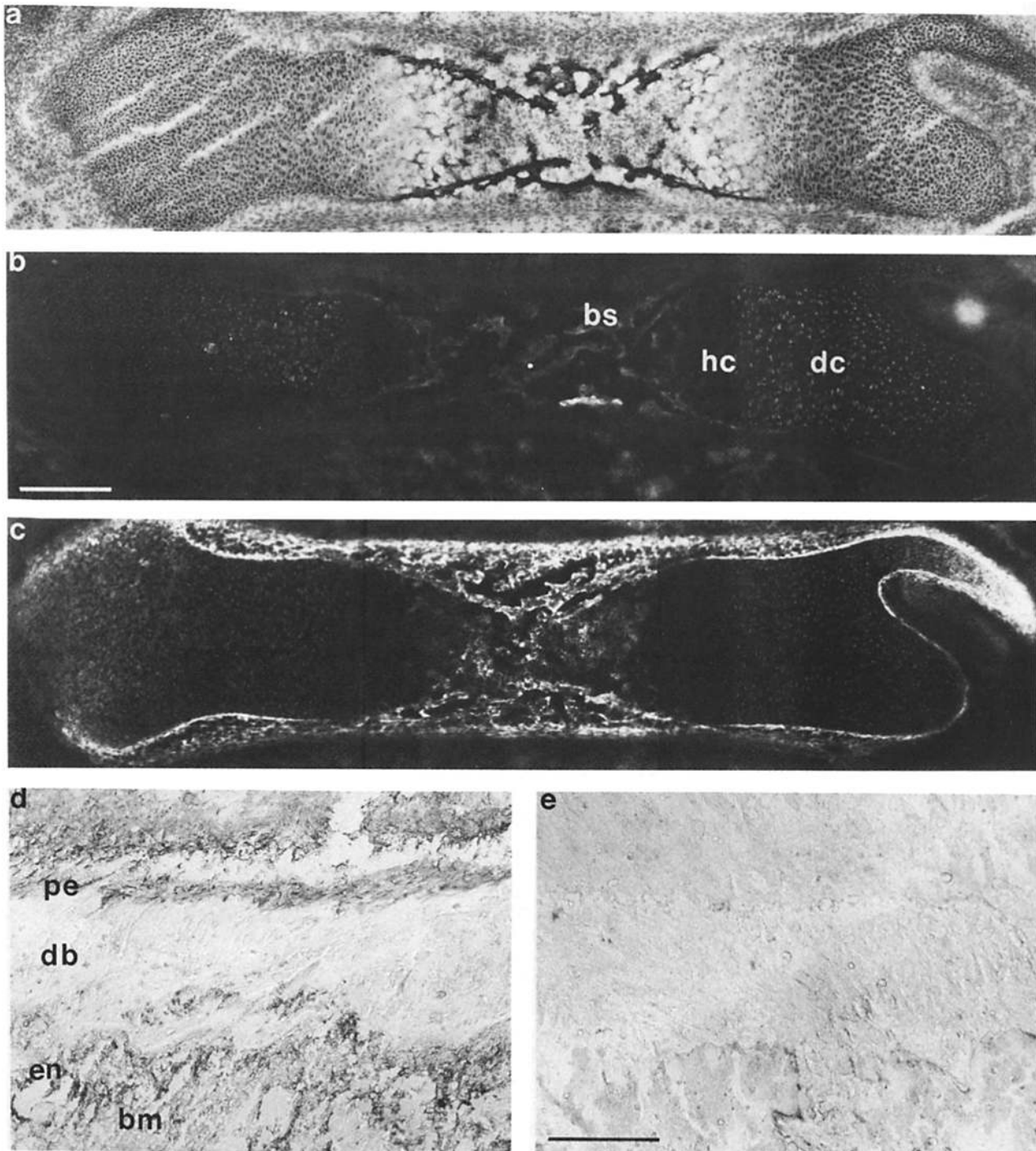
### **Tenascin Promotes Chondrogenesis In Vitro**

When wing buds from 4-d-old chick embryos are dissociated and grown in culture at a cell density higher than confluency, cell aggregates form and differentiate into cartilage nodules, as judged by the presence of an Alcian blue-staining ECM (1). Tenascin was detectable by immunofluorescent staining in 2-d-old cultures in a patchy distribution, whereas fibronectin was present in a rather homogeneous fibrillar distribution (Fig. 5, *a* and *b*). By the fourth day in culture the cartilage nodules stained intensely with anti-tenascin and thick fibrils of tenascin were visible connecting the nodules to each other (Fig. 5 *c*). Fibronectin was not codistributed with tenascin; the thick fibrils seen with anti-tenascin could not be seen with anti-fibronectin (Fig. 5, *d* and *e*). Some individual fibrils which were stained with anti-tenascin could even be seen under phase-contrast microscopy (Fig. 5, *f* and *g*).

Wing bud cells were cultured on tissue culture plastic coated with fibronectin, or tenascin, or without coating, to examine the effect of the different substrates on chondrocyte differentiation. The addition of fibronectin to the culture medium has been shown to inhibit chondrogenesis in such cultures (34). A fibronectin-coated substrate also had an inhibitory effect on the development of Alcian blue-stainable cartilage nodules and more myotubes were discernable than on the other substrates (Fig. 6 *b*). A tenascin-coated substrate had the opposite effect and promoted the formation of cartilage nodules (Fig. 6 *c*). In seven independent experiments ~10-fold more cartilage nodules formed on tenascin than on fibronectin and about three times as many formed on tenascin as on uncoated plastic (Fig. 6, *d-f*). Hyaluronic acid is known to promote in vitro chondrogenesis (22), and tenascin is known to bind to proteoglycans (8). Therefore, in one experiment we treated tenascin, fibronectin, and control (BSA-treated) substrates with hyaluronidase to ensure that the tenascin effect was not due to any associated hyaluronic acid. Hyaluronidase treatment reduced the number of cartilage nodules on all substrates tested (Table I). Since hyaluronidase reduced nodule formation even on the control substrate, it is unlikely that its effect was the result of removal of bound hyaluronate. The effect could perhaps have been due to binding of hyaluronidase to the substrates. Hyaluronidase-treated tenascin still had a significant chondrogenic effect (Table I). The effect of tenascin on wing bud cell attachment was also investigated. The cells were plated on uncoated, fibronectin- or tenascin-coated wells in the presence or absence of exogenous tenascin. The number of cells attached was highest on fibronectin and lowest on tenascin. The addition of tenascin to the medium reduced cell attachment on all substrates, including fibronectin, by ~50% (Table II). Those cells which did attach in the presence of tenascin were rounded and not as well spread as cells in medium without tenascin (not shown).

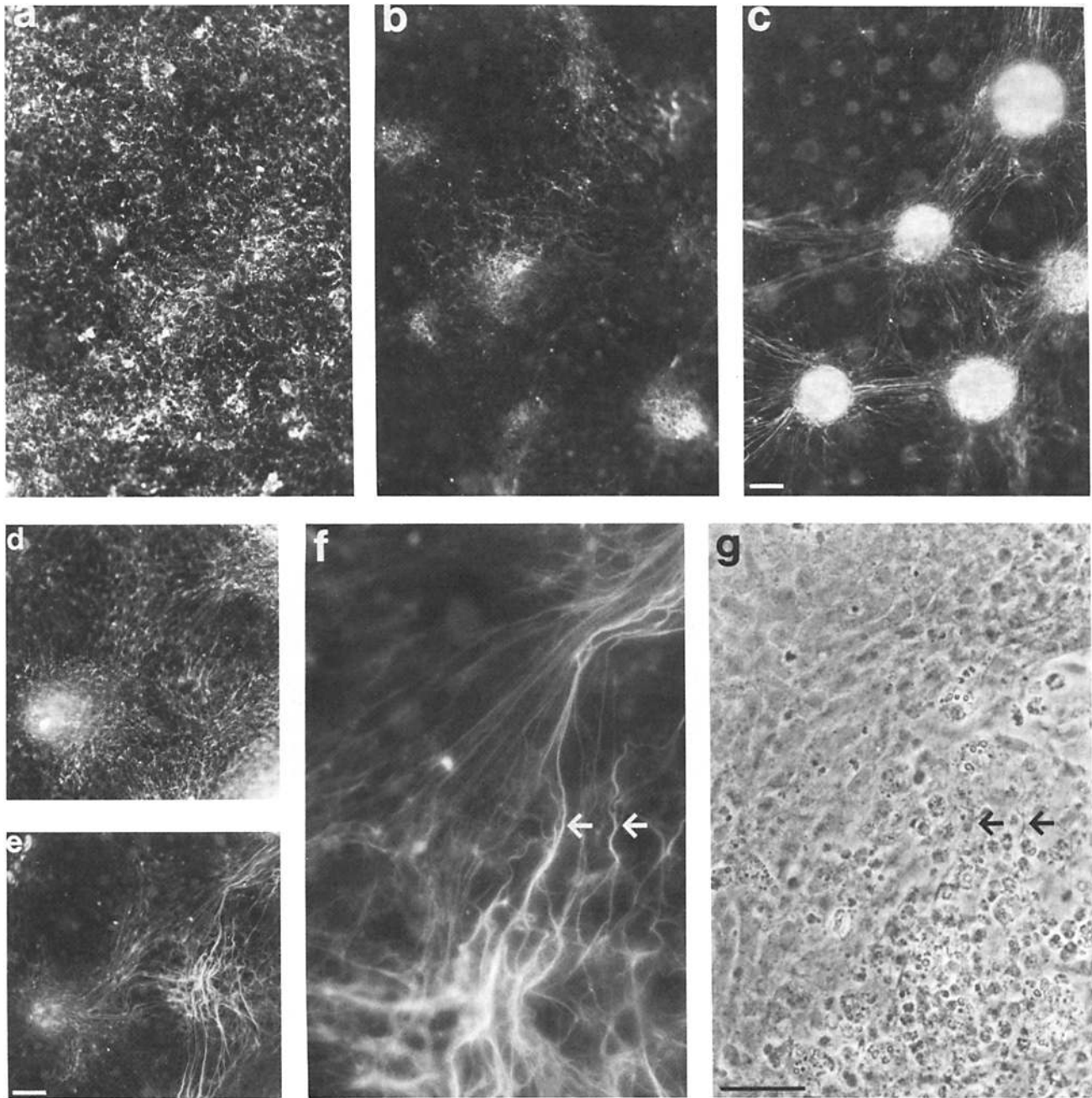






**Figure 4.** Distribution of tenascin in the developing femur and in mature bone. Serial longitudinal cryosections through the femur of a 15-d-old mouse fetus stained with hematoxylin and eosin (*a*), or by indirect immunofluorescence with preimmune (*b*), or anti-tenascin serum (*c*). Cryosections through the ramus of the mandibular bone of a 20-d-old rat stained by the indirect immunoperoxidase method with anti-tenascin (*d*) or normal rabbit serum (*e*). *bm*, bone marrow; *bs*, bone spicules; *db*, dense bone; *dc*, differentiated cartilage; *en*, endosteal surface; *hc*, hypertrophic cartilage; *pe*, periosteum. Bar, 200  $\mu$ m.

**Figure 3.** Distribution of tenascin and fibronectin during endochondral ossification. Serial sagittal cryosections through condensing mesenchyme of ribs and vertebral column of a 13-d-old rat fetus, stained with hematoxylin and eosin (*a*), or stained by indirect immunofluorescence with anti-tenascin (*b*). The area shown in *a* corresponds to the area outlined on the left in *b*. Serial cryosections through a rib (*c-e*) and the vertebral column (*f-h*) of a 17-d-old rat fetus, stained with Alcian blue and counterstained with hematoxylin and picro-ponceau (*c* and *f*), or stained by indirect immunofluorescence with anti-tenascin (*d* and *g*) or anti-fibronectin (*e* and *h*) serum. *bs*, Bone spicules; *hc*, hypertrophic cartilage; *id*, intervertebral disc; *po*, presumptive osteoblasts; *r*, rib; *vb*, vertebral body. Bars, (*a* and *b*) 200  $\mu$ m; (*c-h*) 50  $\mu$ m.



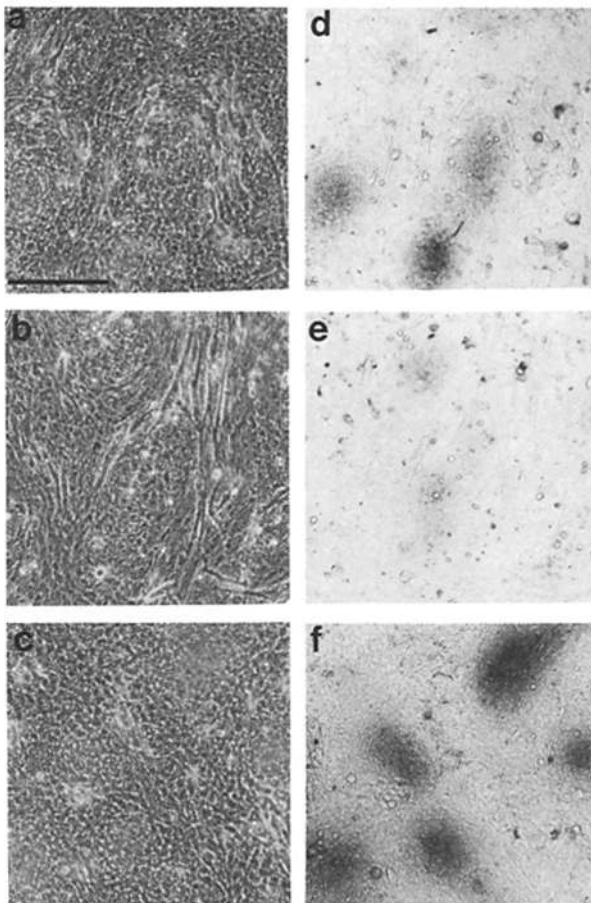
**Figure 5.** Distribution of tenascin and fibronectin in wing bud cultures. Indirect immunofluorescence of wing bud cultures stained 2 d (*a* and *b*), or 4 d (*c-f*) after plating with anti-fibronectin (*a* and *d*) or anti-tenascin (*b*, *c*, *e*, and *f*); *d* and *e* show the same field double-stained with anti-fibronectin and anti-tenascin sera. The arrows in *f* indicate fibrils, stained by anti-tenascin, that can be seen in the same field viewed by phase contrast (*g*). Bar, 50  $\mu$ m.

### Discussion

In this study tenascin was found to be associated with chondrogenic and osteogenic tissues undergoing differentiation. It was present in the early mesenchymal condensations of cartilage and bone anlagen but not in the surrounding mesenchyme. It progressively disappeared with the accumulation of mature cartilage or bone matrix. Tenascin was retained, however, in the perichondrium, which provides a source of cells to differentiate into chondroblasts. The immunofluorescent staining of tenascin in perichondrium was much more intense in the growing fetal cartilages than in adult cartilage

which has the potential for regeneration but is not growing. The analogous situation is seen with tenascin during tooth development. Tenascin is expressed by dental papilla cells early in development but once these cells differentiate into the dentin-producing odontoblasts tenascin is no longer expressed. Tenascin continues to be abundant in the tooth pulp which has the capacity for hard tissue formation (Thesleff, I., E. J. Mackie, S. Vainio, and R. Chiquet-Ehrismann, manuscript submitted for publication). Tenascin is not exclusively present in developing hard tissues. It is found in the dense mesenchyme surrounding the budding epithelial rudiments of mammary gland, hair follicle, and molar tooth (10).





**Figure 6.** Tenascin promotes the formation of cartilage nodules. Wing bud cultures grown on uncoated (a and d), fibronectin-coated (b and e), or tenascin-coated (c and f) wells, viewed under phase-contrast microscopy (a-c) or bright field (d-f) for visualization of Alcian blue-stained nodules. Bar, 200 µm.

It is also present in myotendinous junctions, tendons, and smooth muscle (7). In all these situations, however, the surrounding mesenchyme contains no tenascin.

Tenascin, because of its distribution during hard tissue formation, is unique among the ECM molecules so far described. Collagen type I, which is absent from mature cartilage matrix, is present throughout the limb-bud mesenchyme and becomes more concentrated in cartilage anlagen with mesenchymal cell condensation (11). Tenascin, on the other

**Table I. Cartilage Nodule Formation on Different Substrates**

Coating	Hyaluronidase	No. of cartilage nodules/well	n
Control (BSA)	+	1.8 ± 1.5	6
	-	2.5 ± 1.4	6
Fibronectin	+	0 ± 0 <sup>‡</sup>	6
	-	0.5 ± 0.5 <sup>§</sup>	6
Tenascin	+	5.8 ± 2.3 <sup>§</sup>	9
	-	7.3 ± 2.6 <sup>§</sup>	9

\* Mean ± SD of n wells.

<sup>‡</sup> Significantly different from appropriate control value ( $P < 0.01$ ).

<sup>§</sup> Significantly different from appropriate control value ( $P < 0.005$ ).

**Table II. Inhibition of Cell Attachment by Tenascin**

Substrate	Cell attachment*	
	No addition	20 µg/ml Tenascin addition to medium
	%	%
No coating	59	28
Fibronectin	100	43
Tenascin	48	18

\* Results are expressed as percentage of cells attached/field on a fibronectin-coated substrate without tenascin in the medium, where 363 cells were counted in three fields. For all other combinations of experimental conditions the cells in at least five fields were counted.

hand, was found to be absent from the mesenchyme surrounding cartilage and bone anlagen. A recent report demonstrates that peanut agglutinin binds specifically to an extracellular component of precartilage cellular aggregates both in vivo and in vitro (2). This component appears to have a similar distribution to tenascin and may, indeed, be tenascin.

Fibronectin, like collagen type I, is ubiquitous in mesenchymal tissues and increases in concentration in cartilage anlagen with mesenchymal cell condensation (11). In one study, fibronectin was found to be absent from mature cartilage matrix (11) whereas others have shown that its presence can be unmasked or enhanced by hyaluronidase treatment of sections (14, 37). Our results confirm that, at least in the cartilages we examined, fibronectin is present and its staining can be enhanced by hyaluronidase treatment.

Vaughan et al. (35) have recently suggested that tenascin could serve as a structural component of differentiated cartilage since substantial quantities could be extracted from fetal chick sterna. The results of the present study indicate, however, that tenascin is absent from fully differentiated cartilage matrix and, therefore, is not a structural component of mature cartilage. To confirm this, we examined by immunohistochemistry the presence of tenascin in sterna from chicks of the same stage as those used by Vaughan et al. In sections treated with hyaluronidase, tenascin staining was present around the relatively undifferentiated cells at the growing edges of the cartilage but absent from the matrix produced by the central, differentiated chondrocytes (results not shown).

The results of the immunohistochemical studies presented here demonstrate that tenascin is associated with determined but not fully differentiated, growing areas of developing bone and cartilage. In vitro studies were devised to investigate the function of tenascin during chondrogenesis.

Wing bud cells grown in culture produced tenascin mainly in the cartilage-forming nodules, whereas fibronectin was present throughout the cultures. Zanetti and Solursh (40) have also described staining of the matrix of cartilage colonies by M1, a monoclonal antibody to tenascin. In the present study, thick, tenascin-stained fibrils developed, interconnecting the cartilage nodules. The staining pattern of such cultures with anti-tenascin was reminiscent of the patterns of tension created by fibroblasts or tissue explants in collagen gels described by Harris et al. (16) and Stopak and Harris (33). It is tempting to speculate that tension in nodule-forming wing bud cultures induces the deposition of the

tenascin-stained fibrils in an organized pattern. If this is so, tenascin could participate in translating patterns of mechanical force into a specific protein structure. Such a structure could then serve as a building block for the formation of hard tissue or tendon.

Tenascin as a substrate promoted chondrogenesis by as much as fibronectin inhibited it. The inhibition of chondrogenesis by fibronectin is in agreement with previously published work (27, 34, 38). There are several possible explanations for the chondrogenesis-promoting effect of tenascin. Tenascin may selectively allow the attachment or growth of cells which have already been determined as chondroblasts, or tenascin may actively influence the outcome of determination of wing bud cells. Cultures grown on a fibronectin substrate contained more myogenic cells than on the other substrates examined. The effect on myoblasts is likely to be due to selective attachment, since they are known to be particularly dependent on exogenous fibronectin for attachment (9). The myoblasts are not derived from the limb bud mesenchyme but rather have immigrated from the somite and are already determined at the time of culture (6). The fibroblast and chondroblast phenotypes, on the other hand, are probably not yet determined at the time of culture, since it is known that the transition of chondroblasts into fibroblast-like cells in the presence of fibronectin is reversible (38). It seems likely, therefore, that the increase in the number of chondrogenic nodules on tenascin substrates is due to the promotion of chondroblast differentiation and not due to selection.

The chondroblast phenotype is dependent on cell shape (3, 32). When wing bud cells are grown under conditions which allow them to become rounded they start to produce cartilage-specific matrix components. In addition, when wing bud cells are treated with agents that disrupt the cytoskeleton the cells become rounded and undergo chondrogenic differentiation (39). In the present study, tenascin was able to antagonize the attachment of wing bud cells to fibronectin, and the cells that attached in the presence of tenascin were rounded. Thus it is possible that tenascin interferes with the communication between fibronectin and the cytoskeleton, causing cell rounding and chondrogenic differentiation. The fact that tenascin was able to modulate the interaction of wing bud cells with fibronectin may explain the apparently contradictory information that fibronectin is present in vivo throughout chondrogenesis but inhibits this process in vitro. Perhaps one role of tenascin, which is present in chondrogenic areas but not in the surrounding mesenchyme, is to override the anti-chondrogenic effect of fibronectin.

We have previously found that tenascin is present in the condensed mesenchyme of fetal mammary gland, hair follicle, and molar tooth (10). Since mesenchymal condensation is an essential preliminary step in cartilage formation, and because of the remarkable association of tenascin with condensing mesenchyma, the possibility that tenascin is involved in this process must be considered. Tenascin has been found to cause the aggregation of erythrocytes (10). In the in vitro studies presented here it was not possible to observe an effect of exogenous tenascin on aggregation of wing bud cells since the cells aggregated spontaneously. The results of previous studies suggest that precartilaginous mesenchymal condensation is not a result of increased mitosis but of the movement of cells toward each other (12). Oster et al. (25) have

suggested that the production of hyaluronidase and resultant breakdown of extracellular hyaluronate brings the cells closer together and is theoretically sufficient to cause cartilage condensation. Singley and Solursh (31), however, demonstrated that there is no decrease in hyaluronate in condensing cartilage anlagen. These authors suggested that the retraction of cell processes with cell rounding that they observed in their EM studies results in condensation. Tenascin may contribute to the condensation process through a mechanism analogous to that involved in cell aggregation in vitro, as well as by causing cell rounding.

In conclusion, tenascin was found to be present in a strikingly restricted distribution in chondrogenic and osteogenic tissues, and to promote chondrogenesis in vitro. We suggest that tenascin plays an important role in the differentiation of hard tissue forming cells, possibly by allowing cells to detach from the fibronectin-containing ECM and causing cell rounding and condensation.

The authors would like to thank Drs. M. Chiquet, W. Halfter, R. P. Tucker, and S. Shibahara for critical reading of the manuscript.

Received for publication 10 June 1987, and in revised form 6 July 1987.

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