The recessive mutation nanomelia blocks the synthesis of a large aggregating proteoglycan (aggrecan) by avian embryo chondrocytes. Lack of aggrecan is associated with short stature, multiple morphological defects in cartilage, and embryo lethality. Bony defects have also been described, but were assumed to be a secondary consequence of the cartilage defect. However, two lines of evidence presented in this paper indicate that the aggrecan deficiency directly affects intramembranous bone. First, the morphology (i.e. projected area and shape) of certain membranous bones of nanomelia embryos was abnormal. Second, membranous bone from nanomelia embryos proved to be significantly stiffer in biomechanical tests that measured functional properties of the extracellular matrix. These findings were unexpected because intramembranous bones normally develop from mesenchyme and not from a cartilage intermediate, and they prompted a search for evidence of aggrecan expression in the bone of normal chick embryos. We report that: 1) aggrecan mRNA was identified by PCR analysis of total RNA isolated from day-13 chick embryo calvarium, 2) the PCR method successfully amplified aggrecan mRNA from primary chick embryo osteoblasts in culture, 3) in situ hybridization of membranous bone tissue sections demonstrated aggrecan expression by chick embryo osteoblasts in vitro, and 4) the aggrecan message was identified in Northern blots of calvarial mRNA probed at high stringency. The results of the molecular and biomechanical studies provide evidence that aggrecan is indeed expressed in membranous bone as well as cartilage. Altogether, these results suggest that aggrecan may contribute to the functional properties and the normal growth and development of avian membranous bone.

nanomelia is a recessive, embryonic lethal mutation that was discovered in the F2 generation of a cross between Buff Cochin and White Leghorn chickens (Landauer, 1965). Affected embryos have a parrot-like beak, marked short stature, and multiple morphological defects in cartilage. Nanomelia chondrocytes fail to produce a major chondroitin- and keratan sulfate proteoglycan (aggrecan) that normally interacts with hyaluronic acid and link protein in the cartilage extracellular matrix (Pennypacker and Goetinck, 1976). In the sternum, cell number per cartilage rudiment is normal but the proteoglycan deficiency is reflected in a higher cell density per unit area. Histological studies have also revealed marked disorganization of the growth plate, and the extreme short stature of affected embryos may be a direct consequence of this disorganization. Nanomelia chondrocytes are able to synthesize chondroitin sulfate glycosaminoglycans, but produce only a small amount of the aggrecan core protein mRNA (Stirpe et al., 1987, Goetinck, 1988). Although not yet characterized at the molecular level, the mutation may affect transcriptional regulation of the aggrecan core protein gene. Alternatively, the nanomelia mutation may result in a truncated form of the core protein (O'Donnell et al., 1988).

Defects in the bones of nanomelia embryos have been described, but were thought to arise as a secondary consequence of the cartilage defect. To our surprise, however, membranous bone from nanomelia embryos was found to be significantly stiffer in biomechanical tests that measured functional properties. In addition, the palate and parasphenoid membranous bones of nanomelia embryos were significantly smaller than normal, age-matched counterparts. These functional and morphological defects were unexpected because membranous bone develops directly from mesenchyme and not from a cartilage intermediate. They suggested that aggrecan expression may be more widespread in the skeleton than previously thought. To investigate this possibility, we sought both in vivo and in vitro evidence of aggrecan expression by membranous bone cells of normal chick embryos. We found that aggrecan core protein mRNA could be amplified from the calvarium of normal day-13 chick embryos. In addition, aggrecan expression was detected in chick embryo osteoblasts in vivo using in situ hybridization techniques and in vitro by amplification of RNA isolated from primary bone cells in culture. Finally, a 7.2-9.0-kilobase mRNA species was identified in Northern blots of calvarial mRNA probed at high stringency with an avian probe specific for the aggrecan core protein. These results indicate that the aggrecan core protein is indeed expressed by normal chick embryo bone cells. Aggrecan (cartilage proteoglycan) is known to make an important contribution to the functional properties of cartilage (Mow, 1989). Nanomelia embryos provide an opportunity to study how aggrecan also contributes to the normal development and functional properties of membranous bone.
**Materials and methods**

Detailed methods for much of the work presented here can be found in previous publications. Specifically, methods for preparation of total RNA and DNA, the polymerase chain reaction (PCR) preparation of cDNA probes by the random priming method, M13 cloning, and DNA sequence analysis are described in Patterson et al. (1989). Methods for culture of primary chick embryo osteoblasts and in situ hybridization are described in Majumdar et al. (1991). Mutant embryos, derived from matings between parents heterozygous for the recessive lethal gene nanomelia, were obtained through the courtesy of Dr. Louis J. Pierro, The University of Connecticut, Storrs, CT. 

**Northern Blots—**PolyA-enriched RNA was prepared separately from the skeleton of normal and nanomelia chick embryos on day 14 of development. Samples were quantified by spectrophotometry. Two μg of mRNA were loaded per lane, electrophoresed on a 1.2% agarose/2.2 M formaldehyde gel, and transferred to a nylon membrane (Hybond-N, Amersham). The RNA was cross-linked to the membrane by exposure to a UV light source for 4 min. The membrane was prehybridized for 2.5 h at 65 °C in a buffer solution containing 6 × SSC, 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate, and 0.6 mg of denatured, depurinated salmon sperm DNA. Radiolabeled cDNA probes were generated by random hexanucleotide labeling of a 0.6 mg of denatured, depurinated salmon sperm DNA. Radiolabeled probes were then placed against x-ray film (X-Omat, Kodak) for the indicated changes of 2 × SSC/O.1% sodium dodecyl sulfate at 65 °C. The blot was washed for 30 min at room temperature.

**Hybridization was performed for 16 h at 65 °C. The blot was washed for 2.5 h in three times at −86 °C. Even loading and transfer of mRNA samples was checked by rehybridization of the Northern blot with a 600-bp avian G3 domain of the avian aggregan core protein transcript (Tanaka et al., 1988). Hybridization was performed for 16 h at 65 °C. The blot was washed for 2.5 h in three changes of 2 × SSC/0.1% sodium dodecyl sulfate at 65 °C. The blot was washed for 30 min at room temperature. The method described above. Microspecimens were machined from bone specimens using a micromilling technique described by Choi et al. (1990). The average dimension of a microspecimen was 1500 × 200 × 200 μm. One microspecimen was obtained from each palatine bone, and a uniform specimen orientation relative to the anatomical axis was maintained. The base and height of each specimen were measured with a 25 μm, 50X objective using a video-based image-processing system directly interfaced with a light microscope. Irregular specimens and those that were visibly damaged by the machining process were eliminated from the study. A total of 20 normal and 16 nanomelia beam specimens were tested. The remaining specimens were eliminated for reasons described above. Microspecimens were loaded under four-point bending at a constant displacement rate of 0.1 mm/s until failure. Load and deflection data were acquired using the Labview data acquisition program running on a Macintosh II computer. From these data, an effective modulus (gigapascals) was calculated for each specimen. A Student's t test was performed to test for significant differences between the two groups. After testing, each microspecimen was decalcified for 6 h in 5% formic acid and embedded in paraffin. A 10-μm transverse section from each specimen was stained with hematoxylin and eosin. Five microspecimens were lost or damaged during this procedure. A two-dimensional image of each microspecimen was acquired using a video-based image analysis system, and the microarchitectures of each image was evaluated on the basis of bone area fraction, trabecular plate number, and trabecular plate thickness (Weibel, 1979). Bone area fraction was determined by highlighting the largest possible rectangle within the transverse section. The area of the rectangle occupied by bone tissue was then determined using the thresholding option of the software. Trabecular plate number (counts per μm²) was calculated by placing parallel lines over the image of the section and counting the number of times a grid line entered or exited bone tissue. This was done in two perpendicular directions. Trabecular plate thickness was calculated as the ratio of bone area fraction/trabecular plate number.  

**Image Analysis—**Images of four normal and four nanomelia palatine bones (lying flat) were captured with a MTI series 68 video camera (Dage-MTI Inc, Michigan City, IN) and a DT2828 series single board analog and digital I/O system (Data Translation, Inc., Marlborough, MA). The NIH Image Analysis program (Wayne Rasband, National Institutes of Health, Research Services Branch, National Institute of Mental Health) was used to calculate the two-dimensional projected area of each bone. A Student's t test was used to test for significant differences between the two groups. The procedure was repeated for the parasphenoid, frontal, and squamosal bones. To illustrate differences in shape, a representative bone from each group was traced, and the image of the smaller palatine bone was scaled to have an area equivalent to the normal bone.

**RESULTS**

**Morphological and Functional Defects in Membranous Bone of Nanomelia Embryos—**Some membranous bones from nanomelia embryos were significantly smaller than normal (Fig. 1). The total projected area of the normal palatine bone was 37% greater than its counterpart from the nanomelia embryo. Similarly, for the normal paraspheonid bone the total projected area was 96% greater than its mutant counterpart. Other membranous bones such as the frontal plate and squamosal bones showed no significant difference in projected area.

Image analysis was used to compare the shape of mutant and normal membranous bones from nanomelia embryos to those from normal counterparts. An obvious change in shape was associated with the mutation in the case of the palatine bone (Fig. 2). This result demonstrated that the mutant palatine bone was not simply a smaller version of normal. Similarly, the frontal and squamosal bones from nanomelia embryos showed obvious shape changes despite the fact that their areas were equivalent to normal. The paraspheonid bone differed from its normal counterpart primarily in size, but not in shape.

The mechanical properties of normal and nanomelia membranous bone were measured to determine if the observed differences in morphology were associated with differences in functional properties (Fig. 3). Microspecimens machined from the palatine bones of nanomelia embryos had a 30% higher effective modulus (p = 0.003) compared to age- and site-matched specimens machined from normal palatine bone.

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1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s).
2 Choi, K., and Goldstein, S. A. (1992) *J. Biomech.*, in press.

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**FIG. 1.** Size of membranous bones from day-15.5 normal and nanomelia embryos. The projected area of nanomelia palatine and paraspheonid bones was significantly smaller (about 35% less in both cases) than normal. Upper left, normal palatine bone (top) and nanomelia palatine bone (bottom); upper right, normal paraspheonid bone (left) and nanomelia paraspheonid bone (right). The nanomelia squamosal and frontal plate bones were not significantly different from normal. Lower left, normal squamosal bone (left) and nanomelia squamosal bone (right); lower right, normal frontal plate bone (left) and nanomelia frontal plate bone (right). A millimeter scale is located at the bottom of each figure.
Aggrecan Expression in Membranous Bone

Aggrecan Expression in Membranous Bone

FIG. 2. Shape of membranous bones from day-15.5 normal (shaded) and nanomelia embryos. For this comparison, the area of the nanomelia bones has been scaled by the number shown so that the shape of bones with equivalent projected areas could be compared. Upper left, the scaled nanomelia palatine bone is significantly shorter and broader relative to normal. Upper right, no obvious difference in shape was noted between the parasphenoid bones. Lower left, for the squamosal bones, the bony protrusion seen in the lower left of the figure was significantly truncated. Lower right, the frontal plate from nanomelia embryos was more curved and shorter as compared to normal.

FIG. 3. Biomechanical alterations in membranous bone of nanomelia embryos. Nanomelia specimens demonstrated a significantly increased apparent modulus ($p = 0.003$) when compared to wild-type specimens. This indicated that the mutant specimens were significantly stiffer (i.e. less compliant) than normal.

This result indicated that the mutant bone tissue was significantly stiffer than normal.

In theory, differences in microstructure could account for all or a significant part of the observed differences in mechanical properties (Kuhn et al., 1989). To determine if this was the case, three aspects of microstructure were quantified, e.g. bone area fraction, trabecular plate number, and trabecular plate thickness. A transverse cross-section of the normal and nanomelic palatine bones is shown in Fig. 4A. Nanomelia microspecimens tended to have a higher bone area fraction and a lower trabecular plate number compared to normal microspecimens (Fig. 4B). These differences were not statistically significant. On the other hand, the trabecular plate thickness of nanomelia microspecimens was significantly greater than normal ($p = 0.02$). However, there was no correlation between any of these stereological parameters and the apparent tissue modulus (data not shown).

Aggrecan Is Expressed by Membranous Bone Cells of Normal Avian Embryos—The occurrence of morphological and functional defects in the membranous bones of nanomelia embryos suggested that aggrecan expression may be more widespread in the avian skeleton than previously thought. To investigate this possibility, we sought both in vivo and in vitro evidence of aggrecan expression by normal chick embryo bone cells. Two regions of the avian aggrecan core protein sequence, encoding the G3 and CS-2 domains of the protein, have been reported (Tanaka et al., 1988, and Krueger et al., 1990, respectively). Both regions were successfully amplified from total RNA isolated from day-14 chick embryo calvarium (frontal plate bones, Fig. 5, A and B). In each case, PCR fragments were identified by DNA sequence analysis, and the DNA sequence we obtained was identical to the previously reported sequence (not shown). Total RNA isolated from the femur of day-14 embryos served as a positive control given that long bone at this stage of development contains abundant epiphyseal cartilage (Romanoff, 1960). Water served as the negative control. A previous study has demonstrated that the frontal plate fragments used here do not contain cartilage (Majmudar et al., 1991).

FIG. 6 demonstrates that the G3 domain of the aggrecan core protein message could be amplified from a sample of total RNA harvested from primary chick embryo calvarial osteoblasts (Majmudar et al., 1991). The CS-2 domain was also amplified from this RNA sample (not shown).
Aggrecan Expression in Membranous Bone

Fig. 5. Aggrecan expression by membranous bone cells of wild-type embryos. PCR amplification of mRNA sequences encoding a portion of the G3 and CS-2 domains of chicken aggrecan is shown in A. The G3 amplification product is a 614-bp fragment. PCR primers were based on sequence information originally reported by Tanaka et al. (1988). A nested PCR reaction was performed. The sequence of the primers was as follows: 5'-TGTCTAGGAAGCTGTGCTAG-3' (upstream outer); 5'-TGTGCACCCCTGGCGCCCG-3' (downstream outer); 5'-GAATTCCGCTACCAAGACATCTTT-3' (upstream inner); 5'-GTCGACGCTGTGTCAGACAGC-3' (downstream inner). The CS-2 sequence was a 630 bp amplification product. PCR primers were based on sequence information originally reported by Krueger et al. (1990). Again, a nested PCR reaction was performed. The sequence of the primers is as follows: 5'-ATTCTAGAGATCAGTGGACTG-3' (upstream outer); 5'-TGTGAGGAAGGCTGTTTCTATACCACCTTCTGC-3' (downstream outer); 5'-GAATTCACTGCTACAGGCACTTTT-3' (upstream inner); 5'-GTCGACGGTCTGTGCACGACAC-3' (downstream outer); 5'-TGCTCACCCCTCGCCCCG-3' (upstream outer); 5'-TGTTGAGGAAGGCTGTGCTAG-3' (downstream inner). The identity of all PCR products was determined by DNA sequence analysis of the entire fragment. Note that the PCR product includes sequence of the S103L epitope as described by Krueger et al. (1990). Also note that for both PCR reactions, the femur lane represents a positive control (the epiphysis is filled with cartilage at this stage of development) and the water lane represents a negative control.

Fig. 6. PCR amplification of mRNA sequence encoding a portion of the G3 domain of primary wild-type chick embryo bone cells in culture. The G3 primers described above were used for this PCR reaction. The PCR reaction product from osteoblasts is shown next to an aliquot of the membranous bone sample shown in Fig. 5.

Using biotinylated CDNA probes specific for the G3 domain. As shown in Fig. 7A, a positive hybridization reaction for the core protein mRNA was observed in the cytoplasm of osteoblasts lining the surface of bone and within lacunar spaces. The cytoplasm of epiphyseal chondrocytes from long bone (femur) of day-13 chick embryos hybridized with the G3 probe. In contrast to these results, in situ hybridization of consecutive sections in the paraffin block with a specific type I collagen probe showed positive hybridization in bone cells and negative hybridization in cartilage cells (Fig. 7B). No evidence of positive hybridization was observed in control experiments performed in the absence of probe (not shown). Negative results were also obtained with an irrelevant probe (M13 DNA) labelled with 35S (not shown).

The aggrecan core protein message was also identified in normal embryos on day 14 of development by Northern blot analysis of frontal plate mRNA (Fig. 8, lane 1). A 7.2-9.0-kb mRNA species was specifically hybridized using the G3 PCR fragment as a probe. The size of this bone core protein mRNA is consistent with previous reports of the size of the aggrecan core protein mRNA in cartilage (Krueger et al., 1990). Again, mRNA from day-14 femurs served as a positive control for this experiment (Fig. 8, lane 2). The relative ratio of the aggrecan core protein mRNA in calvarium versus long bone samples was reproduced in one other Northern blot experiment that utilized independently isolated RNA samples (not shown).

Finally, the amount of aggrecan mRNA in membranous bones from normal and nanomelia embryos was also assessed by Northern blotting (Fig. 8, lanes 3 and 4). Aggrecan expression was essentially absent in mRNA prepared from nanomelia membranous bones, a result consistent with previous studies showing decreased aggrecan expression in the cartilage of mutant embryos (Goetinck, 1988).

DISCUSSION

This study has demonstrated that aggrecan mRNA is expressed by osteoblasts in the chick embryo calvarium at day 14 of development and by chick embryo osteoblasts in culture. Nanomelia is an inherited mutation that causes a significant decrease in aggrecan mRNA and protein expression in cartilage (Goetinck, 1988). Given the inherited nature of the defect, it was reasonable to assume that aggrecan mRNA is also decreased in the calvarium of nanomelia embryos. Evidence for this assumption was provided by hybridization of mRNA prepared from membranous bones of normal and mutant embryos. Evidence was also provided in the form of a functional test of the mechanical properties of normal and mutant calvarial bone tissue. The stiffness of mutant bone specimens was significantly increased compared to wild-type, which may reflect the proteoglycan deficit. The inherited defect may also be responsible for significant alterations in the morphology of the calvarium.

Bone may be considered as a nonhomogeneous composite material given that its extracellular matrix is comprised of a highly ordered assembly of collagens, proteoglycans, noncollagenous glycoproteins, and mineral (Robey, 1989). The biomechanical properties of bone reflect the type, relative amount, and arrangement of these constituents within bone tissue (Martin and Burr, 1989). A previous study, using transgenic mice heterozygous for a null mutation that blocks collagen transcription, demonstrated that a decrease in tissue collagen content was associated with a significant decrease in the post-yield behavior of cortical bone (Benadio et al., 1990). This suggested that the mutant bone would be more susceptible to failure with repeated use over time, i.e. would be more
susceptible to "wear and tear" effects, and that type I collagen normally contributes to tissue toughness. The present study extends this work by demonstrating that loss of a bone proteoglycan is associated with a significant increase in bone tissue stiffness. In contrast to collagen, then, the proteoglycan may normally contribute to tissue compliance. Taken together, these results provide a rationale for future studies using animal strains deficient in other matrix constituents. The goal of this work would be to determine the contribution of other matrix molecules to the structure and mechanical properties of skeletal tissue.

The effectively stiffer nature of nanomelia bone specimens may reflect qualitative changes at the level of the extracellular matrix as a result of the aggrecan deficiency. Previous studies which examined the contribution made by aggrecan to cartilage structure and function are relevant in this regard. Aggrecan, the major proteoglycan in cartilage, is a high molecular weight species (est $M_r = 1 \times 10^6$ to $4 \times 10^6$) with a large, extended core protein modified by the addition of $\sim 60$ N- and O-linked oligosaccharides, $\sim 100$ chondroitin sulfate chains, and (depending upon the species) up to 130 keratan sulfate chains (Hassall et al., 1986). Aggrecan forms a ternary complex in the extracellular matrix via noncovalent interactions with hyaluronan and link protein. Typically, many aggrecan molecules bind one hyaluronan chain to form complexes as large as 4,000 nm by 500–600 nm (for reviews, see Barry et al., 1989). The resulting aggregated structure has a high negative charge density, due to the acidic nature of chondroitin- and keratan sulfate glycosaminoglycans. It therefore attracts a large amount of water and may actually occupy a volume of the extracellular matrix 30–50 times its dry weight (Christner et al., 1978). In cartilage, the mechanical properties of the tissue result in large part from proteoglycan-induced tissue swelling under constraint by the fibrillar collagen network (Mow, 1989). The negative fixed charge of glycosaminoglycan groups produces swelling pressures on the order of $0.35$ megapascals which serve to put the fibrillar collagen network under tension (Maroudas, 1979). As cartilage is loaded, interstitial fluid is either exuded or imbibed, and this contributes to time-dependent (viscoelastic) mechanical properties (Mow et al., 1989). One possibility, therefore, is that nanomelia specimens have altered mechanical properties because they contain less water than normal and have a more compact structure.

*nanomelia* appears to affect membranous bone development at two levels. Significant differences were observed in the size...
Fig. 8. Northern blot of mRNA (2 μg/lane) isolated from the skeleton of wild-type and nanomelia chick embryos. A, the Northern blot was probed with a radiolabeled PCR probe specific for the chick aggrecan G3 domain. The blot was exposed to film for 48 h. The result shown in lanes 1 and 2 was reproduced in an independent isolate of total RNA from calvarium and femur (data not shown). Lane 1, mRNA prepared from frontal plate bones of normal embryos; lane 2, mRNA prepared from femur of normal embryos; lane 3, mRNA prepared from membranous bones (including the palatine, parasphenoid, squamosal, and frontal plate bones) of normal embryos; and lane 4, mRNA prepared from membranous bones of nanomelia embryos. B, Northern blot in A reprobed with a radiolabeled PCR fragment encoding the triple helical domain of the avian α1(I) collagen chain. The blot was exposed to film for 1 h.

and/or shape of whole membranous bones from mutant embryos even though the development of these bones does not proceed via a cartilage intermediate (Romanoff, 1960). Aggrecan is thought to function within the extracellular matrix of cartilage as a structural macromolecule (Barry, 1989). The morphological abnormalities observed here may therefore result because the proteoglycan normally plays a similar role in embryonic membranous bone, and its absence leads to a smaller, misshapen structure. It is not clear why the projected areas of certain membranous bones from nanomelia embryos (i.e., the squamosal and frontal plate) were similar to normal when one might expect all bones to be similarly affected. One possibility is that nanomelia is not uniformly expressed in all tissues, resulting in phenotypic variation at sites within the skeleton. Second, a tissue adaptation which compensated for the primary effect of the mutation on bone size may have occurred in the squamosal bone and frontal plate at the time these studies were conducted. Third, alterations in the chondrocranium or in secondary cartilage may have affected the size of certain membranous bones in a selective fashion. Discrimination among these possibilities must await future studies.

Significant differences were also observed in the microarchitectures of primary bone trabeculae of normal and nanomelia palatine bones. These differences are noteworthy because of the documented potential for microstructure to contribute to mechanical properties (Kuhn et al., 1989). However, a strong correlation was not observed between stereological parameters and the tissue modulus values obtained for normal and nanomelia membranous bone microspecimens (data not shown). It therefore appears that microstructural differences did not solely determine the qualitative differences in tissue properties we have observed. This result strongly suggests that the inherent properties of the nanomelia bone matrix were altered in association with the aggrecan deficiency. It thus appears that nanomelia changes aggrecan expression and that this in turn leads to changes in the extracellular matrix, microstructural architecture, and whole bone.

It has become clear recently that the temporal and spatial regulation of extracellular matrix gene expression in vivo is complex. One consequence of this complexity is to expand the number of members in the extracellular matrix gene family. For example, protein variants may be created by transient expression of alternatively spliced forms of mRNA as has been shown for type II collagen (Ryan and Sandell, 1990), type VI collagen (Saitto et al., 1990), and aggrecan (Doeger et al., 1991). In the case of type II collagen, an alternatively spliced version of the protein appears to be expressed in several noncartilaginous tissues during early embryonic development (Kosher and Solursh, 1989; Fitch et al., 1989). Tissue-specific variants may also occur because of the formation of mixed collagen heteropolymers consisting of the subunits of two distinct collagen types (Niyibizi and Eyre, 1989). A molecular basis for the formation of cross-type heteropolymers has not yet been described. The present study extends previous work by providing further evidence that certain matrix genes, once thought to be cell- or tissue-specific, are expressed at low levels in other, noncharacteristic tissues. For example, the link protein that mediates the interaction between aggrecan and hyaluronan is not strictly associated with chondrocytes and cartilage as was previously thought. Link protein gene expression in the absence of aggrecan was recently demonstrated in the developing avian mesenchyme (Stirpe et al., 1990). Similarly, type III collagen and fibronectin were once thought to be excluded from mineralized connective tissues such as bone. However, recent studies have demonstrated the expression of both fibronectin and type III collagen within intramembranous bone of the avian embryo (Majmudar et al., 1991).

The aggrecan core protein mRNA is initially expressed at the onset of chondrogenesis in the chick embryo limb bud, and mRNA production coincides with an increase in expression of the type II collagen, link protein, and cartilage matrix protein genes (Kosher et al., 1986; Stirpe and Goetinck, 1989). Evidence presented here indicates that aggrecan is also expressed by avian embryonic bone cells in vivo and that it plays an important role in normal osteogenesis. Aggrecan therefore may be added to the list of matrix molecules that comprise the phenotype of normal chick embryo osteoblasts. Aggrecan gene expression is likely regulated in a temporal fashion given that this proteoglycan is not synthesized by cultured cells isolated from adult human bone trabeculae (Beresford et al., 1987). An aggrecan-like proteoglycan was isolated from the mesenchyme surrounding fetal membranous bone (Fisher et al., 1983). The molecule was judged to be aggrecan-like on the basis of amino acid composition and immunoreactivity studies. However, this proteoglycan contained larger and fewer chondroitin sulfate side chains than cartilage aggrecan. The relationship between the core protein of this proteoglycan and the protein product encoded by the core protein mRNA studied here remains to be determined.

The results of this study demonstrate that a lack of aggrecan an/d/or a change in aggrecan core protein structure has consequences for the structure, growth, and development of the avian embryo skeleton. It is natural to speculate that mutations in the human aggrecan gene may also be associated with certain of the inherited skeletal disorders. Doeger et al.
(1991) recently reported the complete coding sequence of human cartilage aggrecan and an initial attempt to link the aggrecan gene to familial disorders of cartilage such as achondroplasia and pseudoachondroplasia. For many of these diseases, cartilage is affected as a primary consequence of mutation, whereas bone is affected as a secondary consequence, e.g. endochondral bones may be abnormally short as a result of growth plate failure. The present study suggests that inherited disorders in which cartilage and membranous bone are affected as a primary consequence of mutation may also represent good candidates for linkage studies.

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