Developmental regulation of mRNA species for types II, IX and XI collagens during mouse embryogenesis

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Several techniques were used to study the co-ordination of mRNA levels for five constituent chains of cartilage collagen fibrils during mouse development. Short cDNA clones were first constructed for mouse and human $\alpha 3(\text{IX})$ and for mouse $\text{pro}\alpha 1(\text{XI})$ collagen mRNA species. Northern analysis of developing mouse embryos revealed that the mRNA species for $\alpha 1$, $\alpha 2$ and $\alpha 3$ chains of type IX collagen peaked earlier than those for $\text{pro}\alpha 1(\text{II})$ and $\text{pro}\alpha 1(\text{XI})$ collagen chains. Quantification of these mRNA species by slot-blot hybridization confirmed this developmental regulation: the mRNA ratios for type II/type IX/type XI collagens changed from 5.7:1:0.6 (at embryonic day 12.5) to 10.6:1:0.9 (in newborn mice). However, the genes coding for the three chains of type IX collagen seemed to be

under more co-ordinated regulation during mouse development. In addition to high mRNA levels in cartilages and the eye, low levels of type IX collagen transcripts were identified in brain and skin of newborn mouse using RNase protection and reverse transcriptase–PCR assays. Finally, hybridization *in situ* revealed identical tissue distributions of the three type IX collagen mRNA species during early chondrogenesis but somewhat more widespread expression of the $\alpha 1(\text{IX})$ and $\alpha 3(\text{IX})$ mRNA species during endochondral ossification at day 16.5 of embryonic development. These results suggest a relatively tight co-ordination of the $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$, and $\alpha 3(\text{IX})$ collagen mRNA species in chondrocytes, but a lack of co-ordination in several non-cartilaginous tissues.

INTRODUCTION

Hyaline cartilage contains a network of collagen fibrils that provide structural strength to the tissue and entrap the proteoglycan molecules responsible for furnishing cartilage with resilience [1,2]. The fibrils form a multicomponent network where type II collagen, a homotrimer of $\alpha 1(\text{II})$ chains, is the major component, and types IX and XI are minor components. Type XI collagen is a heterotrimeric fibrillar collagen that in cartilage contains $\alpha 1(\text{XI})$, $\alpha 2(\text{XI})$ and $\alpha 3(\text{XI})$ chains in a 1:1:1 ratio [2]. The $\alpha 1(\text{XI})$ and $\alpha 2(\text{XI})$ chains are products of *COL11A1* and *COL11A2* genes, whereas the $\alpha 3(\text{XI})$ chain is a product of the *COL2A1* gene coding also for the type II collagen chains [2]. Type IX collagen, a heterotrimer of $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ chains, is a fibril-associated collagen attached laterally on the fibril surface [3] through covalent cross-links to type II and XI collagens [4]. Small proteoglycans, decorin and fibromodulin, as well as cartilage matrix protein, have also been shown to interact with collagen fibrils in cartilage [2]. Interestingly the collagen fibrils in the vitreous and cornea of the eye share with cartilage a similar composition of collagen types II, IX and XI, except that the $\alpha 2(\text{V})$ chain replaces the $\alpha 2(\text{XI})$ chain and only one of the two alternative forms of the $\alpha 1(\text{IX})$ collagen chains is present [2,5,6].

The function of type IX collagen remains unknown. Its structure and location on the fibril surface have led to suggestions that type IX collagen has an important role in regulating the diameter of cartilage collagen fibrils and/or in mediating the interactions between the collagen network and proteoglycans. However, transgenic mice carrying a homozygous knock-out mutation of the $\alpha 1(\text{IX})$ gene have not been reported to exhibit any abnormalities in skeletal morphogenesis and growth, suggesting that type IX collagen is not essential for the formation of

skeletal elements [7]. Development of early osteoarthritic lesions in these mice would instead suggest a structural function for type IX collagen in maintaining mechanical stability in articular cartilage. Osteoarthritic lesions and abnormally thin, randomly oriented collagen fibrils were observed in mice harbouring cDNA-based *Col9a1* transgenes with a large deletion mutation [8]. Recently, type XI collagen has been implicated in the regulation of collagen fibril diameter in cartilage [9]. Taken together, the results available suggest that a correct balance of types II, IX and XI collagens, which in embryonic chick cartilage is 8:1:1 [3], is important for the maintenance of the correct fibril diameter.

Although much is known about the composition of cartilage collagen fibrils, the regulatory mechanisms controlling their synthesis are poorly understood. Maintenance of a correct balance of types II, IX and XI collagen molecules for fibrillogenesis presents interesting questions concerning the regulation of the corresponding genes (*COL2A1*, *COL9A1*, *COL9A2*, *COL9A3*, *COL11A1* and *COL11A2*), which reside on four different chromosomes [10]. Molecular biological characterization of the type IX collagen has focused on the chick system because cDNA clones have been available for all three chains. Lack of cDNA or genomic clones for mammalian $\alpha 3(\text{IX})$ collagen has prevented systematic analyses of the regulation of type IX collagen gene expression in mammalian species. We have previously used reverse transcriptase–PCR (RT–PCR) technology to construct short cDNA clones for mouse and human $\alpha 1(\text{IX})$ and $\alpha 2(\text{IX})$ collagen mRNA species [11–13]. Here we report the sequences of the cDNA clones for the mouse and human $\alpha 3(\text{IX})$ collagens and mouse $\text{pro}\alpha 1(\text{XI})$ collagen. These and other clones were used for the determination of cartilage-specific collagen mRNA species and their ratios during mouse development.

Abbreviations used: COL, collagenous; NC, non-collagenous; RT–PCR, reverse transcriptase–PCR.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers X91012 (pMCol9a3-1), X91013 (pHCOL9A3-1) and X91014 (pMCol11a1-1).

EXPERIMENTAL

RNA extraction, cDNA synthesis and amplification by PCR

Total RNA species were extracted from several newborn mouse tissues including rib and epiphyseal cartilages, from mouse embryos at 9.5, 10.5, 12.5, 14.5, 16.5 and 18.5 days of development, and from human fetal cartilage, with the guanidinium isothiocyanate method [14]. Random hexamers and oligo(dT) were used to prime reverse transcription of 1 µg of total RNA by Moloney Murine Leukaemia Virus reverse transcriptase under conditions recommended by the supplier (Gibco BRL). Aliquots of cDNA were used for amplification by PCR (AmpliTaQ[®]; Perkin Elmer) with oligonucleotide primers MP-23 [5'-GA(G/A)CAACA(C/T)AT(C/T/A)AGAGA(G/A)CT-3'] and MP-24 [5'-GCA(A/G)GCTGATGTGTCACA-3']. The reactions were cycled through denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min, and extension at 72 °C for 2 min. After 30 amplification cycles, aliquots of the reactions were fractionated on 1.5 % (w/v) agarose gels and the specific fragments were purified and cloned by blunt-end ligation into the *EcoRV* site of the Bluescript KS⁻ (Stratagene) vector. The cloned fragments were sequenced with the Sanger dideoxy method (Sequenase[®] reagent kit; U.S. Biochemical).

Northern hybridization

For Northern hybridization 10 µg of total RNA from mouse embryos were denatured with formamide, subjected to electrophoresis on 0.8 % agarose gels and transferred to Pall Biodyne nylon membranes in 10 × SSC [1 × SSC is 0.15 M NaCl/0.015 M sodium citrate (pH 7.0)]. Membranes were hybridized with ³²P-labelled cDNA probes in 5 × SSC/1 × Denhardt's solution/50 % (v/v) formamide/0.1 % SDS/250 µg/ml denatured calf thymus DNA at 42 °C overnight. Membranes were washed at high stringency and autoradiographed with intensifying screens at -70 °C.

RT-PCR analysis

Total RNA extracted from several tissues of newborn mice was used as templates for cDNA synthesis as described above. Reverse transcription reaction mixtures (5 µl) were used for PCR reactions for α1(IX), α2(IX) and α3(IX) with the primers complementary to the non-collagenous NC1 and NC2 domains of each chain under conditions also described above. Reaction products were subjected to electrophoresis on 1.5 % (w/v) agarose gels.

RNase protection assay

Linearized cDNA probes for α1(IX), α2(IX) and α3(IX) collagens were used as templates in transcription reactions with T3 or T7 RNA polymerase (Promega) and [α -³²P]UTP (800 Ci/mmol; Amersham) as the labelled nucleotide. Full-length transcripts were purified on a denaturing polyacrylamide gel. Dried RNA samples (10 µg) were dissolved in 10 µl of hybridization buffer [80 % (v/v) formamide/40 mM Pipes (pH 6.4)/400 mM NaCl/1 mM EDTA] containing (2–5) × 10⁵ c.p.m. of the cRNA probe. Hybridization was performed at 55 °C overnight, after which the samples were incubated for 1 h at 37 °C with 300 µl of digestion mixture (300 mM sodium acetate/5 mM EDTA/12 µg/ml RNase A/30 units/ml RNase T1). The digestion was terminated by adding 50 µg of proteinase K and 3.5 µl of 20 % (w/v) SDS followed by incubation for 15 min at 37 °C, phenol/chloroform (1:1, v/v) extractions and ethanol precipitation. The precipitates were dissolved, denatured and subjected to electro-

phoresis on 8 M urea/4.5 % polyacrylamide gels. Dried gels were subjected to autoradiography at -70 °C for 1–8 days.

Slot-blot analysis

For RNA slot-blot analysis duplicate filters were prepared containing dilution series of cDNA inserts for α1(IX), α2(IX), α3(IX), α1(XI) and α1(II) collagens and total RNA species from newborn mouse limb cartilage and from 12.5-day and 16.5-day embryos. Yeast tRNA was also blotted as a negative control. Samples were blotted on Hybond N⁺ (Amersham) filters with a vacuum manifold (Schleicher & Schuell). The filters were hybridized with each of the probes under the same conditions as described above. Densitometric analyses of the hybridization signals of the dilution series were performed on an Ultrosan XL densitometer (LKB) to measure the molar amount of each mRNA in the RNA samples.

Histology and hybridization *in situ*

For histological examination and hybridization *in situ* the dissected limbs from mouse embryos were fixed in 4 % (v/v) formalin in PBS at room temperature overnight, or in 4 % (w/v) paraformaldehyde in PBS at 4 °C for 3–19 h, dehydrated in a graded ethanol series and embedded in paraffin for sectioning.

Details of the *in situ* hybridization technique used have been published earlier [15]. The paraffin sections were pretreated with proteinase K (3.5 µg/ml for 30 min at 37 °C) and acetylated. The hybridizations were performed at 52 °C for 18 h with sense and anti-sense cRNA probes prepared as above except that [³⁵S]uridine 5'-[α -thio]triphosphate was used as the radiolabelled nucleotide. Hybridization was followed by washes and autoradiography for 2.5–14 days and by staining of the sections with haematoxylin.

Hybridization probes

The following mouse cDNA clones were used as hybridization probes: pMCol1a1-1 for proα1(I) collagen, pMCol2a1-1 for proα1(II) collagen, pMCol3a1-1 for proα1(III) collagen, pMCol9a1-1 for α1(IX) collagen [11], pMCol9a2-1 for α2(IX) collagen, pMCol10a1-1 for α1(X) collagen [12], pMCol9a3-1 for α3(IX) collagen and pMCol11a1-1 for α1(XI) collagen. For detection of the long form of type II collagen mRNA a genomic subclone containing exon 2 was used as the probe [16].

RESULTS

cDNA cloning of mouse and human α3(IX) collagen mRNA species

Two oligonucleotide primers (MP23 and MP24) were designed for the non-collagenous NC1 and NC2 domains of the α3 chain of the type IX collagen based on the available chick *Col9a3* cDNA and bovine amino acid sequences [4,17–19]. The same degenerate oligonucleotides were used for the amplification of both mouse and human α3(IX) collagen cDNA covering sequences coding for the collagenous (COL) domain COL1 and parts of the non-collagenous domains NC1 and NC2. A sequence comparison of the 438 bp clones pMCol9a3 (mouse) and pHCOL9A3 (human) revealed nucleotide and amino acid sequence identities of 93 % and 82 % respectively between mouse and human (Figure 1). The length of the COL1 domain in mouse and human α3(IX) chains is 112 residues, as in the chick α3(IX)

1	MO	<u>EOHIRELCGG</u>	MISEQIAQLA	AHLRKPLAPG	SIGRPGPAGP	PGPPGPPGSI
	HU					
	CH	E	..ND.....	..N.....S	MT.....	AT..V
51	MO	GHPGARGPPG	YRGPTGELGD	PGPRGSQGDR	GDKGATGAGL	DGPAGDQGYQ
	HU	*		N.....	A.....	E.....P
	CH			DT.EK	PA.Q.I	D.....P
101	MO	GPQGVPGISK	DGRDGAHGEP	GLPGDPGLPG	AAGAQTPTGI	<u>CDTSAC</u>
	HU	T.....	Q.....P.....	P.....	I.....	
	CH	..LP.....	..N.....Q.....	T.....	V.....	

Figure 1 Deduced amino acid sequences of clones pMCol9a3-1 and pHCOL9A3-1 for the mouse and human $\alpha 3(\text{IX})$ collagens respectively

MO, deduced amino acid sequence of the mouse $\alpha 3(\text{IX})$ collagen clone and the corresponding human (HU) and chick (CH) sequences shown only at the positions where they differ from the mouse sequence. The oligonucleotides used for PCR amplification are underlined. The asterisk at residue 55 denotes Ala $\rightarrow$ Thr polymorphism detected in the human sequences analysed.

Figure 2 Northern analysis of mRNA species for types I-III and IX-XI collagens during mouse embryo development

Total RNA was isolated from whole mouse embryos of days 9.5 (lane a), 10.5 (lane b), 12.5 (lane c), 14.5 (lane d), 16.5 (lane e) and 18.5 (lane f) and from newborn mice (lane g). Aliquots were fractionated on 0.8% agarose gels and transferred to Pall Biotrans membranes, which were subsequently hybridized with cDNA probes for the following mRNA species: pro $\alpha 1(\text{I})$ collagen (A), pro $\alpha 1(\text{III})$ collagen (B), pro $\alpha 1(\text{II})$ collagen (C), exon 2 of pro $\alpha 1(\text{II})$ collagen (D), $\alpha 1(\text{IX})$ collagen (E), $\alpha 2(\text{IX})$ collagen (F), $\alpha 3(\text{IX})$ collagen (G), $\alpha 1(\text{X})$ collagen (H) and pro $\alpha 1(\text{XI})$ collagen (J). Hybridization was detected by autoradiography for 1-7 days using X-ray films. (K) Ethidium bromide-stained gel for detection of the rRNA species.

chain. Two interruptions in the Gly-Xaa-Yaa repeat structure are also conserved between the three species [18,19].

Further sequence comparisons of the mouse pMCol9a3 clone were performed with the corresponding domains in the mouse

$\alpha 1(\text{IX})$ and $\alpha 2(\text{IX})$ collagen cDNA [11,12]. Sequence similarities of 53% between the $\alpha 3$ and $\alpha 1$ chains and 51% between the $\alpha 3$ and $\alpha 2$ chains at nucleotide level were observed; at the levels of translated amino acids the similarity was 43% in both comparisons. After these clones had been sequenced and the data deposited in EMBL/GenBank, Brewton et al. [20] published a full-length cDNA sequence for the human $\alpha 3(\text{IX})$ collagen. This sequence contains one nucleotide difference from the corresponding region of our clone, which results in an Ala $\rightarrow$ Thr amino acid change (Figure 1).

Nucleotide sequence of the mouse $\alpha 1(\text{XI})$ collagen cDNA

The 562 bp mouse $\alpha 1(\text{XI})$ collagen cDNA clone encoding a 3' region of the triple helical domain was obtained accidentally in an RT-PCR reaction. The clone was discovered during the sequencing of putative RT-PCR clones for the 5' end of the mouse $\alpha 2(\text{IX})$ collagen cDNA. The oligonucleotide MH-12 (5'-CTGGCCCTTGACTCCAGG-3') for mouse $\alpha 2(\text{IX})$ collagen seemed to prime the amplification of the $\alpha 1(\text{XI})$ collagen cDNA from both the 3' and the 5' directions. Comparison of the mouse $\alpha 1(\text{XI})$ collagen sequence with the corresponding human sequence revealed an amino acid identity of 93% and nucleotide identity of 85%. Amino acid identity with the chick gene is 94% and nucleotide identity 79%. After clone pMCol1a1 had been sequenced and the data deposited in EMBL/GenBank, Yoshioka et al. [21] published the entire coding sequence of the mouse *Col11a1* cDNA. In comparison with our cDNA fragment this sequence contains six nucleotide differences that result in two amino acid changes (results not shown).

Expression of genes for cartilage-specific collagens in developing mouse embryos

Specific probes for types I, II, III, IX, X and XI collagen chains were first used to study how their expression is co-ordinated at the mRNA level. Northern analysis of total RNA isolated from 9.5-18.5-day mouse embryos and from newborn mice (Figures 2 and 3) revealed several different expression patterns. The mRNA levels for the three major fibrillar collagens (types I, II and III) increased rapidly from day 10.5 to day 14.5. Thereafter the levels of type I and III collagen mRNA remained relatively constant, but type II collagen mRNA continued to increase. The mRNA level of pro $\alpha 1(\text{XI})$ collagen paralleled that of pro $\alpha 1(\text{II})$ collagen, whereas those for the $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ chains reached their maximal level earlier than type II and type XI collagen mRNA species. Very little mRNA for the $\alpha 1(\text{X})$ collagen was detected until day 14.5, after which a rapid increase in the transcription of this gene was observed, peaking at day 16.5. On extended exposure, the Northern analyses exhibited low levels of types II, IX and XI collagen mRNA species as early as day 9.5.

The two forms of the $\alpha 1(\text{IX})$ collagen mRNA arising from alternative promoters were developmentally regulated: the shorter approx. 3 kb $\alpha 1(\text{IX})$ mRNA appeared with type II collagen as early as day 9.5 and peaked at day 12.5, whereas the longer approx. 4 kb mRNA peaked at days 14.5-16.5. The size difference of the two forms of $\alpha 1(\text{IX})$ collagen mRNA allowed us to directly determine their ratio at the different time points (Figure 3F), whereas the two alternatively spliced pro $\alpha 1(\text{II})$ collagen mRNA species could not be separated in Northern blots. The probe pMCol2a1-1 used for Figures 2(C) and 3(C) detected both the long and the short forms of pro $\alpha 1(\text{II})$ collagen mRNA. The long form of pro $\alpha 1(\text{II})$ collagen mRNA was detected

Figure 3 Developmental expression of cartilage-specific collagen genes in mouse embryos

Graphs summarizing the densitometric analysis of several exposures of Northern blots (Figure 2). The panels show the levels of mRNA species for (A) pro $\alpha 1(I)$ collagen, (B) pro $\alpha 1(III)$ collagen, (C) pro $\alpha 1(II)$ collagen, (D) long form of pro $\alpha 1(II)$ collagen containing exon 2 sequences, (E) long form of $\alpha 1(IX)$ collagen containing the large NC4 domain ($\triangle$) and short form of $\alpha 1(IX)$ collagen without the large NC4 domain ($\blacklozenge$), (G) $\alpha 2(IX)$ collagen, (H) $\alpha 3(IX)$ collagen, (J) $\alpha 1(X)$ collagen and (K) pro $\alpha 1(XI)$ collagen from embryonic day 9.5 to newborn. All values have been corrected for variations in RNA loading (levels of 28 S rRNA). (F) Short form of $\alpha 1(IX)$ collagen mRNA as a percentage of the total $\alpha 1(IX)$ collagen mRNA.

with a separate probe specific for exon 2 (Figures 2D and 3D) and was found to peak much earlier than total type II collagen mRNA.

RT-PCR and RNase protection assays

We wished next to analyse the tissue distribution of the three type IX collagen mRNA species in different newborn mouse tissues by both RT-PCR and RNase protection assays (Figures

Figure 4 Detection of $\alpha 1(IX)$, $\alpha 2(IX)$ and $\alpha 3(IX)$ mRNA species in different tissues by RT-PCR amplification

RNA samples from cartilage (lanes C), skin (lanes S), kidney (lanes K), eye (lanes E), muscle (lanes M), brain (lanes B) and liver (lanes L) were used as templates for random hexamer- and oligo(dT)-primed reverse transcription. Reactions without template RNA (lanes o) and with yeast tRNA (lanes t) were included as controls. Aliquots of cDNA were used for PCR amplification with specific primers corresponding to the NC1 and NC2 domains of the $\alpha 1(IX)$ (A), $\alpha 2(IX)$ (B) and $\alpha 3(IX)$ (C) collagen chains. Amplification products were subjected to electrophoresis on 1.5% (w/v) agarose gels stained with ethidium bromide. Lanes λ , λ DNA size markers.

4 and 5). For RT-PCR, specific primers for the NC1 and NC2 domains of each chain were used. Approximately equal amounts of PCR products for $\alpha 1(IX)$, $\alpha 2(IX)$ and $\alpha 3(IX)$ collagens were obtained from total RNA prepared from cartilage and the eye. From brain RNA, PCR products were detected only for $\alpha 2$ and $\alpha 3$ chains, but not for $\alpha 1$ chain. No expression of type IX collagen chains was detected in skin, kidney, muscle or liver with this technique (Figure 4).

To verify the tissue distribution of the three chains of type IX collagen, RNase protection analysis was performed (Figure 5). As well as in cartilage and the eye, low levels of type IX collagen

Figure 5 Detection of $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ mRNA species in mouse tissues by RNase protection assay

Total RNA (10 μg) isolated from cartilage (lanes C), brain (lanes B), kidney (lanes K), eye (lanes E), skin (lanes S) and liver (lanes L) were hybridized with the cRNA probes for the $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ collagens. Protected fragments were subjected to electrophoresis on 8 M urea/4.5% (w/v) polyacrylamide gels with end-labelled MspI fragments of pBR322 as standards (lanes st). The 524 bp $\alpha 1(\text{IX})$ probe was protected as a 444 bp fragment, the 506 bp $\alpha 2(\text{IX})$ probe as a 444 bp and the 513 bp $\alpha 3(\text{IX})$ probe as a 437 bp fragment. Undigested probes (lanes p) were run on the gel, as were the probes hybridized with yeast tRNA (lanes t). The gel was exposed for 6 days (A) or 1 day (B).

transcripts were identified in brain and skin. The ratios of the three type IX collagen mRNA species in the eye seemed to differ from those in cartilage. In brain RNA the protected fragments for the $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ chains were more abundant than that for the $\alpha 1(\text{IX})$ chain. In skin more mRNA for $\alpha 3(\text{IX})$ collagen was detected than for the other two chains of type IX collagen. Trace amounts of protected $\alpha 2(\text{IX})$ mRNA were detected in kidney RNA, whereas no hybridization signal for any of the type IX chains was detected in liver RNA.

Quantification of mRNA levels of cartilage-specific collagen mRNA species and determination of their ratios during mouse development

To determine more exactly the ratios of types II, type IX and type XI collagen mRNA species during mouse development, slot-blot hybridization analyses were performed. Dilution series of cDNA inserts for $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$, $\alpha 3(\text{IX})$, $\alpha 1(\text{XI})$ and $\alpha 1(\text{II})$ collagens were blotted on the same filters as dilution series of total RNA species from newborn mouse cartilage and from 12.5-day and 16.5-day embryos (Figure 6). The filters were hybridized consecutively with each of the probes. To measure the molar amount of each mRNA in the three RNA samples the hybridization signals of the corresponding cDNA insert were compared with the signals of different RNA species by densitometric analyses. This allowed us to determine the molar amount of each mRNA per total RNA and mRNA ratios in the samples (Table 1). The ratio of mRNA species for $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ collagens in 12.5-day embryos and newborn mouse limb cartilages was close to 1:1:1, but in 16.5-day embryos the number of $\alpha 2(\text{IX})$ mRNA molecules was less than half of that of the other two chains. If equal translational efficiencies of the mRNA

Figure 6 Quantification of mRNA levels of type II, type IX and type XI collagen mRNA species

Duplicate hybridization filters containing dilution series of cDNA inserts for $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$, $\alpha 3(\text{IX})$, $\alpha 1(\text{XI})$ and $\alpha 1(\text{II})$ collagens (from top to bottom: 5×10^{-17} , 10^{-17} , 5×10^{-18} , 10^{-18} , 5×10^{-19} , 10^{-19} and 5×10^{-20} mol of each of the inserts) were prepared. Total RNA species from newborn mouse cartilage and from 12.5-day and 16.5-day embryos (from top to bottom: 4.0, 2.0, 1.0, 0.5, 0.2, 0.1 and 0.05 μg of each RNA) were blotted on the same filters. Similar amounts of yeast tRNA were also blotted as a control. The blotting arrangement is shown in (A). The figure shows one of the filters after consecutive hybridizations with the probes for the $\alpha 1(\text{IX})$ (B), $\alpha 2(\text{IX})$ (C), $\alpha 3(\text{IX})$ (D), $\alpha 1(\text{XI})$ (E) and $\alpha 1(\text{II})$ (F) collagen mRNA species.

Table 1 Quantification of the $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$, $\alpha 3(\text{IX})$, $\alpha 1(\text{XI})$ and $\alpha 1(\text{II})$ collagen mRNA species

The results are given in 10^{-19} mol/ μg of total RNA as calculated from densitometric analyses of the slot-blot hybridizations of total RNA species extracted from mouse embryos at days 12.5 and 14.5 of development, and from limbs from newborn mice.

mRNA	Collagen mRNA quantity (10^{-19} mol/ μg of total RNA)		
	Day 12.5	Day 16.5	Newborn
$\alpha 1(\text{IX})$	4.2	7.7	12
$\alpha 2(\text{IX})$	3.3	2.8	11
$\alpha 3(\text{IX})$	4.4	8.2	13
$\alpha 1(\text{XI})$	2.4	3.1	11
$\alpha 1(\text{II})$	68	120	380

species for cartilage collagens are assumed, the following ratios would be predicted for the type II/type IX/type XI collagens produced: 5.7:1:0.6 at day 12.5, 6.4:1:0.5 at day 16.5, and 10.6:1:0.9 in the newborn mouse limbs.

Localization of type IX collagen transcripts in mouse embryos by hybridization *in situ*

As a final approach to analysing the co-ordination of $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ collagen mRNA species, hybridizations of mouse limbs were performed *in situ* at 12.5, 14.5, 16.5 and 18.5 days of embryonic development. During initial chondrogenesis the expression patterns of the three mRNA species seemed identical in the cartilaginous anlage of bones (results not shown).

On subsequent endochondral ossification the distribution within the chondrocytes of epiphyseal growth plates remained similar; however, the mRNA species for the $\alpha 1(\text{IX})$ and $\alpha 3(\text{IX})$ collagens, but not that for the $\alpha 2(\text{IX})$ collagen mRNA, were also detected in periosteal cells (Figure 7).

DISCUSSION

In the present study we have examined the expression of genes coding for the constituent chains of types II, IX and XI collagens by determining their mRNA levels during mouse embryogenesis with several different techniques. The results indicate that the ratio of type II/type IX/type XI collagen mRNA species varies during embryonic development, whereas the three mRNA species coding for the $\alpha 1$, $\alpha 2$ and $\alpha 3$ chains of type IX collagen exhibit considerable co-ordination in cartilage but not in other tissues. In addition, the results reveal the extraskeletal expression of type IX collagen mRNA species during embryonic development analogous to the other two 'cartilage-specific' collagen types II and XI.

Non-co-ordinated expression of types II, IX and XI collagen mRNA species

We first used several hybridization techniques to determine the expression patterns and molar ratios of types II, IX and XI collagen mRNA species during mouse embryonic development (Figure 6 and Table 1). Assuming equal translational efficiency of the different mRNA species we extrapolated the ratio of types II, IX and XI collagens produced in newborn mouse limb cartilages to be 10.6:1:0.9, which is relatively close to that

Figure 7 Localization of $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ collagen mRNA species in 16.5-day mouse embryo limbs

Serial sections through the antebrachium were analysed by hybridization *in situ* with cRNA probes for the three mRNA species. Dark-field images of the sections reveal overlapping expression patterns of $\alpha 1(\text{IX})$ (a), $\alpha 2(\text{IX})$ (c) and $\alpha 3(\text{IX})$ (e) collagen mRNA species in chondrocytes. The arrows in (a) and (e) highlight the periosteal expression of $\alpha 1(\text{IX})$ and $\alpha 3(\text{IX})$ collagen mRNA species. (b, d, f) Light microscopic views of the dark-field images on the left. The scale bar in (f) represents 100 μm .

(8:1:1) reported by Vaughan et al. [3] for these proteins in 17-day embryonic chick cartilages. In bovine vitreous humour, where type XI collagen is present as heterotypic molecule containing $\alpha 1(\text{XI})$ and $\alpha 2(\text{V})$ chains [6], a ratio of 15:3:2 was obtained for type II/type IX/types V/XI collagens [22].

Several recent studies have suggested that the relatively even diameter of 20–50 nm of collagen fibrils in hyaline cartilage and the vitreous humour [1] depends on the correct ratio of types II, IX and XI collagens. The fibril reconstitution experiments *in vitro* of Eikenberry et al. [23] demonstrated that the assembly of collagen fibrils of the correct diameter required the presence of types II, IX and XI collagens; type II collagen alone resulted in the formation of thick tactoidal fibrils. The natural mouse mutant *cho/cho* does not produce any $\text{pro}\alpha 1(\text{XI})$ collagen chains and suffers from a lethal chondrodysplasia with characteristic thick bundles of collagen fibrils throughout the cartilaginous matrices [9]. Overproduction of normal type II collagen in transgenic mice has also resulted in thick bundles of collagen fibrils in hyaline cartilages [24]. Interestingly, no alterations in cartilage collagen fibril diameter were reported in transgenic mice where both *Col9a1* alleles were inactivated [7], whereas abnormally thin and randomly oriented collagen fibrils were seen in cartilages of transgenic mice producing shortened $\alpha 1(\text{IX})$ collagen chains [8].

Northern analyses of total RNA from 9.5–18.5-day mouse embryos revealed, however, that the mRNA species for types II, IX and XI collagens exhibited different expression patterns (Figures 2 and 3). If constant translational efficiency is assumed, this indicates that the ratio of these collagen types produced varies during development. The mRNA species for the $\alpha 1$, $\alpha 2$ and $\alpha 3$ chains of type IX collagen peak as early as days 12.5–14.5 of development. A similar expression pattern for $\alpha 1(\text{IX})$ collagen mRNA was observed in RT-PCR analysis of mouse limb buds [25]. This is different from the levels of type II or type XI collagen mRNA species, which increase in parallel until day 18.5 [26] (Figures 2 and 3). The biological significance of these observations remains unknown at present.

An additional complexity in the expression of cartilage collagen genes stems from the alternative splicing of exon 2 of type II collagen and from the use of two alternative promoters for the $\alpha 1(\text{IX})$ chain, which are both clearly developmentally regulated also in the mouse [16,26–28]. These studies have suggested that the short form of $\alpha 1(\text{IX})$ collagen mRNA and the long form of $\alpha 1(\text{II})$ collagen mRNA are predominantly associated with expression in extraskeletal tissues, prechondrogenic mesenchyme and immature chondrocytes, whereas the long form of $\alpha 1(\text{IX})$ collagen mRNA and the short form of $\alpha 1(\text{II})$ collagen mRNA are characteristic of mature cartilage. Our results fit well with these observations and suggest further that the alternative splicing of type II collagen transcripts correlates at least temporally with the alternative promoter usage of the $\alpha 1(\text{IX})$ collagen gene.

Type X collagen has been shown to be a major product of terminally differentiated hypertrophic chondrocytes before replacement of the mineralized cartilaginous matrix by bone [29]. In developing mice the level of type X collagen mRNA was low until day 12.5; thereafter a rapid increase occurred until day 16.5 of embryonic development (Figures 2 and 3). This coincides with the period of widespread endochondral ossification [30] and is in agreement with earlier observations.

Co-ordinated regulation of type IX collagen mRNA species in cartilage

Because each type IX collagen molecule is a heterotrimer of $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ chains, the synthesis of these molecules

presents interesting questions about their co-ordinated regulation to yield a relatively constant mRNA ratio of 1:1:1. The gene for the human $\alpha 1(\text{IX})$ collagen (*COL9A1*) has been mapped to chromosome 6q12–q13 [31], whereas gene *COL9A2* is localized in chromosome 1p32.3–p33 [32] and *COL9A3* in chromosome 20q13.3 [20]. In mouse, the $\alpha 1(\text{IX})$ gene (*Col9a1*) has been mapped to chromosome 1 [31] and the gene for the $\alpha 2(\text{IX})$ to chromosome 4 [32]. The chromosomal localization of the mouse $\alpha 3(\text{IX})$ collagen chain remains unknown. Unfortunately the *Col9a2* gene is at present the only gene for which a mammalian promoter sequence is known [33]. Thus no comparisons or functional analyses between regulatory elements can be performed. Although the availability of new cDNA probes makes it possible to measure transcript levels of these genes in different tissues and developmental stages, functional analyses of the promoters are needed to understand how the type IX collagen genes are regulated.

Hybridization analyses *in situ* revealed that the mRNA species for $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$, $\alpha 3(\text{IX})$ collagen mRNA species were very similarly distributed in the cartilaginous anlage of long bones as well as in the epiphyses and growth plates during endochondral ossification. The presence of $\alpha 1(\text{IX})$ and $\alpha 3(\text{IX})$ collagen mRNA species in the perichondrium and periosteum of embryonic long bones was surprising, although the expression of $\alpha 1(\text{IX})$ collagen mRNA has been shown to be an early phenotypic feature of osteogenic cells in rat calvarial bones [34]. As type IX collagen is believed to exist only as a heterotrimer of three different chains, no protein product would be expected to be secreted by these cells. This suggests that the presence of the $\alpha 2(\text{IX})$ chains might be a limiting factor for type IX collagen production in some tissues. Interestingly, differential regulation of the three genes coding for type XI collagen has also been observed during mouse embryonal development [35].

Non-chondrogenic expression of type IX collagen mRNA species

In Northern analyses faint hybridization signals of types II, IX and XI collagen mRNA species were seen as early as day 9.5 (Figures 2 and 3). The expression of type II collagen gene has previously been reported in prechondrogenic mesenchyme and in extraskeletal locations during embryonic development [26,36]. In the present study we also detected low levels of type IX collagen transcripts in brain, skin and kidney of newborn mice by RT-PCR and RNase protection assays (Figures 4 and 5). As the ratios of the $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$ collagen mRNA species seemed to vary considerably in these tissues it is possible that no type IX collagen protein is actually synthesized in these tissues. Interestingly, transient low-level expression of type II collagen has also been detected in brain, skin and kidney, as well as in several other tissues of mouse embryos [36,37]. Extraskeletal expression of $\alpha 1(\text{IX})$ collagen mRNA has also been reported in mouse heart [38]. It remains to be determined whether this expression of type IX and other cartilage collagens represents illegitimate transcription or serves some unknown biological function. The expression of the mRNA species for types II, IX and XI collagen in the eye will be reported elsewhere (M. Savontaus, T. Ihanamäki, M. Perälä, M. Metsäranta, M. Sandberg-Lall and E. Vuorio, unpublished work).

Implications for the function of type IX collagen

Molecular cloning of type IX collagen cDNA species and genes forms the basis for studies on their molecular genetic analyses. The genes for type IX collagen are clearly candidate genes in various hereditary diseases affecting cartilage (chondrodysplasias) and degenerative diseases of joints and spine. Recently

the first mutation affecting type IX collagen causing multiple epiphyseal dysplasia (EDM2) was characterized in the $\alpha 2(\text{IX})$ collagen gene [39].

Transgenic mice harbouring specific mutations of type IX collagen genes have also been used for defining the role of this collagen type in cartilage and other tissues. A dominant negative mutant of the $\alpha 1$ chain of type IX collagen [8] and $\alpha 1(\text{IX})$ gene inactivation in mice [7] are characterized by degenerative joint disease and mild chondrodysplasia. An osteoarthritic phenotype has been observed in transgenic mice harbouring *Col9a2* transgenes with a deletion mutation in the COL2 domain (M. Perälä, unpublished work). The availability of molecular probes for all three constituent chains of mouse type IX collagen and the slot-blot technique employed here allow more reliable quantitative analyses of mRNA levels (including statistical analyses) and should be of use for detailed analyses of existing and future transgenic mice and thus direct us to a better understanding of the roles of the individual constituent molecules of cartilage.

We thank Merja Lakkisto and Tuula Oivanen for expert technical assistance. This study was financially supported by the Academy of Finland, the EU Biotechnology funds and the Sigrid Juselius foundation.

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