

Tenascin Is Associated With Articular Cartilage Development

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ABSTRACT The roles of tenascin in cartilage development and function remain unclear. Based on the observation that tenascin is particularly abundant at the epiphyseal extremities of developing cartilaginous models of long bones in chick and mouse embryo, we tested the hypothesis that tenascin is involved in articular cartilage development. Immunofluorescence analysis revealed that tenascin was first localized in the cell condensation region of Day 4 chick embryo limb buds, where the cartilaginous models form. With further development, tenascin gene expression became indeed restricted to the articular cap of the models. Tenascin persisted in the articular cartilage of postnatal chickens but appeared to decrease with age. The protein was also abundant in embryonic and adult tracheal cartilage rings which, like articular cartilage, persist throughout postnatal life. Similar patterns of tenascin expression were seen in mouse. Using monoclonal antibodies to avian tenascin variants, we found that the bulk of articular cartilage contained the shortest tenascin variant (Tn190), whereas the largest variant (Tn230) was present in tissues associated or interacting with articular cartilage (ligaments and meniscus). The protein and its mRNA, however, were undetectable in growth plate cartilage undergoing maturation and endochondral ossification. This inverse correlation between chondrocyte maturation and tenascin production was corroborated by the finding that tenascin gene expression decreased markedly during maturation of chondrocytes in culture and during formation of a secondary ossification center within the articular cap in vivo. Thus, tenascin is intimately associated with the development of articular cartilage and other permanent cartilages whereas absence or reduced amounts of this matrix protein characterize transient cartilages which undergo maturation and are replaced by bone.

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Key words: Articular cartilage, Tenascin, Growth plate, Limb development, Chondrocytes, Extracellular matrix

INTRODUCTION

Tenascin is a large extracellular matrix glycoprotein present in a restricted number of embryonic tissues, including cartilage (Erickson and Bourdon, 1989; Chiquet-Ehrismann, 1990). When isolated in its native form, tenascin consists of six subunits associated into a disulfide-bonded "hexabrachion" structure (Vaughan et al., 1987; Erickson and Taylor, 1987). Each subunit contains a series of structural domains, including N-terminal EGF-like repeats, fibronectin type III repeats, and a C-terminal Ca^{2+} -binding domain (Jones et al., 1989; Spring et al., 1989). Tenascin variants differing in molecular size have been found in both mammals and avians; they are the product of alternative splicing and differ in the number of fibronectin type III repeats (Spring et al., 1989; Gulcher et al., 1989; Prieto et al., 1990; Saga et al., 1991; Siri et al., 1991; Weller et al., 1991). In chick the largest tenascin has 13 such repeats and an Mr of 230,000 (Tn230), whereas variants with only nine or eight repeats exhibit Mr's of 200,000 (Tn200) and 190,000 (Tn190), respectively (Spring et al., 1989; Chiquet-Ehrismann et al., 1991).

Despite sharing structural features with fibronectin, tenascin actually possesses anti-adhesion and anti-spreading properties. Tenascin interferes with attachment and spreading of a number of cell types in culture (Erickson and Bourdon, 1989; Lightner and Erickson, 1990; Chiquet-Ehrismann et al., 1988), in good correlation with its transient expression in migrating cells during morphogenesis in vivo (Crossin et al., 1986; Chuong et al., 1987; Bonner-Fraser, 1988). The anti-adhesion properties of tenascin mainly reside in its N-terminal half, as revealed by experiments with truncated forms of the protein (Spring et al., 1989; Prieto et al., 1992). Interestingly, the C-terminal half, which contains fibronectin type III repeats, has adhesion-promoting effects on cultured cells (Spring et al., 1989). Thus, both anti-adhesion and pro-adhesion signals co-exist on tenascin.

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The role(s) of tenascin in cartilage remains largely unknown but data are available on its distribution during development. Crossin et al. (1986) found that tenascin is homogeneously distributed in the matrix of early, newly formed vertebral cartilage in Day 7 chick embryo; with further development, however, tenascin was no longer detected in the cartilage but persisted in the surrounding perichondrium. The same group found that tenascin is present only in the matrix of peripherally located chondrocytes in Day 14 chick embryo sternum and is largely undetectable in centrally located chondrocytes (Hoffman et al., 1988). Similarly, in cartilaginous models of femur, tibia, and humerus in rat or chick embryos, tenascin and its mRNA were detected only in the chondrocytes located at each epiphyseal end (Chiquet and Fambrough, 1984; Mackie et al., 1987; Prieto et al., 1990; Mackie and Tucker, 1992). A major conclusion reached in these previous studies was that tenascin is involved in the early stages of chondrocyte development, characterizes only immature, poorly differentiated chondrocytes, and is no longer produced by fully differentiated chondrocytes.

The presence of tenascin at each epiphyseal end of the cartilaginous long bone models prompted us to analyze an additional hypothesis. The tenascin-positive chondrocytes located in this area must largely represent those that give rise to articular cartilage. In the avian embryo and juvenile chicken, each epiphyseal end of the model forms the so called articular cap, which includes a top thin layer of fibrocartilage facing the synovial cavity and a thicker underlying layer of articular cartilage (Lutfi, 1974; Howlett, 1979). The caps are attached to very extensive growth plates that extend deep into the diaphysis (Wolbach and Hegsted, 1952; Wise and Jennings, 1973; Howlett, 1979; Stocum et al., 1979). Our hypothesis was that the epiphyseal tenascin-rich chondrocytes may in large part represent developing articular chondrocytes and, thus, that tenascin may be specifically involved in articular cartilage development. To test this hypothesis, we analyzed the temporal and spatial distribution of tenascin both in fetal articular cartilage as it forms during chick embryogenesis and in definitive postnatal articular cartilage. The observations described below lend support to our hypothesis.

RESULTS

Tenascin Distribution in Developing Articular Cartilage

Longitudinal frozen sections of hind limbs from Day 4 through Day 19 chick embryos were processed for immunofluorescence using the M1 monoclonal antibody; this antibody recognizes each isoform of chick tenascin. We found that in the Day 4 embryo tenascin was enriched in the core of the limb bud, representing the region of chondrogenic cell condensation and differentiation (Fig. 1A,B). In Day 6 embryo, tenascin was present in a wider mesenchymal region but was clearly enriched in the incipient femoral and tibial models fac-

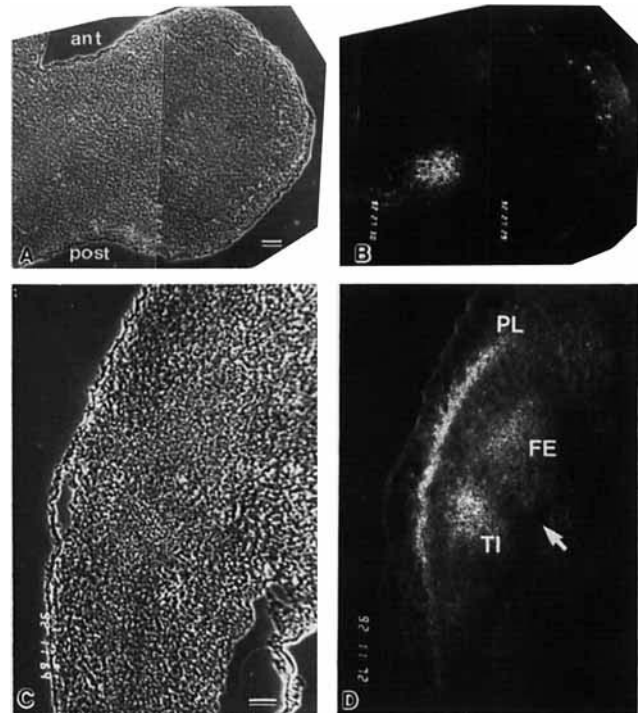


Fig. 1. Distribution of tenascin in developing limb buds. Phase (A and C) and immunofluorescence (B and D) micrographs of longitudinal sections of Day 4 (A,B) and Day 6 (C,D) chick embryo hind limbs stained with the M1 antibody. At Day 4, tenascin is enriched in the core of the limb. At Day 6 tenascin is detected in the newly formed femoral (FE) and tibial (TI) cartilaginous models separated by a rudimentary joint interzone (arrow); it is also rich in the presumptive patella ligament (PL). ant, anterior; post, posterior. Bar, 70 μ m.

ing a rudimentary joint interzone, as well as in the presumptive patella ligament (Fig. 1C,D). In Day 12 and Day 18 embryos, in which the articular cap and the overall knee joint had become well defined, tenascin was abundant in the articular cap of both tibia (TI) and femur (FE) but was present at low to background levels in the underlying growth plate (Figs. 2A,B, 3A,B, respectively). Particularly in the Day 18 embryo, the articular cap had become clearly demarcated from the growth plate on the basis of cell morphology. Thus, the tenascin-positive chondrocytes in the cap were all round whereas the tenascin-negative chondrocytes in the first top zone of growth plate were quite flat (Fig. 3A,B).

The demarcation between articular cap and growth plate was confirmed by pulse-labeling of Day 18 chick embryo tibias in organ-culture with [3 H]thymidine followed by autoradiography. Whereas the round chondrocytes within the cap were largely unlabeled, the underlying flat chondrocytes were heavily labeled (Fig. 3C,D) and, thus, represented the proliferative zone of growth plate (see Stocum et al., 1979; Howlett, 1979).

In addition to the articular cap, tenascin was present in fibrocartilage (FC), cruciate ligament (CL), menisci

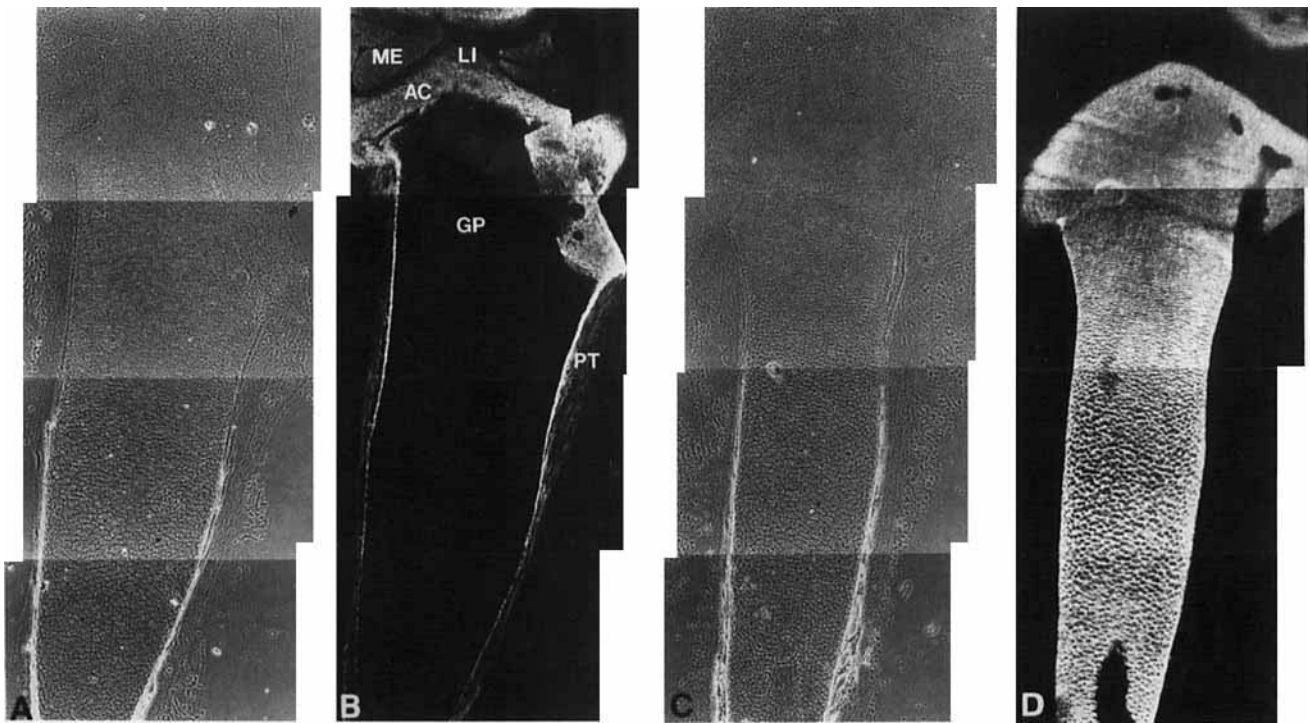


Fig. 2. Distribution of tenascin in Day 12 chick embryo tibia. Phase (A,C) and immunofluorescence (B,D) micrographs of longitudinal sections stained with the M1 antibody (A,B) or chick aggrecan antiserum (C,D). A,B: Tenascin is enriched in the developing articular cap (AC), meniscus (ME), cruciate ligament (LI) and the perichondrial tissue (PT)

but is undetectable in the growth plate (GP), skeletal muscles and other connective tissues. Note that some of the tissue at the upper right corner was accidentally folded after sectioning. C,D: Aggrecan is uniformly distributed over the entire cartilaginous model but is undetectable in the surrounding tissues. Bar, 375 μ m.

(ME) and perichondrial connective tissue (PT) adjacent to the growth plate; however, the protein was undetectable or very low in most surrounding tissues such as skeletal muscles. It was, however, detected in the osteoid collar surrounding hypertrophic cartilage (Fig. 2A,B, lower portion); because the collar forms by intramembranous ossification (Fell, 1925), tenascin appears to be associated with this process (see Mackie and Tucker, 1992).

To verify the selectivity of tenascin distribution, longitudinal knee sections from Day 12 embryo were stained with an antiserum to aggrecan, the major cartilage proteoglycan. We observed that each area of the cartilaginous model stained intensely whereas none of the non-cartilaginous tissues forming the joint, perichondrium, skeletal muscle or other tissues stained (Fig. 2C,D). Identical data were obtained with sections from Day 18 embryos (not shown).

To corroborate the immunofluorescence data, whole cellular RNAs were isolated from Day 19 chick embryo femur articular cartilage and from the underlying growth plate. The results of northern blot analysis showed that articular cartilage contained high steady-state levels of the approximately 6.5 Kb tenascin mRNA whereas growth plate chondrocytes contained undetectable amounts of it (Fig. 4A). In contrast, the

growth plate cells contained large amounts of type X collagen mRNA, a collagen type associated with chondrocyte maturation and endochondral ossification (Gibson et al., 1984), whereas this RNA was absent in articular cartilage (Fig. 4B). Both tissues contained distinct amounts of mRNAs encoding other cartilage-characteristic macromolecules, namely types II and IX collagen and aggrecan (Fig. 4C-E).

Tenascin Distribution in Postnatal Articular Cartilage and Tracheal Rings

Histological analysis of longitudinal sections of 2-month-old chicken proximal tibia revealed that the articular cap now displayed histological features typical of definitive functional articular cartilage. Thus, the articular chondrocytes formed characteristic isogenic groups separated by very abundant extracellular matrix (Fig. 5). The chondrocytes beneath fibrocartilage were smaller in size (Fig. 5A) than those present in the medial portion of articular cartilage (Fig. 5C), whereas those in the lower portion of the tissue and immediately adjacent to the underlying growth plate were flat (Fig. 5E). Immunofluorescence staining with the M1 antibody showed that tenascin was present in articular cartilage and fibrocartilage (Figs. 5B,D,F). Intensity of staining was higher in the upper and lower

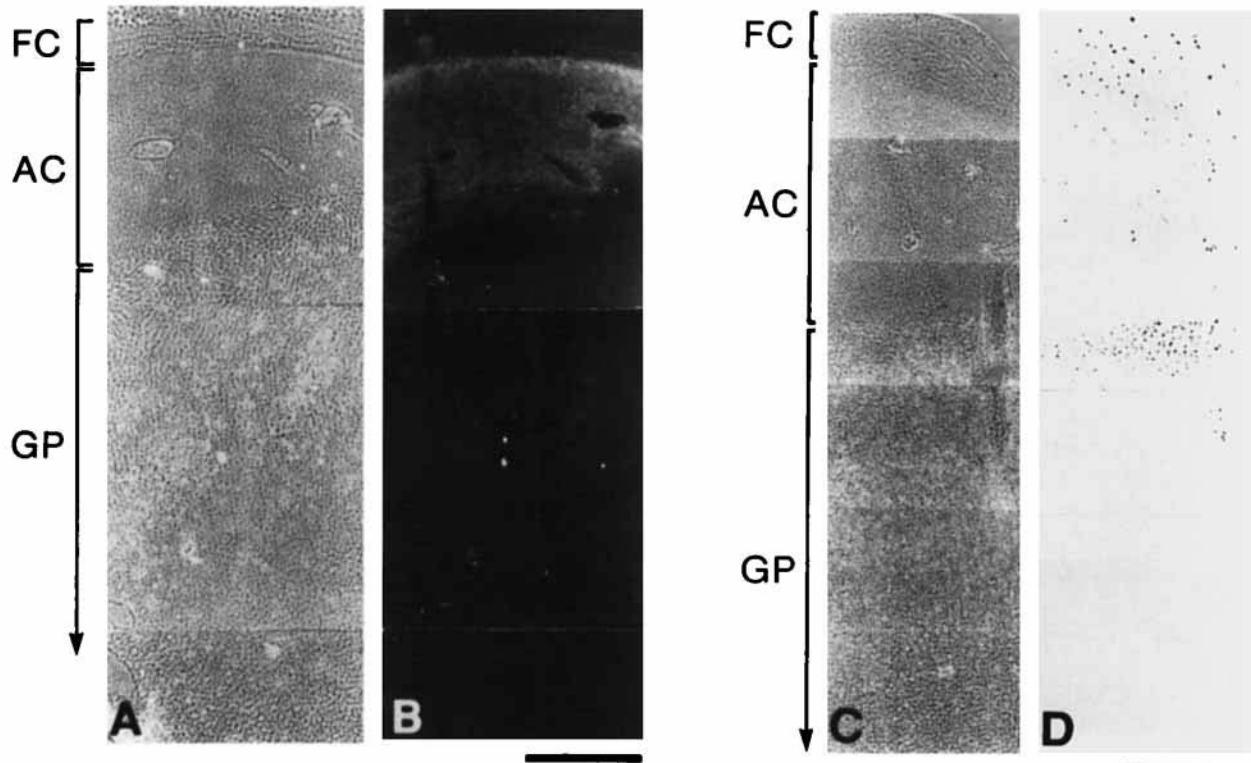


Fig. 3. Localization of tenascin and proliferating chondrocytes in Day 18 chick embryo femur. **A,B**: Phase and immunofluorescence micrographs, respectively, of a longitudinal section stained with the M1 antibody. Tenascin is clearly detectable in articular cap (AC) and fibrocartilage (FC) but not in growth plate (GP). Bar, 230 μ m. **C,D**: Phase and bright field micrographs, respectively, of a longitudinal section from Day

18 chick embryo femur which was labeled for 3 hr with [³H]thymidine, sectioned and then processed for autoradiography. Note the thin zone of radiolabeled, proliferating, irregularly shaped chondrocytes at the beginning of the growth plate (GP) and the relatively few proliferating chondrocytes in the articular cap (AC). Bar, 200 μ m.

portions of articular cartilage than in the middle portion. Moreover, the protein appeared to be preferentially distributed in the matrix within and around each isogenic group and less abundant in the interterritorial matrix (Fig. 5A–F). Tenascin was very scarce around the flat articular chondrocytes located immediately above the growth plate (Fig. 5E,F). Although reduced in size and near its final closure, the growth plate in the 2-month-old juvenile animal displayed a typical histological organization and, like its embryonic counterpart, did not stain with the M1 antibody (Figs. 5E,F).

Like articular cartilage, the cartilaginous rings of trachea persist throughout postnatal life. To determine whether tenascin may also participate in the development of this permanent cartilage, transverse sections of trachea from Day 20 chick embryo and 2-month-old chicken were stained with the M1 antibody. In embryonic trachea, tenascin was abundant and homogeneously distributed throughout the matrix of the rings (Fig. 6A,B). In the older tissue, however, the overall level of immunostaining was lower (Mackie et al., 1987) and the protein appeared to be enriched in the pericellular matrix of the round enlarged chondrocytes

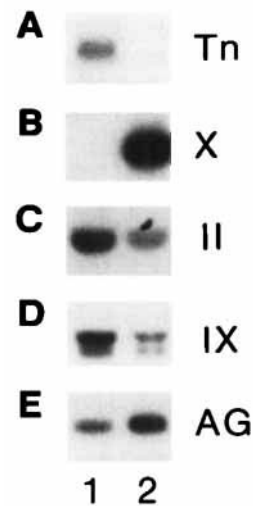


Fig. 4. Northern blot analyses. Autoradiograms show the steady-state levels of mRNAs encoding (A) tenascin, (B) type X collagen, (C) type II collagen, (D) type IX collagen, and (E) aggrecan in Day 18 chick embryo femoral articular cartilage (lane 1) and underlying growth plate (lane 2). Lanes contained identical amounts of total RNA.

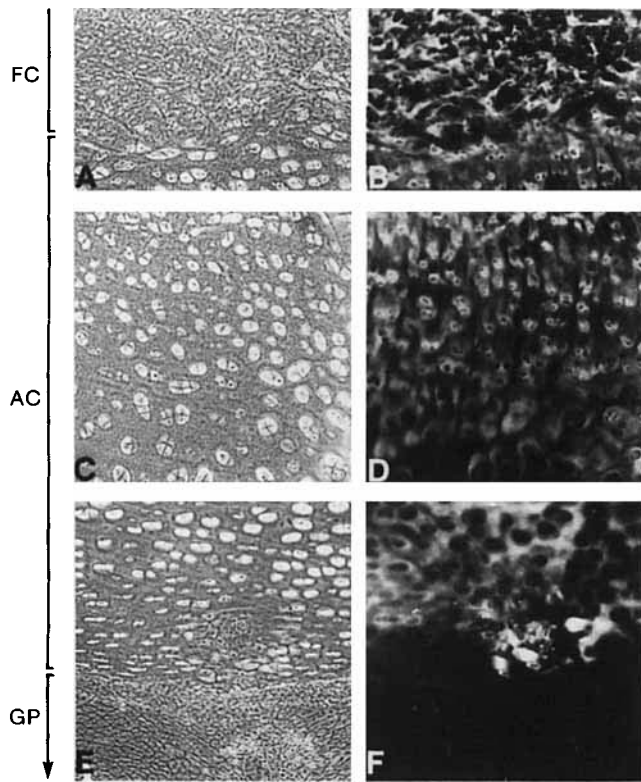


Fig. 5. Distribution of tenascin in adult articular cartilage. Phase (A,C,E) and immunofluorescence (B,D,F) micrographs of a longitudinal section through the articular plateau of 2-month-old chicken proximal tibia stained with the M1 antibody. A,B: Top region of articular cartilage (AC) and overlying fibrocartilage (FC), both of which contain abundant tenascin. Note the preferential pericellular localization of the protein. C,D: Central portion of articular cartilage showing gradual decrease of immunostaining. E,F: Lower portion of articular cartilage and top zone of growth plate (GP). Note the reduced immunostaining around the flat chondrocytes adjacent to the growth plate. Bar, 45 μm .

located in the medial area of the rings (Fig. 6C,D). The perichondrium also stained.

Tenascin and Secondary Ossification Centers

Starting during late embryogenesis, secondary ossification centers begin to form within the articular caps. In chicken proximal tibial articular cap, the center is restricted to a very peripheral area of the cap and does not involve the articular plateau (our unpublished observations). To determine whether formation of a secondary ossification center was accompanied by a change in tenascin distribution, longitudinal sections of proximal tibial articular cap from prenatal and postnatal animals were stained with the M1 antibody. Interestingly, barely detectable levels of immunofluorescence were observed in the incipient secondary ossification center in Day 19–20 chick embryo proximal tibia whereas strong tenascin staining persisted in the surrounding articular cartilage (Fig. 7A,B). Hypertrophic chondrocytes producing type X collagen ap-

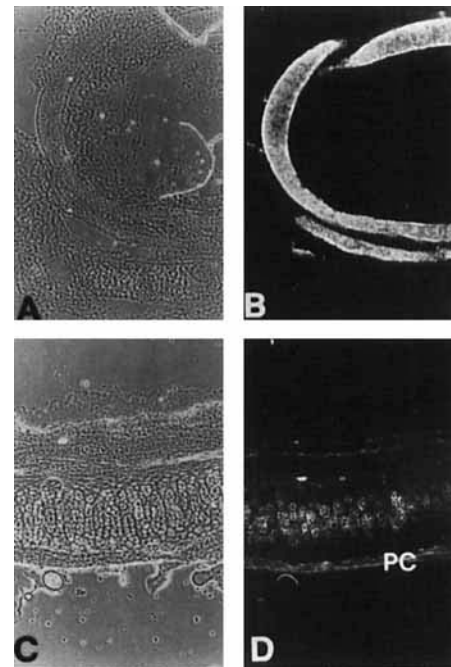


Fig. 6. Distribution of tenascin in trachea. Phase (A,C) and immunofluorescence (B,D) micrographs of transverse sections of tracheal cartilage from Day 20 chick embryo (A,B) and 2-month-old chicken (C,D) stained with the M1 antibody. Note that the embryonic rings stain strongly and uniformly with the antibody whereas in the adult the stain is more prominent around the centrally-located chondrocytes as well as in perichondrium (PC). Bar, 255 μm .

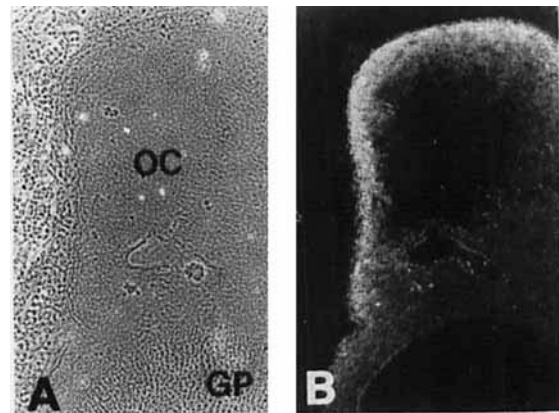


Fig. 7. Tenascin and development of secondary ossification centers. Phase (A) and immunofluorescence (B) micrographs of a longitudinal section through the peripheral portion of newborn chick proximal tibial articular cap; section was stained with the M1 antibody. Note the reduced immunostaining in the area of articular cartilage in which a secondary ossification center (OC) is forming whereas positive staining persists in the surrounding articular cartilage. GP, growth plate. Bar, 265 μm .

peared within this tenascin-negative center starting at 2 to 4 weeks post-hatch (not shown). A similar marked decrease in tenascin staining was observed in the much

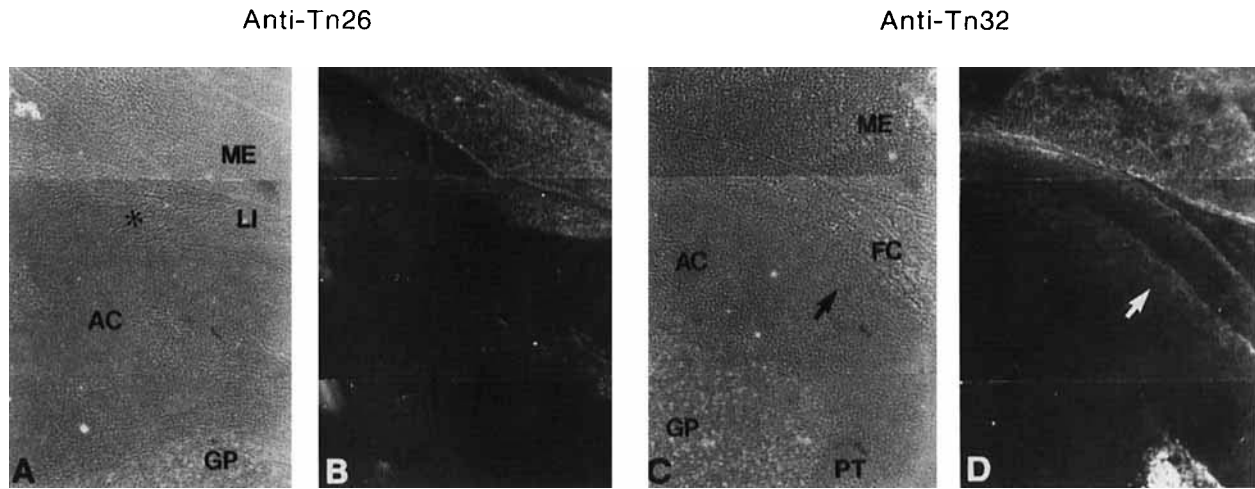


Fig. 8. Tenascin isoforms in the developing joint. Phase (A,C) and immunofluorescence (B,D) micrographs of longitudinal sections through Day 19 chick embryo proximal tibia articular cap stained with anti-Tn26 (A,B) or anti-Tn32 (C,D) antibodies. A,B: Note that the anti-Tn26 antibody stains the meniscus (ME) and cruciate ligament (LI). However, no appreciable stain is observed in fibrocartilage (FC) including where the cru-

ciate ligament inserts into it (asterisk), in articular cartilage (AC) and growth plate (GP). C,D: Note that the anti-Tn32 antibody stains meniscus and fibrocartilage as well as the most peripheral articular chondrocytes (arrow). Strong stain is also present in perichondrial tissue (PT). Bar, 170 μ m.

larger secondary ossification center forming in distal tibial cap of Day 20 chick embryo; contrary to its proximal counterpart, however, we found that this center already contained a few type X collagen-positive hypertrophic chondrocytes (see Schmid and Linsenmayer, 1985).

Tenascin Isoforms

The three isoforms of chick tenascin, Tn230, Tn200, and Tn190, display tissue-specific distributions in vivo (Prieto et al., 1990; Chiquet-Ehrismann et al., 1991). To determine which tenascin isoform was present in the tissues examined above, longitudinal sections of Day 19 chick embryo knee were processed for immunofluorescence using anti-Tn26 and anti-Tn32 monoclonal antibodies (Spring et al., 1989). Anti-Tn26 only recognizes the largest tenascin isoform, Tn230, whereas anti-Tn32 recognizes both Tn230 and Tn200.

We found that anti-Tn26 failed to react with articular cartilage and fibrocartilage (Fig. 8A,B), whereas anti-Tn32 produced consistent staining of the most peripheral articular chondrocytes and fibrocartilage (Fig. 8C,D). Both anti-Tn26 and anti-Tn32 stained the meniscus and the cruciate ligament (Figs. 8A-D). With regard to perichondrial tissue, the inner layer (IL) (characterized by variously shaped cells and very scarce matrix) was strongly stained by both anti-Tn26 and anti-Tn32; in contrast, the outer layer (OL) (with a string-like appearance) stained weakly and only with anti-Tn32 (Fig. 9C-F). Both layers were stained by the M1 antibody (Fig. 9A,B).

The overall results obtained with the various tenascin antibodies are summarized in Table 1. We found that the bulk of avian articular cartilage contains the

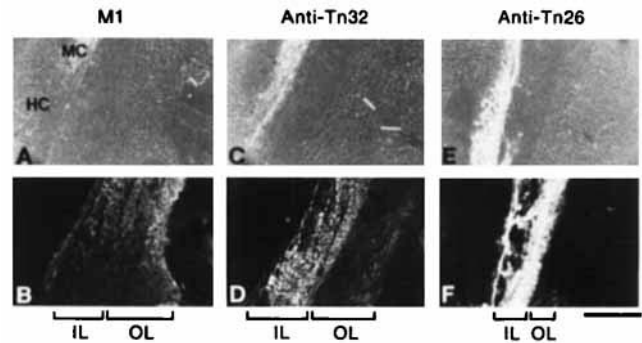


Fig. 9. Tenascin distribution in perichondrial tissue. Phase (A,C,E) and immunofluorescence (B,D,F) micrographs of longitudinal sections of Day 19 chick embryo tibial growth plate and perichondrial tissue. A,B: M1 antibody stains both inner layer (IL) and outer layer (OL) of perichondrial tissue. C,D: Anti-Tn32 stains IL and, to a lesser extent, OL. E,F: Anti-Tn26 stains only the IL. HC, hypertrophic cartilage; MC, mineralized cartilage. Bar, 130 μ m.

TABLE 1. Distribution of Tenascin Isoforms in Chick Tissues

Tissue ^a	Tn190	Tn200	Tn230
Meniscus	+	+	+
Ligaments	+	+	+
Fibrocartilage	+	+	-
Peripheral A.C.	+	+	-
Bulk of A.C.	+	-	-
Secondary O.C.	-	-	-
Inner P.T.	+	+	+
Outer P.T.	+	±	-

^aA.C., articular cartilage; O.C., ossification center; P.T., perichondrial tissue.

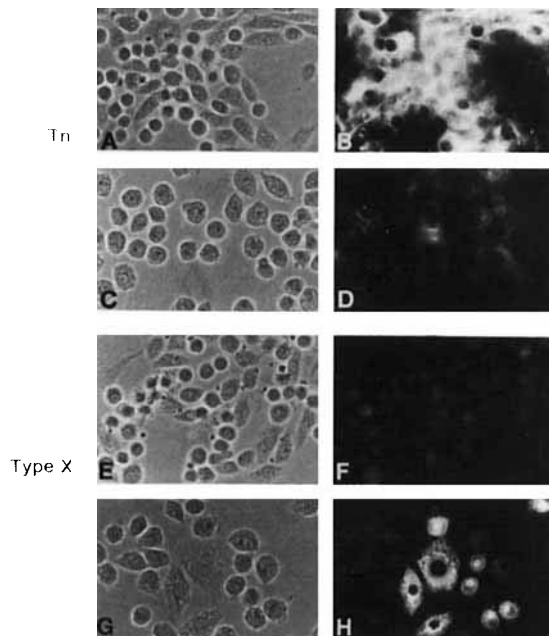


Fig. 10. Tenascin in cultured chondrocytes. Phase (A,C,E,G) and immunofluorescence (B,D,F,H) micrographs of cultured articular chondrocytes stained with the M1 antibody (Tn) or type X collagen antiserum. A,B: 5-day-old primary chondrocytes exhibiting abundant pericellular tenascin. C,D: 4-week-old 3rd-passage chondrocytes showing significantly reduced levels of tenascin. E,F: 5-day-old chondrocytes negative for type X collagen. G,H: 4-week-old chondrocytes several of which are type X collagen-positive. Bar, 35 μ m.

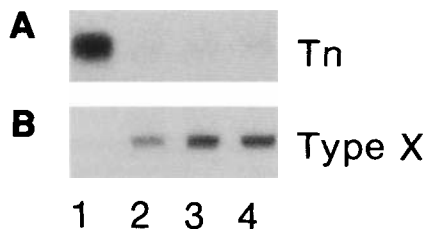


Fig. 11. Northern blot analyses of cultured chondrocytes. Autoradiograms show the steady-state levels of mRNAs encoding (A) tenascin and (B) type X collagen in articular chondrocyte cultures grown for 5 days (lane 1), 2 weeks (lane 2), 3 weeks (lane 3), and 4 weeks (lane 4).

smallest isoform of tenascin, Tn190, whereas the peripheral articular chondrocytes adjacent to fibrocartilage, fibrocartilage itself and the outer layer of perichondrial tissue contain both Tn190 and the intermediate form of tenascin, Tn200. Tn230 was instead found only in meniscus, joint ligaments and the inner layer of perichondrium; thus, these three tissues probably contain all isoforms of tenascin.

Tenascin Gene Expression in Cultured Articular Chondrocytes

We showed previously that when 2-month-old chicken articular chondrocytes are grown in culture for

3 to 5 weeks, they are induced to initiate the process of maturation and give rise to hypertrophic, type X collagen-producing cells (Pacifci et al., 1991a). Thus, we studied the pattern of tenascin gene expression in similar cultures and its relationship to the maturation process. Chondrocytes isolated from 2-month-old chicken proximal tibial articular plateau were grown for up to 4 weeks in standard culture conditions. Immunofluorescence staining with the M1 antibody revealed an abundant tenascin-rich pericellular matrix in 5-day-old primary cultures, particularly around the round-shaped chondrocytes (Fig. 10A,B). Staining, however, was markedly reduced in 4-week-old, third-passage cultures (Fig. 10C,D). An opposite pattern of staining was obtained with type X collagen antiserum. None of the 5-day-old cells stained positively with the type X collagen antiserum, indicating that the cells were still immature (Fig. 10E,F), whereas several of the 4-week-old chondrocytes did so and also exhibited increased cell size (Fig. 10G,H).

To verify these morphological observations, whole cellular RNAs were isolated from companion 5-day-, 2-week-, 3-week-, and 4-week-old cultures and processed for northern blot analysis. Clearly, the steady-state levels of tenascin mRNA decreased with increasing age of the cultures whereas type X collagen mRNA levels increased (Fig. 11).

Tenascin Distribution During Articular Cartilage Development in Mouse

In a final set of experiments, we determined whether tenascin also participates in the development of mammalian articular cartilage. Longitudinal sections of proximal femoral epiphysis from newborn, 1-month- and 4-month-old mice were stained with a mouse tenascin antiserum. We found that, as in chick, tenascin immunostaining was strong around the most epiphyseal chondrocytes in newborn mice but very weak in underlying growth plate (Fig. 12A,B). In 1-month-old animals, tenascin immunostaining characterized the thin layer of articular chondrocytes at the femoral head (Fig. 12C,D). However, reduced levels of staining were observed with increasing age (Fig. 12E,F); although our standard de-masking procedure was also used in these experiments, we cannot exclude that this decrease reflected at least in part antigen masking. At each developmental stage, strong staining was present in perichondrial tissue, meniscus and joint ligaments (not shown).

DISCUSSION

The results of our study indicate that tenascin is closely associated with the development of articular cartilage. We find that in the early chick embryo the protein is first restricted to the most epiphyseal but ill-defined portion of long bone cartilaginous models in limb bud (Chiquet and Fambrough, 1984). As development continues and the articular cap becomes morphologically and phenotypically distinguishable from the

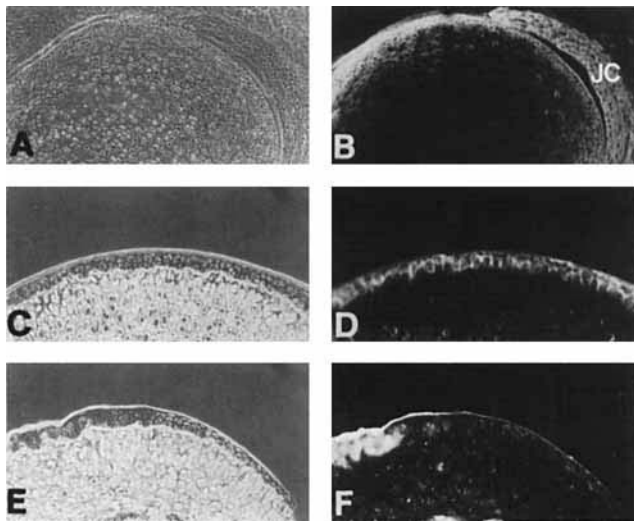


Fig. 12. Distribution of tenascin in developing mouse joint. Phase (A,C,E) and immunofluorescence micrographs (B,D,F) of longitudinal sections through proximal femoral epiphysis of mice of different ages. A,B: Newborn mouse. Note that tenascin is abundant in the matrix surrounding the most epiphyseal femoral chondrocytes but markedly reduced in the underlying growth plate cartilage. The protein is also present in the joint capsule (JC). C,D: 1-month-old mouse. Tenascin is present in the definitive articular cartilage. E,F: 4-month-old mouse. Tenascin immunostaining of articular cartilage appears reduced and not uniform. Bar, 140 μ m.

underlying growth plate, tenascin characterizes and is homogeneously distributed throughout the cap itself but is absent in the growth plate. The absence of detectable tenascin in growth plate correlates well with similar data in previous studies (Chiquet and Fambrough, 1984; Mackie et al., 1987; Hoffman et al., 1988; Prieto et al., 1990; Mackie and Tucker, 1992). We also find that tenascin persists as a component of functional articular cartilage in newborn and juvenile chicken and mice but its levels appear to decrease with age.

The presence of tenascin in developing articular cartilage and its absence from the growth plate suggest that there is an inverse relationship between the process of chondrocyte maturation during endochondral ossification and tenascin gene expression. They also indicate that tenascin characterizes chondrocytes which persist throughout life (permanent chondrocytes) but is not a major gene product of growth plate chondrocytes destined to be replaced by bone (transient chondrocytes). These conclusions are sustained by our finding that tenascin is present in another permanent cartilage, i.e., tracheal rings, in both chick embryo and postnatal animal. They are also sustained by our observations that tenascin decreases in the area of the articular cap in which a secondary ossification center forms, and that tenascin gene expression is down-regulated during long-term culture and maturation of avian articular chondrocytes.

Our proposal that tenascin characterizes permanent

chondrocytes appears to be at variance with conclusions reached in both earlier and most recent studies (Chiquet and Fambrough, 1984; Mackie et al., 1987; Hoffman et al., 1988; Prieto et al., 1990; Mackie and Tucker, 1992). In all those studies, tenascin was proposed to characterize only "poorly-differentiated," "immature" chondrocytes and to be no longer a product of "fully-differentiated," "mature" chondrocytes. Regrettably, these and similar terms were never defined in those studies and were never assigned to defined stages of chondrocyte development or specific subpopulations of chondrocytes; consequently, they remain rather ambiguous and possibly misleading. Despite this limitation, it is clear that the most-epiphyseal tenascin-rich chondrocytes in long bone models were categorized as poorly-differentiated immature chondrocytes whereas the more central, diaphyseal, tenascin-negative cells were viewed as fully differentiated cells. As reiterated in our study, however, the most-epiphyseal cells represent developing articular chondrocytes (Lutfi, 1974; Howlett, 1979). Because these cells persist throughout life, perform highly specialized functions and, as shown here, are tenascin-rich, it follows that tenascin does in fact characterize also fully differentiated, functional chondrocytes and, in particular, permanent chondrocytes.

The bulk of avian articular cartilage exhibits the shortest isoform of tenascin, Tn190, an isoform also found in early vertebral cartilage in Day 7–10 chick embryos (Prieto et al., 1990; Tucker, 1993). In addition, we demonstrate that the more peripheral articular chondrocytes and the overlaying fibrocartilage share the intermediate variant, Tn200, whereas the inner layer of perichondrial tissue contains the largest variant, Tn230. Such exquisite differential distribution of tenascin variants suggests that the different forms of tenascin may serve different functions. By being associated with permanent chondrocytes, Tn190 and Tn200 may serve with other matrix components and factors to maintain the cells in a stable phenotype, prevent their maturation, and allow them to persist throughout life. On the other hand, by being associated with the inner layer of perichondrial tissue, Tn230 may have a role in the differentiation of perichondrial mesenchymal cells into chondrocytes and, thus, may be involved in appositional growth of the cartilaginous model. Indeed, differentiation in culture of chick embryo limb mesenchymal cells into chondrocytes is stimulated by exogenous tenascin added to the medium (Mackie et al., 1987) or present on the substrate (Chuong et al., 1993), whereas treatment of similar cultures with M1 antibody inhibits it (Brooks and Tanzer, 1992; Chuong et al., 1993).

How can Tn190 and Tn200 aid in maintaining the stable phenotype of articular chondrocytes? One possibility is suggested by the very different morphology of articular and growth plate chondrocytes. The articular cells are quite round in shape whereas the chondrocytes in the top zones of growth plate are flat to irregularly shaped. Tenascin has been shown to prevent at-

tachment and spreading of cells in culture and, as a consequence, to maintain the cells in a round configuration (Erickson and Bourdon, 1989; Lightner and Erickson, 1990; Chiquet-Erismann et al., 1988). Thus, tenascin could exert a similar role in articular cartilage and help the cells retain a round shape; this cell configuration is known to favor a stable phenotype in chondrocytes (Benya and Shaffer, 1982; Pacifici and Oettinger, 1985). On the other hand, the absence of tenascin in the growth plate, particularly in the early zones, would be associated with flattening of the cells, rapid cell proliferation, and initiation of the maturation process.

A second possibility relates to the fact that integrins have been identified as receptors for tenascin (Mendler et al., 1991). Signal transduction mechanisms triggered by tenascin-integrin interactions at the articular chondrocyte's cell surface could lead to the differential behavior of these cells compared to tenascin-free chondrocytes in the growth plate. One last possibility is suggested by the ability of tenascin to bind heparan sulfate proteoglycan (Salmivirta et al., 1991). This proteoglycan is a cell surface component and serves as a receptor for important factors and cues, such as basic fibroblast growth factor (bFGF) (Moscatelli, 1987). Both articular and growth plate chondrocytes in culture are exquisitely sensitive to bFGF treatment (Iwamoto et al., 1991), and both articular and growth plate cartilage contains bFGF (our unpublished observations). By binding to heparan sulfate proteoglycan, tenascin could affect the interactions between this receptor and bFGF (or other factors) at the cell surface and, as a consequence, modulate chondrocyte behavior and developmental fate.

While tenascin is abundant in embryonic and neonatal articular and tracheal cartilage, it appears to decrease with postnatal age. We interpret this finding to indicate that tenascin may be particularly important during the embryonic development of permanent cartilage. However, once the fate and phenotypic properties of this tissue are established, tenascin-dependent mechanisms may become less important to maintain the phenotype of the cells for the remainder of postnatal life. Alternatively, other matrix proteins may replace or supplement tenascin action. This latter possibility is broadly sustained by a recent report by Saga et al. (1992). The authors found that transgenic mice with a disrupted tenascin gene and no tenascin production develop normally. They propose that tenascin-like molecules, such as tenascin-MHC or restrictin (Matsumoto et al., 1992; Norenberg et al., 1992), may compensate for tenascin and allow for normal development. Genes which are of fundamental importance to development usually have redundant mechanisms (Matsumura et al., 1992) and this may indeed be the case for tenascin. In their study, Saga et al. did not determine whether articular cartilage develops normally in the tenascin-free transgenic mice. If it does, it will be of interest to determine which tenascin-like molecules compensate

for tenascin during articular cartilage development and whether articular cartilage, meniscus and associated tissues are able to maintain a normal phenotype and function throughout postnatal life or may actually be more prone to disease with age.

EXPERIMENTAL PROCEDURES

Immunohistochemistry

This procedure was carried out as described previously (Oettinger et al., 1985; Pacifici et al., 1990). Hind limbs isolated from chick embryos, chickens and mice were embedded in Tissue-Tek II. Longitudinal 8 μ m-thick frozen sections were mounted on gelatin-coated glass coverslips and fixed for 2 min with 2% paraformaldehyde in phosphate buffered saline (PBS). To remove matrix-associated proteoglycans which may mask antigenic determinants, sections from embryonic tissue were treated with 5 mg/ml hyaluronidase for 1 hr at 37°C (Schmid and Linsenmayer, 1985) in the presence of 10% normal bovine serum; sections from postnatal tissue were instead treated for 3 to 16 hr because they contained a much more abundant extracellular matrix. Sections were first incubated for 60 min in 10% normal goat serum in PBS (blocking solution) to minimize nonspecific antibody binding and then overnight with appropriate dilutions of primary antibody in blocking solution at room temperature in a humid chamber. Sections were rinsed for 30 min with blocking solution and were exposed to a 1:250 dilution of rhodamine-conjugated secondary antibodies (Cappel) for 60 min; after rinsing, sections were mounted in 60% glycerol and viewed under epifluorescence. The primary antibodies used were: the monoclonal antibody M1 (Chiquet and Fambrough, 1984) to chick tenascin obtained from the Developmental Studies Hybridoma Bank; the monoclonal antibodies anti-Tn26 and anti-Tn32 to chick tenascin variants (Spring et al., 1989), kindly provided by Dr. Ruth Chiquet-Ehrismann, Friedrich Miescher Institut, Switzerland; the rabbit antiserum to mouse tenascin, kindly provided by Drs. T. Sakakura and M. Kusakabe, Tsukuba Life Science Center, Japan; the rabbit antiserum to chick cartilage aggrecan (Pacifici et al., 1983); and the rabbit antiserum to chick type X collagen (Pacifici et al., 1991b).

Chondrocyte Cultures

Cultures of adult articular chondrocytes were prepared as detailed previously (Pacifici et al., 1991a). Proximal tibial articular caps isolated from 2-month-old chickens were first cut longitudinally into two equal portions. The one-half portion containing the secondary ossification center was discarded, while the other portion (that includes the tibial articular plateau) was used to isolate chondrocytes. Under a dissecting scope, the bulk of articular cartilage was surgically separated from the overlaying layer of fibrocartilage; in addition, the bottom part of the tissue, adjacent to

the growth plate, was shaved off and discarded. The resulting pure articular cartilage tissue was finely minced and incubated overnight at 37°C in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 0.25% trypsin, 0.1% collagenase and antibiotics. The released cells were separated from tissue fragments by filtration through 20 µm Nitex, recovered by centrifugation, suspended in complete medium (see below), and counted in a hemacytometer. Cells were plated at 7.5×10^5 cells/35 mm tissue culture dish and 7.5×10^6 cells/100 mm dish and grown in primary monolayer cultures for 5 days. Cultures on 35 mm dishes were processed for immunofluorescence with tenascin antibodies or type X collagen antiserum as described (Pacifci et al., 1991a) whereas a portion of the cultures on 100 mm dishes were used for RNA isolation. The remaining cultures were subcultured into secondary cultures after detachment of the cells by treatment for 5 min at 37°C with 0.1% trypsin/0.02% EDTA. Cells were maintained for a total of 4 weeks with weekly subculturing. Floating chondrocytes appearing in the cultures were harvested from the medium by centrifugation and plated back onto their original dishes; this procedure was carried out at each feeding. Immunofluorescence and RNA isolation were repeated with 2-, 3-, and 4-week-old cultures. The complete medium used consisted of high glucose DMEM (Gibco), containing 10% defined fetal bovine serum (Hyclone), 2 mM L-glutamine, 50 U/ml each of penicillin and streptomycin, and 10 µg/ml gentamycin (Pacifci et al., 1983).

For autoradiography, Day 19–20 chick embryo tibias were organ-cultured for 3 hr in serum-free DMEM in the presence of 50 µCi/ml [³H]thymidine (80 Ci/mmol; Amersham). After rinsing, the tissue was quickly frozen and longitudinal sections were processed for autoradiography with Kodak NTB-2 emulsion as described previously (Pacifci et al., 1983).

RNA Isolation and Analysis

Whole cellular RNAs were isolated from chondrocyte cultures by the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987) as detailed previously (Pacifci et al., 1991b). For RNA isolation from tissue, the distal one-half of femur was microscopically dissected from Day 19 chick embryos. The thin layer of fibrocartilage attached to the articular cartilage and the perichondrium surrounding the growth plate were shaved off with a scalpel and discarded. The articular cap was then separated from the underlying growth plate, and RNA was isolated directly from the two tissues by the method of Smale and Sasse (1992) or after enzymatic digestion of the tissues into single cell suspensions by the method above. RNA samples were denatured by glyoxalation, electrophoresed on agarose gels, transferred to Hybond-N membranes (Amersham) by capillary blotting, and hybridized to nick-translated [³²P]-

labeled cDNA probes. Blots were washed at high stringency ($0.1 \times$ SSPE at 65°C) and exposed to Kodak films at -70°C for various lengths of time; quantitative analyses were carried out by video digitization. Blots were stripped at 90°C in $0.05 \times$ SSPE for 5 min and rehybridized to an 18S ribosomal RNA probe (Wilson et al., 1982) to control for variations in RNA concentrations, gel loading, and transfer efficiency. The plasmids used were the chick type X collagen cDNA pDLr10 (Leboy et al., 1988), a 197-bp subclone of pYN3116 (Ninomiya et al., 1986); pCTN230, a full length chick tenascin cDNA clone (Spring et al., 1989); pCPG.1, a plasmid containing a 1.6 kb cDNA coding for a portion of the C-terminal end of chick cartilage aggrecan (Oettinger and Pacifci, 1990); pCs2, a plasmid containing a 1.2 kb cDNA representing a portion of the 3' end of chicken $\alpha 1(\text{II})$ collagen (Young et al., 1984); and pYN1738, a plasmid containing a 3.3 kb cDNA encoding a portion of chicken $\alpha 1(\text{IX})$ collagen (Ninomiya and Olsen, 1984).

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