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Differential co-expression of long and short form type IX collagen transcripts during avian limb chondrogenesis in ovo

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Summary

Using RNA blot analysis of developmentally staged avian limb buds, we demonstrate that transcripts of several cartilage marker genes appear in limb tissue prior to overt chondrogenesis. Type II collagen mRNA, cartilage proteoglycan core protein mRNA, α2(IX) collagen mRNA,and transcripts of the short form α1(IX) collagen chain derived from the downstream promoter are co-expressed in limb tissue approximately 24 - 36 hours before the appearance of the respective polypeptides in differentiating cartilagenous tissue. Transcripts of the long form α1(IX) collagen chain derived from the upstream promoter appear somewhat later in development; nearly coincident with the immunolocalization of type IX collagen in the cartilage elements of the limb. The spatial distribution of type II and type IX collagen transcripts was analyzed by *in situ* hybridization. Type II collagen and the long form α1(IX) collagen transcripts co-localized in the chondrogenic elements of the developing forelimb. In contrast, short form α1(IX) collagen transcripts which lack the 5′ region encoding the NC4 globular amino-terminal domain were distributed throughout the non-chondrogenic, non-myogenic mesenchymal regions of the limb and were not detectable above background levels in the limb chondrogenic elements. The precocious appearance of several cartilage marker gene transcripts prior to chondrogenesis suggests that multiple levels of gene regulation including alternative promoter use, alternative RNA splicing, alternative polyadenylation, and other post-transcriptional as well as translational mechanisms are active prior to, and during avian limb chondrogenesis.

Key words: type IX collagen, cartilage, extracellular matrix, avian limb.

Introduction

Chondrogenesis involves the synthesis and assembly of a number of distinct extracellular matrix components to form the cartilagenous skeleton with its characteristic physical properties (Heinegard and Paulsson, 1987). While many of these components are known to interact with each other, little is known about the regulation of their accumulation or assembly. The developing limb bud has been a useful system for analysis of chondrogenesis (Solursh, 1986), since several cartilage matrix components accumulate sequentially during normal development (Franzen et al., 1987). The present study represents an additional effort to obtain insights into the developmental significance of the sequential appearance of particular extracellular matrix components, notably type IX collagen.

Our interest in type IX collagen expression during limb chondrogenesis follows the recent discovery that type II and type IX collagen are covalently cross-linked in cartilage and that this association may influence such physical properties as the regulation of type II collagen fibril diameter or interactions between the type II - type IX collagen heterofibrils and other components of the extracellular matrix (reviewed by Gordon and Olsen, 1990). In particular, it is the presence or absence of the type IX collagen NC4 amino-terminal globular domain which appears to influence the interaction of type II/type IX collagen with other matrix components (Nishimura et al., 1989). These observations raise the possibility that the association of type IX collagen with type II collagen fibrils and with other components of the extracellular matrix may influence diverse structural properties of the developing avian limb as well. Type II collagen expression in the limb bud has been well documented by solution hybridization (Kravis and Upholt, 1985), cytoplasmic dot blot hybridization (Kosher et al., 1986a), *in situ* hybridization (Nah et al., 1988; Devlin et al., 1988; Swalla et al., 1988), and immunological analyses (von der Mark et al., 1976; Dessau et al., 1980). While the detection of type IX collagen transcripts has been limited to avian limbs undergoing overt chondrogenesis (Kimura et al., 1988; Kulyk et al., 1991), a broad temporal study of avian limb type IX collagen expression *in vivo* has not been reported. Hence the relationship between type II collagen and type IX collagen expression during early limb cartilage development is not known.

Overt biochemical chondrogenesis in the avian forelimb is characterized by the synthesis of type II

collagen by limb bud mesenchymal cells. Transcripts of type II collagen, a homotrimeric triple helical polypeptide composed of three identical chains [α1 (II)]$_3$, have been detected in wing buds as early as stage 18/19 (3-3 1/2 days *in ovo*); at least 12-24 hours prior to the pre-cartilagenous condensations of mesenchymal cells observed at stage 22 (Fell and Canti, 1934). Initially, type II collagen mRNA is detected at low levels throughout the limb mesenchyme and subsequently is localized to the pre-cartilage condensations by stage 23 (Swalla et al., 1988). The early appearance and localization of the transcripts over the future cartilagenous zones precede immunodetection of type II collagen which occurs at late stage 24 (4 1/2 days, Dessau et al., 1980, Aulthouse and Solursh, 1987). At this time, type II collagen gene transcription is enhanced and α1(II) mRNAs are translationally active (Kravis and Upholt, 1985; Kosher et al., 1986a). Type II collagen is present throughout chondrogenesis until chondrocyte hypertrophy, the onset of type X collagen synthesis (LuValle et al., 1989), and ultimate replacement by bone (Sandberg and Vuorio, 1987).

Type IX collagen is a trimeric protein [α1(IX) α2(IX) a3(IX)] composed of three non-identical polypeptide chains encoded by three separate genes (van der Rest et al., 1985; Mayne et al., 1985). Each chain consists of three triple helical domains (COL I, COL 2, COL 3) interspersed with four non-collagenous domains (NC1, NC2, NC3, NC4) (Ninomiya and Olsen, 1984; van der Rest et al., 1985). The NC3 domain of all three chains is a flexible hinge which allows type IX collagen to project its COL 3 and amino terminal NC4 domain from the surface of type II collagen fibrils into the surrounding perifibrillar space (Vaughn et al., 1988). Attached to the NC3 domain of the α2(IX) collagen subunit is a glycosaminoglycan chain (chondroitin or dermatan sulfate; Irwin et al., 1986, McCormick et al., 1987, and Huber et al., 1988) which may interact with the type II collagen fibril in the heterofibril configuration or project from non-fibril associated type IX collagen for potential interactions with other extracellular matrix molecules.

The presence or absence of the pivotal 266 amino acid NC4 globular domain of the α1(IX) collagen chain is the result of tissue specific alternative promoter use and alternative RNA splicing (Nishimura et al., 1989). While both promoters are active in chick sternal cartilage and cornea (Nishimura et al., 1989) and in whole mouse embryos and in human fetal tissue (Muragaki et al., 1990), the relative abundance of transcripts derived from each promoter is regulated in a tissue-specific manner. In sternal cartilage, the most abundant α1(IX) collagen mRNAs are transcribed using the upstream promoter and generate transcripts which include the NC4 globular domain encoded in exons 1-7. In the sterna, type II collagen fibrils are organized in a parallel array, thus imparting the sternal cartilage with its typical tensile strength. The presence of type IX collagen on the surface of the type II collagen fibrils increases the potential for perifibrillar ECM interaction in this tissue (Nishimura et al., 1989). In the primary corneal stroma, however, the most abundant α1(IX) collagen mRNAs are transcribed using the downstream promoter and a cryptic exon (exon 1*) which is located in the intron between the cartilage form exons 6 and 7. Alternative RNA splicing eliminates exons 1-7 (and hence the 266 amino acid NC4 cartilage domain), giving rise to a truncated α1(IX) collagen.

Although there is no direct evidence for crosslinking between type II and the truncated type IX collagen in the non-cartilagenous primary corneal stroma, both are found in this corneal extracellular matrix. Type I and type II collagen fibrils form orthogonal fibrillar arrays (Hay and Revel, 1969) typical of a transparent visual matrix, while type IX collagen may regulate the compactness of the corneal matrix (Fitch et al., 1988) and may also influence the organization of the vitreous (Yada et al., 1990).

Since α1(IX) collagen transcripts that have the NC4 globular domain and those that lack it are co-expressed in both avian sternal cartilage and cornea (Nishimura et al., 1989), and in developing limb buds as demonstrated herein, we will refer to α1(IX) collagen that possesses the NC4 globular domain as "long form" and that lacks the domain as "short form." In this report, we present new evidence from RNA blot hybridization, *in situ* hybridization, and immunological studies that several cartilage marker gene transcripts, notably type IX collagen, appear in avian limb buds prior to overt chondrogenesis *in ovo*. In conjunction with earlier studies of the sterna and cornea, we provide additional support for a complex regulatory pathway of cartilage marker gene expression during avian chondrogenesis.

Materials and methods

RNA isolation and blot analysis

Total cellular RNA was isolated from White Leghorn chick embryo limb buds (staged according to Hamburger and Hamilton, 1951) and from day-14 embryo caudal sternal cartilage using guanidine thiocyanate (Chomczynski and Sacchi, 1987). Poly(A) RNA was selected by oligo(dT) cellulose chromatography (type 7, Pharmacia), electrophoresed in 0.8% agarose gels containing formaldehyde, and transferred to nitrocellulose (BA-85, Schleicher and Schuell) according to Maniatis et al. (1982). Hybridization probes were gel-purified, nick-translated inserts of the plasmids described below and diagrammed in Fig. 1.

(A) pYN 1738, a 2 kb *Xho*I/*Xba*I cDNA fragment which corresponds to sequences encoding the seven carboxy-terminal amino acids of the chicken α1(IX) collagen COL3 domain through 450 bp of the 3′ untranslated region (Ninomiya and Olsen, 1984; The kind gift of Drs. B R. Olsen and Y. Ninomiya) This cDNA recognizes both long and short form α1(IX) collagen mRNAs.

(B) pIN 321; a 412 bp *Eco*RI/*Eco*RI cDNA fragment which corresponds to sequences encoding the 5′ one-third of the NC4 globular domain of the long-form of chicken α1(IX) collagen (Vasios et al , 1988; The kind gift of Dr I Nishimura). This probe hybridizes solely to long form α1(IX) transcripts.

(C) pIN 212, a 100 bp *Eco*RI/*Apa*I cDNA sub-fragment which corresponds to sequences encoding the 5′ end and a portion of the signal peptide of the short form of chicken α1(IX) collagen (Nishimura et al., 1989, The kind gift of Dr I.

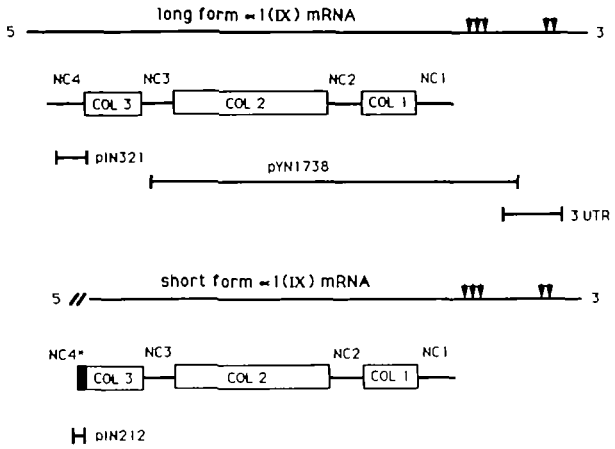

Fig. 1. Diagram of chicken cDNAs encoding the long and short form α1(IX) collagen chains used in this study Arrowheads above the 3′ untranslated region of the mRNA indicate the proximal and distal polyadenylation signal clusters. Non-helical domains of the polypeptide diagrammed below are indicated by lines (NC1, NC2, NC3, NC4) The short form α1(IX) chain *NC4 domain arising from an alternative transcription site located in the intron between long form exons 6 and 7 is indicated by an asterisk (*). Triple-helical domains of the polypeptide (COL1, COL2, COL3) are indicated by boxes. (Adapted from Nishimura et al., 1989).

Nishimura). These sequences make up part of exon 1*, the alternative exon for the 5′ end of the short form α1(IX) collagen which is located in the intron between exons 6 and 7 of the α1(IX) gene (as counted from the 5′ end of the gene). This probe hybridizes solely to short form α1(IX) transcripts.

(D) 3′ UTR; a 600 bp *Xba*I/*Pvu*II restriction fragment from the 3′ untranslated portion of the cDNA insert of pYN 1738. This fragment hybridizes to the 700 bp longer transcript of the long form (4.2/3.5 kb) mRNA doublet and of the short form (3.5 kb/2.8 kb) α1(IX) mRNA doublet by virtue of alternative polyadenylation using the 3′ most distal polyadenylation signals (Svoboda et al., 1988).

(E) pYN 1731; a 1450 bp *Hin*dIII/*Pvu*II cDNA which corresponds to sequences encoding the non-collagenous domain NC1 through part of the collagenous domain COL2 of chicken α2(IX) collagen (Ninomiya et al., 1985; The kind gift of Drs. B. R. Olsen and Y Ninomiya).

(F) pBSCA 12; an 890 bp *Bam*HI/*Pst*I genomic DNA fragment corresponding to sequences encoding the 3′ untranslated region and a portion of the C-propeptide of chicken type II collagen (Nah et al., 1988; The kind gift of Dr. W. B Upholt)

(G) ST-1; a 1 kb *Pst*I/*Eco*RV cDNA which corresponds to the carboxy-terminal end of the chicken cartilage-specific proteoglycan core protein (Sai et al., 1986, The kind gift of Dr. M. L. Tanzer).

Hybridization using the α1(IX) and α2(IX) collagen and cartilage-specific proteoglycan core protein probes was for 16 hours at 42°C in 50% formamide, 5× SSC (SSC is 0.15 M NaCl, 0 015 M sodium citrate), 20 mM sodium phosphate buffer (pH 7.2), 0.02% Denhardt's solution, 0 2% SDS, 100 μg/ml sheared salmon sperm DNA and 10% dextran sulfate. Following hybridization, the filters were washed three times for 15 min. each at room temperature in 2× SSC/0 1% SDS followed by three rinses for 15 min. each at 50°C or 65°C in 0.1× SSC/0.1% SDS. Hybridization using the type II collagen probe was carried out under more stringent conditions to avoid cross-hybridization with other fibrillar collagen transcripts. Hybridization was in 50% formamide, 1× SSC, 50 mM sodium phosphate buffer (pH 7.2), 0.02% Denhardt's solution, 0.2% SDS, 100 μg/ml salmon sperm DNA at 52°C. Washes were carried out at room temperature in 2× SSC/0 1% SDS followed by rinses in 0 1 × SSC/0.1% SDS at 65°C (Nah et al., 1988). Kodak X-Omat XAR-5 film was exposed at −70°C with Dupont Cronex Lightning Plus intensifying screens. RNA length standards (0.4-9 5 kb RNA ladder, Bethesda Research Lab) were used to determine RNA nucleotide lengths. Following autoradiography, nitrocellulose filters were stripped of radioactivity and rehybridized with a ^{32}P-labeled cDNA probe for glyceraldehyde-3-phosphate dehyrogenase (GAPDH; Dugaiczyk et al , 1983; the kind gift of Dr. R. J Schwartz) to confirm that an equivalent mass of RNA was loaded in each lane (data not shown) Densitometric analysis was done using the Dendron program created by SVUI (Iowa City, IA) Autoradiogram images were entered into a Macintosh FX computer using a Sharp JX-450 color scanner followed by averaging of pixel intensities and graphic analysis. The area between the baseline and peaks of the graph were measured and plotted (Fig. 6)

In situ hybridization

Procedures for *in situ* hybridization were carried out according to Angerer et al. (1987) with some modifications Briefly, forelimbs and heads were dissected and fixed at 4°C in Bouins solution (15% picric acid, 5% formalin, 1% glacial acetic acid). Fixed tissues were dehydrated through increasing concentrations of ethanol, cleared in xylenes and embedded in Paraplast (Paraplast Plus, Oxford Labware). Sections of 7 μm were cut and mounted onto TESPA- (aminopropyltriethoxydilane, Aldrich) coated slides and dried for 48 hours at 38°C. Rehydrated sections were treated with Proteinase K, acetylated, and dehydrated through ethanols.

The cDNA inserts of the 5′ end specific probes (plasmids pIN 321 and pIN 212, respectively) were subcloned into the transcription vector pIBI 30 (International Biotechnologies, Inc.) and were subsequently designated pIBIN 321 and pIBIN 212, respectively. Plasmid pIBIN 321 was linearized with *Bam*HI to prepare a cRNA probe using T7 RNA polymerase. Plasmid pIBIN 212 was linearized with *Hin*dIII to prepare a cRNA probe using T7 RNA polymerase. The type II collagen cRNA probe was prepared by linearizing plasmid pBSCA 12 with *Bam*HI followed by transcription by T3 RNA polymerase (Nah et al., 1988). High specific activity RNA probes were synthesized using ^{35}S-labeled UTP (<3000 Ci/M mole, New England Nuclear) under standard conditions. Hybridization conditions and post-hybridization rinses were carried out according to Angerer et al. (1987).

For autoradiography, the slides were dipped in Ilford K-5D emulsion, dried overnight, and exposed for 7-12 days at 4°C Slides were developed for 3 min. at 10°C in Kodak D-19 developer, stopped in water and fixed in Kodak Rapid Fix for 20 min Sections were stained with hematoxylin, dehydrated, cleared in xylenes and mounted under coverslips with Permount.

Immunohistochemistry

Wing buds were embedded in OCT compound, frozen, and frozen sections were cut at 10 μm as described previously (Solursh and Jensen, 1988). Type IX collagen was localized by indirect immunofluorescence using monoclonal antibodies directed at the NC2 domain of the polypeptide (2C2) and the NC4 globular domain (4D6) according to Irwin and Mayne

(1986); generously provided by Dr R. Mayne Type II collagen was detected using a rabbit polyclonal, affinity purified antibody against type II collagen, generously provided by Dr. T. F. Linsenmayer, diluted 1/10 (Shinomura et al., 1990).

Results

Temporal appearance of cartilage marker gene transcripts during limb development

Type II collagen, α2(IX) collagen, and cartilage-specific proteoglycan core protein

To compare the temporal appearance of cartilage marker gene transcripts during chondrogenesis, total cellular poly(A) RNA was isolated for RNA blot analysis from staged forelimbs ranging from pre-differentiated mesenchymal tissue (stages 22 and 23; 3 1/2-4 days *in ovo*) which includes a cellular population capable of differentiation into chondrocytes, myoblasts and fibroblasts, to the more developmentally advanced stage 37 forelimb (day 11 *in ovo*) which exhibits well-established skeletal elements and musculature. Poly(A) RNA from day-14 caudal sterna was included in each blot as a representative of differentiated cartilagenous tissue and to confirm probe specificity. Following hybridization with a type II collagen genomic DNA probe under conditions that prevent cross-hybridization to other fibrillar collagens, the characteristic type II collagen 5.6 kb mRNA and 7 kb precursor were observed (Fig. 2, upper panel). As expected, type II collagen mRNA was readily detectable in pre-chondrogenic stage 22 to 24 (and stage 21) forelimbs and exhibited an increase in steady state mass with advancing chondrogenesis. These data are in agreement

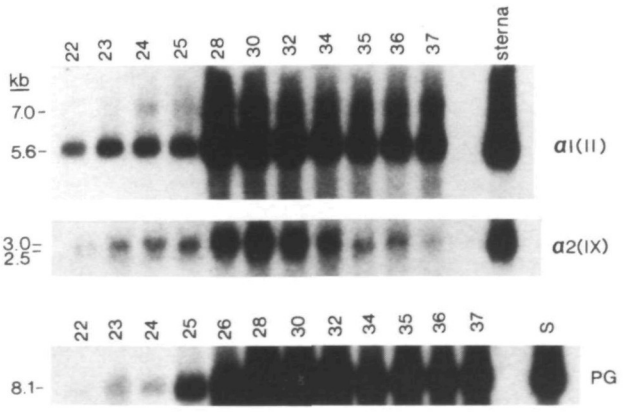

Fig. 2. Temporal appearance of type II collagen, α2(IX) collagen and cartilage-specific proteoglycan core protein (PG) transcripts during forelimb development. RNA blot analysis of 1 μg limb poly(A) RNA and 0.1 μg sternal (S) cartilage poly(A) RNA hybridized to ^{32}P-labeled inserts of a type II collagen probe pBSCA12 (upper panel) and an α2(IX) collagen cDNA probe pYN1731 (middle panel). RNA blot analysis of 4 μg limb poly(A) RNA hybridized to a ^{32}P-labeled insert of cartilage specific PG cDNA probe ST-1 (lower panel). Autoradiography was for 24 hours, 48 hours, and 8 days, respectively.

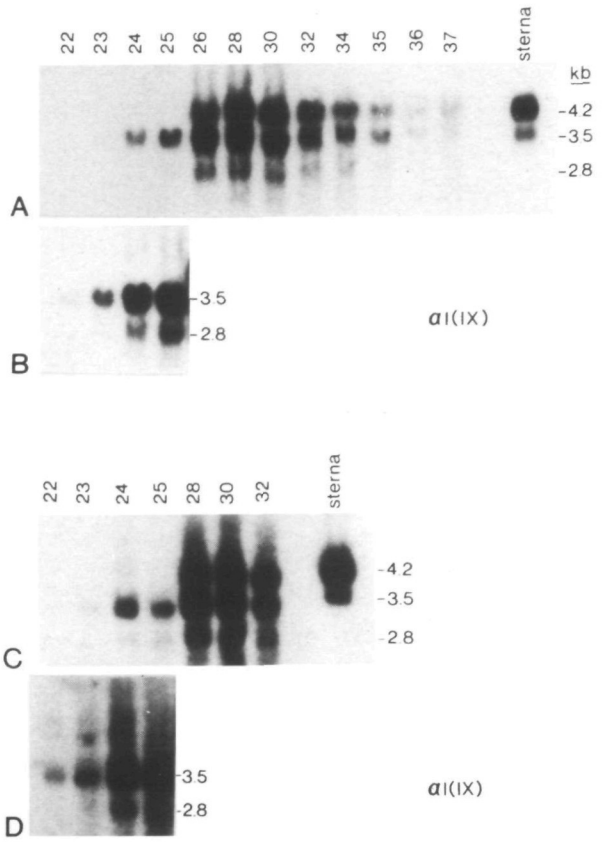

Fig. 3. Temporal appearance of α1(IX) collagen transcripts during forelimb and hindlimb development. RNA blot analysis of 4 μg limb poly(A) RNA and 0.1 μg sternal cartilage poly(A) RNA of forelimb (A,B) and hindlimb (C,D) hybridized to a ^{32}P-labeled *Xho*I/*Xba*I insert of the α1(IX) collagen cDNA probe pYN 1738 which is capable of hybridizing to both long and short form α1(IX) transcripts Autoradiography time of A and C (24 hours) was doubled in B and D to enhance detection of transcripts in pre-chondrogenic stages.

with the results of Kravis and Upholt (1985) and Kosher et al. (1986a) and underscore the precocious appearance of type II collagen mRNA approximately 12-24 hours prior to overt biochemical chondrogenesis at late stage 24.

In contrast to an earlier report which described the initial appearance of type IX collagen transcripts at overt chondrogenesis, notably at stage 26 (Kimura et al., 1988), we detected α2(IX) collagen transcripts in pre-chondrogenic tissue as early as stage 22 (Fig. 2, middle panel). The characteristic 3.0/2.5 kb mRNA doublet observed in the sterna and in the limb was not detectable in stage 21 forelimbs (data not shown). These data confirm and extend the results of a related study by Kulyk et al. (1991) of the distribution of type IX collagen transcripts in the chondrogenic central core of stage 25 limb buds and their absence from peripheral non-chondrogenic tissue. Notably, α2(IX) collagen transcripts can be detected in early limb bud tissue as early as stage 22 (Fig. 2, middle panel). Similarly, the 8.1 kb transcript of the cartilage-specific proteoglycan

core protein (PG) was present in pre-chondrogenic limb buds as early as stage 22 (Fig. 2, lower panel); well before immunodetection of protein at stage 25 (Shinomura et al., 1984, 1990; Oettinger et al., 1985; Mallein-Gerin et al., 1988).

α1(IX) collagen

Due to the complex pattern of α1(IX) collagen alternative promoter use/RNA splicing/and alternative polyadenylation observed in cartilage and corneal tissue (Svoboda et al., 1988, Nishimura et al., 1989), the mass of developmentally staged forelimb bud poly(A) RNA was increased from 1 μg to 4 μg in order to detect the presence of both long and short forms of α1(IX) collagen transcripts and their alternative polyadenylated counterparts by RNA blotting.

By selecting the *Xho*I/*Xba*I insert of plasmid pYN 1738 which can hybridize to both long and short form α1(IX) collagen transcripts, we observed transcripts typical of each form in developing forelimb (Fig. 3A,B) and hindlimb (Fig. 3C,D) buds. As in the case of type II collagen, α2(IX) collagen, and cartilage-specific proteoglycan core protein, prechondrogenic limb tissue exhibited low levels of 3.5 kb and 2.8 kb mRNAs, similar in length to the transcripts of the α1(IX) collagen short form (3.5/2.8 kb doublet). At stage 26 and developmental timepoints thereafter when cartilage matrix is being actively synthesized, the steady state level of the 3.5/2.8 kb mRNAs increased and a 4.2 kb mRNA was also observed. In sternal cartilage, however, this probe hybridized specifically to a 4.2/3.5 kb α1(IX) collagen mRNA doublet which is characteristic of the long form α1(IX) collagen mRNA. Based on the established lengths of the long form (4.2/3.5 kb) and short form (3.5 kb/2.8 kb) α1(IX) collagen mRNAs (Svoboda et al., 1988; Nishimura et al., 1989), our data suggest that both forms appear in a non-temporally coordinated manner during limb bud development, and that the intensity of the 3.5 kb RNA hybridization signal seen at stage 26 and timepoints thereafter is actually the sum of the 3.5 kb mRNAs derived from both long and short α1(IX) collagen transcripts. The following experiments confirm this suggestion.

α1(IX) collagen alternative polyadenylation

Nucleotide sequence analysis of the long form α1(IX) collagen cDNA established two clusters of polyadenylation signals separated by about 600 nucleotides in the 3′ untranslated region. Subsequent RNA blot hybridization studies of two differentiated tissues confirmed that the 700 nucleotide difference between the 4.2 and 3.5 kb mRNAs of the sternal cartilage (long form), and between the 3.5 and 2.8 kb mRNAs of the cornea (short form) are the result of alternative polyadenylation signal usage. The longer transcript of each form results from the utilization of a 3′ distal polyadenylation signal, while the shorter transcript of each doublet arises from the use of a more 5′ proximal signal (Svoboda et al., 1988). A cDNA fragment corresponding to the most 3′ distal polyadenylation signal region of the α1(IX) collagen 3′ untranslated region (Fig. 1) was used as a hybridization probe of forelimb, hindlimb and sternal cartilage mRNAs (Fig. 4A,B). As expected, only the 4.2 kb member of the 4.2/3.5 kb doublet in sternal cartilage hybridized to this probe. In differentiating forelimb and hindlimb bud tissue alternatively polyadenylated α1(IX) collagen transcripts were also observed. The 4.2 kb mRNA was observed initially at stage 26 and in more developmentally advanced tissue. In pre- chondrogenic as well as in more advanced stage

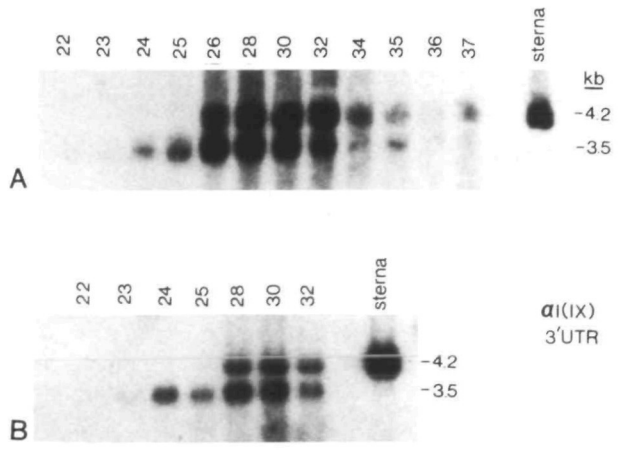

Fig. 4. RNA blot analysis of 4 μg poly(A) RNA from forelimbs (A) and hindlimbs (B) and 0.1 μg sternal cartilage poly(A) RNA hybridized to a ^{32}P-labeled cDNA subfragment of the α1(IX) collagen probe pYN1738 corresponding to the most 3′ distal polyadenylation signal cluster. The detection of more than the single 4 2 kb mRNA of the long form α1(IX) 4.2/3.5 kb RNA doublet suggests that the 3.5 kb RNA detected here is the 700 nucleotide longer RNA of the short form α1(IX) 3 5/2.8 kb RNA doublet Autoradiography was for 9 days

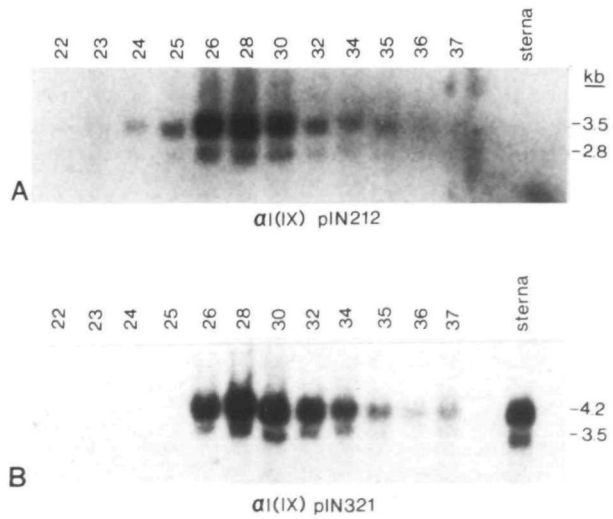

Fig. 5. RNA blot analysis of 4 μg poly(A) RNA and 0.1 μg sternal cartilage poly(A) RNA hybridized to 5′ end specific ^{32}P-labeled cDNA inserts of the short form α1(IX) collagen probe pIN212(A) and of the long form α1(IX) collagen probe pIN321(B) confirming the transcription start site origins of the limb α1(IX) collagen mRNAs. Autoradiography was for (A) 11 days and (B) 9 days

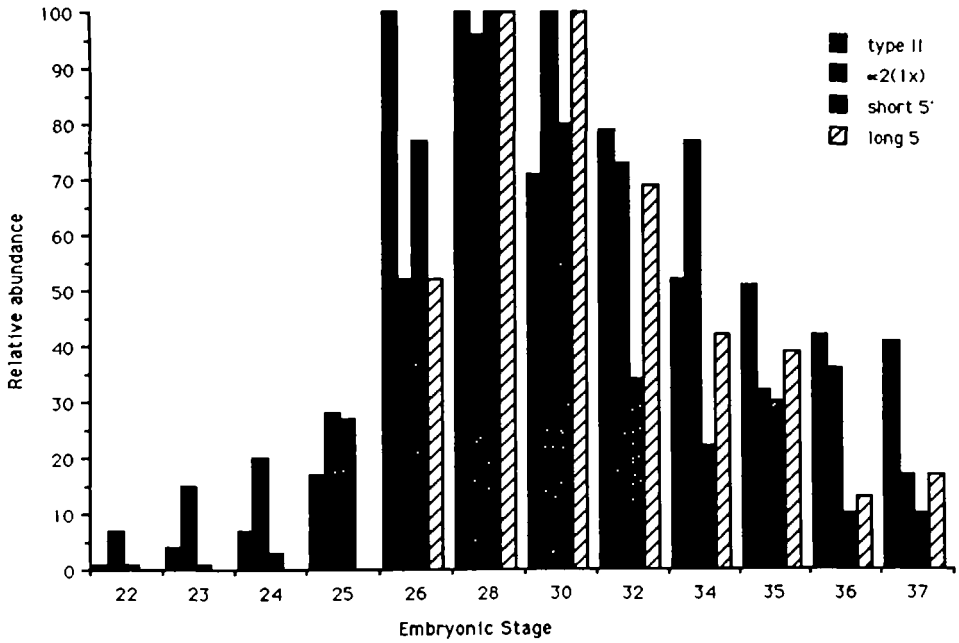

Fig. 6. Graphic presentation of the temporal appearance of type II collagen, short form α1(IX) collagen, long form α1(IX) collagen, and α2(IX) collagen transcripts during embryonic limb chondrogenesis. RNA blots were analyzed by densitometric tracing and computer-generated quantitative analysis. Type II collagen and α2(IX) collagen RNA levels at stage 26 were obtained independently from autoradiograms not shown in the text.

tissue, however, a 3.5 kb mRNA also hybridized to this probe, suggesting that the earliest α1(IX) collagen mRNAs seen in the limb are transcribed from the downstream promoter to yield the short form α1(IX) collagen mRNAs (3.5/2.8 kb doublet). Therefore, at stages 26 through 37, the 3.5 kb mRNA seen during limb development using the cDNA probe pYN 1738 which is capable of detecting both long and short form α1(IX) transcripts mRNAs (Fig. 3) is the sum of the 3.5 kb transcripts of both the short and long forms of α1(IX) collagen.

α1(IX) collagen long form/short form 5′ end specific probes

To further verify that both upstream and downstream α1(IX) collagen gene promoters are active during limb development, poly(A) RNA from staged forelimb buds was hybridized to two mutually exclusive cDNA probes which encode the 5′ one-third of the NC4 globular domain of the long form α1(IX) collagen (pIN 321) and the 5′ end and a portion of the signal peptide of the non-globular amino-terminal domain of the short form α1(IX) collagen (pIN 212). As predicted from the data of the previous figures, the earliest limb α1(IX) collagen mRNAs are transcribed from the downstream promoter, hybridize to the probe pIN 212, and give rise to the 3.5/2.8 kb short form mRNA doublet (Fig. 5A). The weak hybridization signal of stages 22-24 RNA is probably due to the relatively short length of the cDNA insert probe (100 bp). A stronger signal was observed when comparable limb stage poly(A) RNA was hybridized with the 2 kb *Xho*I/*Xba*I insert of the long/short form general α1(IX) collagen cDNA probe pYN 1738 (Fig. 3A,B). As expected, no hybridization signal was detectable in sterna poly(A) RNA using the short form specific pIN 212 probe.

The striking initial appearance of the long form α1(IX) collagen doublet (4.2/3.5 kb) at stage 26 is shown in Fig. 5B, following hybridization of developmentally staged forelimb bud poly(A) RNA to the 5′ end specific probe of the long form α1(IX) collagen chain (pIN 321). As expected, this probe hybridized to the 4.2/3.5 kb mRNA doublet in sternal cartilage. Thus, both long and short forms of α1(IX) collagen appear in the steady state RNA population during limb chondrogenesis, albeit in a non-temporally coordinated manner.

The temporal pattern of cartilage marker gene transcript accumulation observed for type II collagen, cartilage-specific proteoglycan core protein, and type IX collagen (summarized graphically in Fig. 6) suggests a common pattern of precocious transcription in pre-chondrogenic tissue. At the time of chondrogenesis, transcription and translation are enhanced in chondrocytes as the characteristic elements of the limb cartilage extracellular matrix are established and organized.

Localization of type II and type IX collagen mRNAs by in situ hybridization

Following the detection of precocious short form type IX collagen mRNAs in pre-chondrogenic limb buds, we investigated the spatial distribution of type II and type IX collagen mRNAs in developing wing buds by *in situ* hybridization. Antisense, or cRNA probes for type II collagen, and for the long form (5′ end) and short form (5′ end) of α1(IX) collagen were prepared and hybridized with stage 25 forelimb bud cross-sections. At this stage, only type II collagen transcripts were detectable in the chondrogenic regions of the limb (data not shown). Transcripts of the short form α1(IX) collagen, although detectable by RNA blotting, did not give a hybridization signal above background, possibly due to their relatively low abundance (compare RNA blots of Figs 2A and 5A) or perhaps due to their homogenous distribution throughout the limb at this stage. As predicted from the RNA blot of Fig. 5B, the long form α1(IX) collagen transcripts did not give a

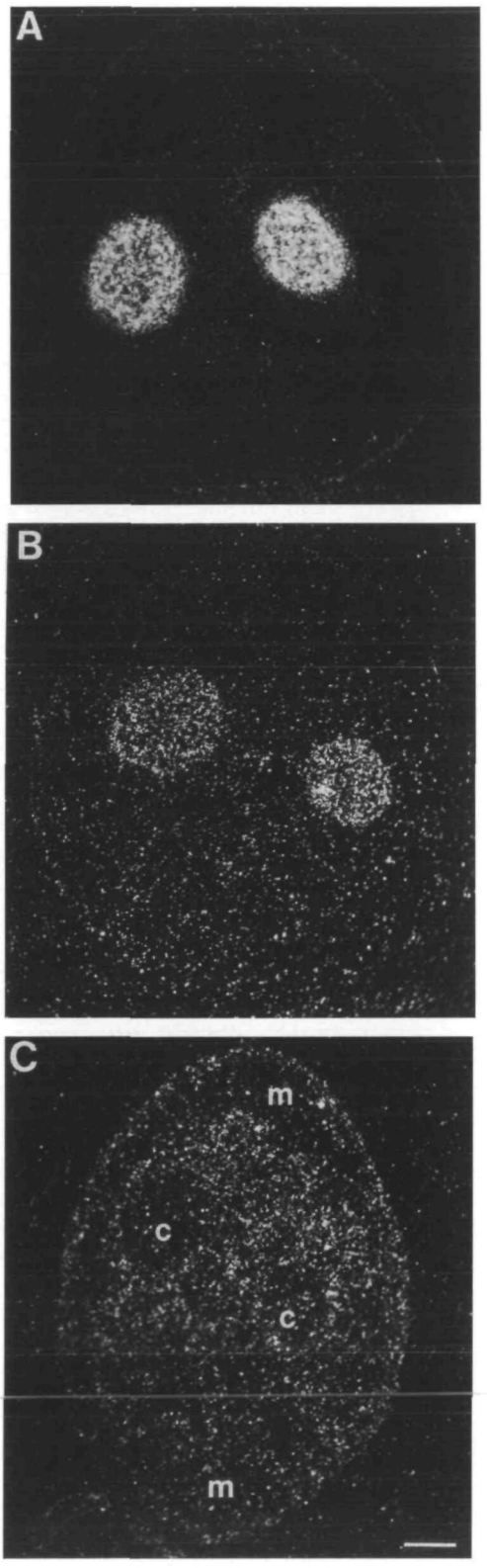

Fig. 7. Spatial localization of (A) type II collagen (B) long form α1(IX) collagen, and (C) short form α1(IX) collagen transcripts in cross-sections of stage 30 forelimbs at the level of the future radius and ulna by *in situ* hybridization. ^{35}S-labeled antisense probes were synthesized using transcription vectors containing inserts for type II collagen (pBSCA12), long form α1(IX) collagen (pIBIN321), and short form α1(IX) collagen (pIBIN212) (c=cartilage, m=muscle mass). Bar equals 100 μm.

signal above background at stage 25. By stage 30 when chondrogenesis is well underway, transcripts of type II collagen and the long form α1(IX) collagen co-localized over the well-defined cartilage elements of the future radius and ulna (Fig. 7A,B). Transcripts of the short form α1(IX) collagen, however, were distributed throughout the non-chondrogenic, non-myogenic, mesenchymal regions of the limb and were not detectable above background levels in the chondrogenic elements (Fig. 7C). This distribution of short form α1(IX) collagen transcripts in the limb contrasts markedly with the localization of long form transcripts solely in the cartilage elements. As a control for the specificity of hybridization of the long and short α1(IX) collagen probes, sections through stage 28 eyes were tested. As expected, the long form α1(IX) collagen probe hybridized to the presumptive ciliary body region of the neural retina adjacent to the lens (Fig. 8B), while the short form α1(IX) collagen probe hybridized to the cornea (Fig. 8C). These results are in agreement with the *in situ* data of Linsenmayer et al. (1990). Our data further refine this earlier study using the "general" α1(IX) collagen probe pYN1738 to detect transcripts in the eye. By use of the α1(IX) 5′-end-specific probes we are able to assign the ciliary body hybridization signal to the long form and the corneal signal to the short form. Sense probes were also included as negative controls and showed only background hybridization to either forelimb or eye tissue (data not shown).

Type IX collagen immunolocalization in forelimbs

The precocious appearance of type II collagen transcripts in the avian forelimb prior to overt biochemical chondrogenesis has been well documented (Kosher and Solursh, 1989; Fitch et al., 1989). Evidence from the type IX collagen RNA blots and *in situ* localization data presented here suggests that transcription from the short form α1(IX) and α2(IX) collagen genes precedes phenotypic differentiation in forelimb mesenchymal cells as well. In order to correlate the temporal appearance of type IX collagen transcripts and the polypeptide, early limb buds were assayed for type IX collagen using immunohistochemical analysis.

Immunofluorescence of the monoclonal antibody 4D6 that recognizes the NC4 globular domain of the long form type IX collagen (Irwin et al., 1985; Brewton et al., 1991), and of the monoclonal antibody 2C2 directed at the NC2 domain of both long and short form type IX collagen (Irwin et al., 1985) was negative in stage 24 forelimbs. At this stage, only type II collagen was detectable in the early chondrogenic elements (data not shown). This result suggests that the α2(IX) collagen and short form α1(IX) collagen transcripts detected by RNA blotting may not be translated in the early limb bud, or that the level of 2C2-immunoreactive type IX collagen is too low to detect.

Cross-sections of stage 28 forelimb buds cut at the

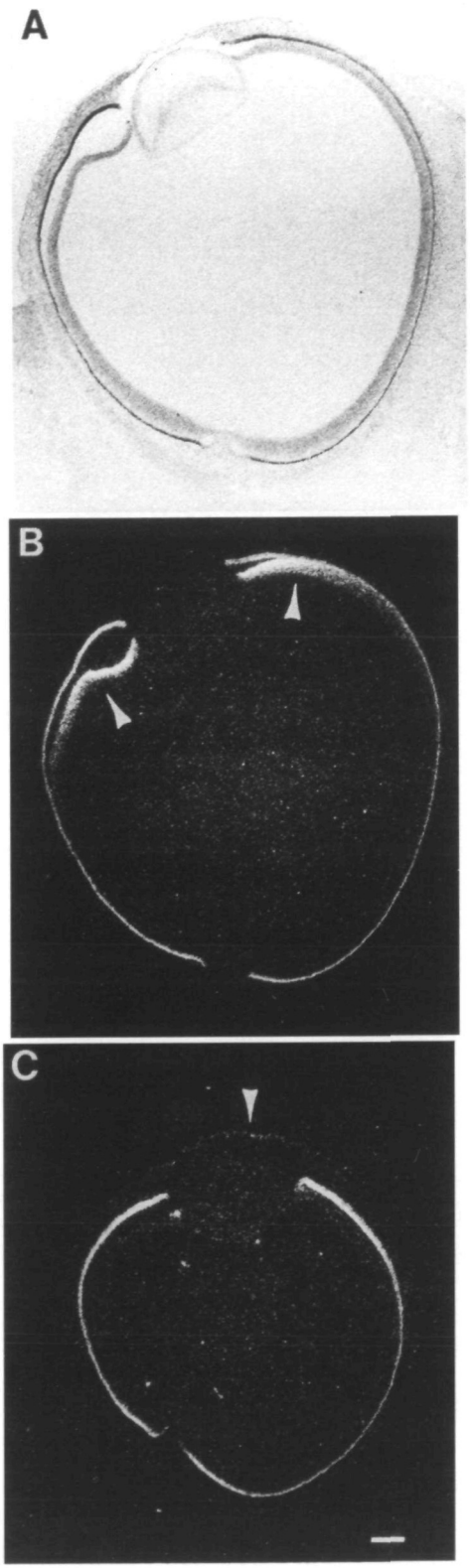

Fig. 8. Spatial localization of long and short form α1(IX) collagen transcripts in stage 28 eyes cut in cross-section with respect to the embryo by *in situ* hybridization. (A) bright-field illumination. (B) detection of long form α1(IX) collagen transcripts in the presumptive ciliary body/neural retina (arrows) using an ^{32}S-labeled antisense probe prepared from plasmid pIBIN321. (C) localization of short form α1(IX) collagen transcripts in the cornea (arrow) using an ^{32}S-labeled antisense probe prepared from plasmid pIBIN212. Bar equals 100 μm. The artifactual appearance of a positive signal in the pigmented portion of the retina is due to the presence of pigment granules.

level of the future radius and ulna reacted positively in the cartilage elements for type II and for type IX collagen, using both 4D6 and 2C2 monoclonal antibodies (Fig. 9). Evidence for the translation of the long form α1(IX) transcripts in stage 28 limb buds comes from the immunoreactivity of 4D6 which recognizes an epitope of the α1 chain NC4 domain and the NC4 domain of the α3 chain (Irwin et al., 1985; Brewton et al., 1991). Based on the *in situ* hybridization results shown in Fig. 7C, the 2C2 immunoreactivity in stage 28 limbs may result either from translation of the low amount of short form α1(IX) collagen mRNA present in the cartilage elements, or the 2C2 antibody may recognize the NC2 domain of the long form type IX collagen which is common to both long and short forms.

Discussion

We present the first evidence that both short and long form α1(IX) collagen transcripts, which are typically expressed in a tissue-specific manner in the developing avian cornea and cartilage, respectively, are co-expressed during avian limb bud chondrogenesis in a non-temporally coordinated manner. The most developmentally precocious type IX collagen transcripts to appear in embryonic pre-chondrogenic tissue encode the short form α1(IX) collagen and α2(IX) collagen chains of the type IX collagen trimer. By *in situ* hybridization and immunohistochemical analyses, neither short form α1(IX) collagen transcripts nor the type IX collagen polypeptide were detectable in the early chondrogenic elements of stage 25 limb buds. At this time, we cannot come to a conclusion about the translational fate of the short form α1(IX) chain and α2(IX) chain mRNAs. The absence of detectable antigen may be the result of polypeptide levels below the limits of detection, or type IX collagen proteolysis. Despite the temporal increase in the steady state short form α1(IX) collagen mRNA levels measured by RNA blotting, our failure to localize the transcripts in the chondrogenic regions of stage 30 limbs suggests several different potential fates of the short form α1(IX) mRNAs. Since these mRNAs were localized in non-chondrogenic, non-myogenic mesenchymal regions of the limb, these short form α1(IX) mRNAs probably do not contribute to chondrogenesis, perhaps as a consequence of their lack of the NC4 globular domain typically associated with cartilagenous regions. The absence of the NC4 domain in α1(IX) mRNAs of the non-chondrogenic mesenchymal regions of the limb may serve to maintain a loosely organized extracellular matrix favorable to mesenchyme morphogenesis. Alternately, selective type IX collagen proteolysis may

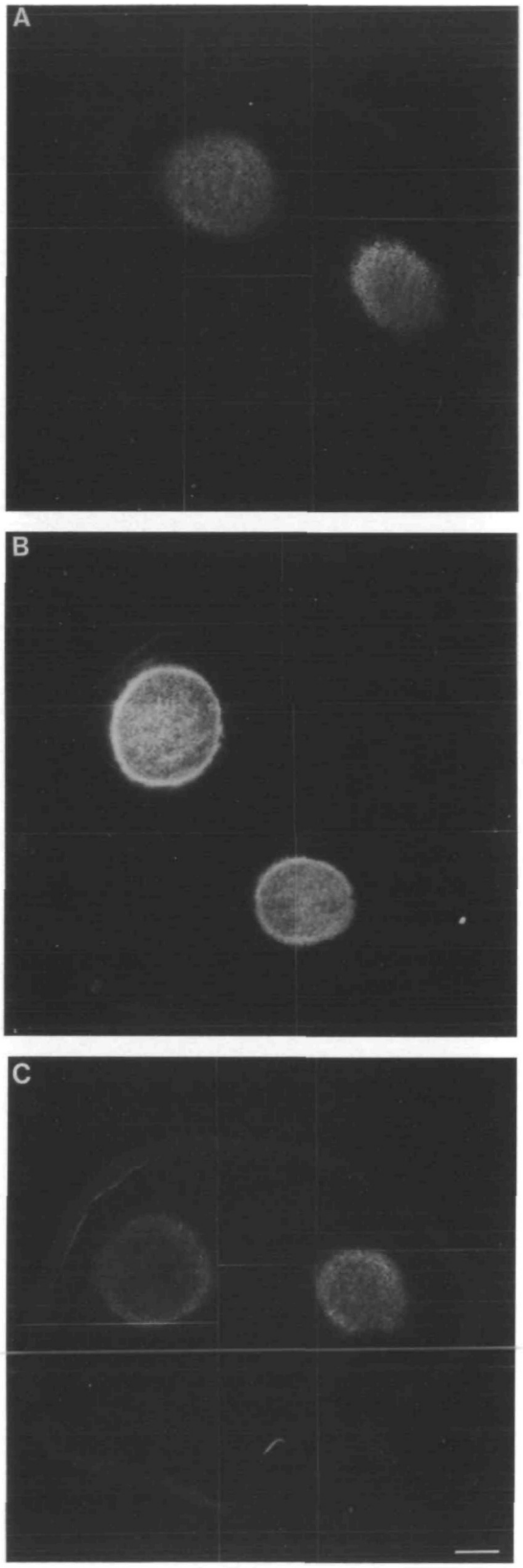

Fig. 9. Immunohistochemical localization of type II and type IX collagen in stage 28 forelimbs. Immunofluorescence is localized in the future radius and ulna after staining with a polyclonal antibody to type II collagen (A), with monoclonal antibody 4D6 specific for long form type IX collagen (B), and with monoclonal antibody 2C2 capable of detecting both long and short form type IX collagen (C). The detection of immunofluorescence associated with the epithelial basement membrane in (C) is non-specific and due to high background at this interface. Bar equals 100 μm.

remove the antigen from regions of the limb where such a polypeptide is not required. Similar reasoning has been used to offer an explanation for the brief appearance of short form type IX collagen in the developing primary corneal stroma despite the persistence of the short form transcripts for several days afterwards (Svoboda et al., 1988; Fitch et al., 1989).

In contrast to the early appearance of short form α1(IX) and α2(IX) collagen mRNAs, a more coordinated temporospatial expression pattern of long form α1(IX) collagen transcripts and polypeptide was observed during early limb chondrogeneis. Long form α1(IX) collagen mRNAs were initially detected in stage 26 limb buds by RNA blotting, coincident with the initial detection of metabolically radiolabeled type IX collagen as reported by Kimura et al. (1985, 1988). Our *in situ* hybridization and immunohistochemical analyses concluded that both transcripts and polypeptides were co-localized to the cartilagenous regions of the limb. Therefore, it appears that the expression of the long form α1(IX) collagen gene may be under the influence of a different set of temporospatial expression instructions than those that control the precocious transcription of the type II collagen gene, α2(IX) collagen gene, and cartilage-specific proteoglycan core protein gene in pre-chondrogenic stages.

While these experiments have emphasized the complex nature of type IX collagen gene expression during limb bud chondrogenesis, the picture remains incomplete in the absence of information on the α3(IX) collagen chain expression pattern. Since the α3(IX) collagen chain is found in both long and short form type IX collagens, its temporal transcript expression pattern may resemble that of the α2(IX) collagen chain; another member of the type IX collagen trimer found in both type IX long and short forms. Efforts are underway in a number of laboratories to isolate cDNA probes for the α3(IX) collagen chain and complete our understanding of type IX collagen gene regulation during limb chondrogenesis.

As in all developing systems, the question of what stimulus/stimuli induces overt type IX biochemical chondrogenesis remains unanswered. Ongoing *in vitro* experiments in this laboratory, however, suggest a role for exogenous factor(s) in the inductive process of hyaline cartilage formation in avian limb buds (Geduspan and Solursh, unpublished data). When prechondrogenic (stage 18) limb buds are cultured adjacent to comparable stage mesonephric tissue, a highly organized type II collagen/type IX collagen immunoreactive cartilage is formed, while in the absence of the mesonephros, type II collagen and a minor amount of type IX collagen can be detected in a highly disorgan-

ized cartilage. A comparable extension of this phenomenon may exist in the developing limb bud.

Comparison of the results from previous studies using differentiating avian limbs (Kulyk et al., 1991; Kimura et al., 1988; Kosher et al., 1986b; Sai et al., 1986) and the results of the present study on early pre-chondrogenic limb buds suggests a similar pattern of cartilage marker gene expression in pre-chondrogenic tissue. While type II collagen mRNA has been detected in stage 18 limb buds (Kravis and Upholt, 1985), the antigen has been found to be associated with a number of non- chondrogenic cells during early embryogenesis (Kosher and Solursh, 1989; Fitch et al., 1989). Nonetheless, type II collagen is the prototype of the well-established model of pre-chondrogenic transcription/relative translational inactivity followed by enhanced transcription/translation at overt chondrogenesis in the avian limb bud (Kravis and Upholt, 1985; Kosher et al., 1986a). In the present study, similarities were observed for the α2(IX) collagen, cartilage-specific PG, and for short form α(IX) collagen; although the translational fate of the latter remains unresolved at present. Low levels of these transcripts were detectable by RNA blot analysis at least 24-36 hours prior to the appearance of detectable polypeptides. While type II collagen transcripts can be localized in pre-chondrogenic aggregates as early as stage 23 (Swalla et al., 1988), cartilage-specific PG transcripts may be present at sufficiently low levels in early limb buds so that they cannot be localized in the pre- chondrogenic aggregates (Mallein-Gerrin et al., 1988; Stirpe and Goetinck, 1989). Interestingly, all of these cartilage marker gene transcripts are detected by stage 22 when the pre-cartilage humeral condensation is also first detected (Fell and Canti, 1934).

After the formation of pre-cartilage aggregates, when transcription of a number of genes for cartilage extracellular matrix components has been initiated, there is a progressive increase in the steady state levels of mRNAs for these components. While the present study suggests that the initiation of transcription of many of these genes begins early, long before these components can be detected in the extracellular matrix, others appear to be activated later (Stirpe and Goetinck, 1989; LuValle et al., 1989). With progressive development, matrix components which undergo early transcriptional activation gradually accumulate in the extracellular matrix, while others which might play a role in organizing the extracellular matrix appear sequentially (Shinomura et al., 1984, 1990; Franzen et al., 1987). As a distinct chondrogenic event occurring by stage 26, the long form of the α1(IX) collagen gene is transcriptionally activated. This event is likely to effect important changes in the physical structure of the cartilage extracellular matrix.

Altogether, the evidence from earlier studies of the structural/functional role of long and short form type IX collagen in cartilage and corneal extracellular matrices fit well into the temporospatial expression pattern of type IX collagen gene expression during avian limb chondrogenesis. Further experimentation will refine our understanding of the developmentally regulated, tissue-specific regulatory mechanisms controlling this event.

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