Co-expression of collagens II and XI and alternative splicing of exon 2 of collagen II in several developing human tissues

Minna M. SANDBERG, Harri E. HIRVONEN, Kati J. M. ELIMA and Eero I. VUORIO

Department of Medical Biochemistry, University of Turku, SF-20520 Turku, Finland

Northern analyses, RNAase protection assays and *in situ* hybridizations were used to study the expression of the mRNA for the $\alpha 2$ chain of collagen XI and the two different mRNAs generated from the collagen II gene through alternative splicing of exon 2 in several different tissues of 15–19-week-old fetuses. The highest expression levels of procollagen $\alpha 2(\text{XI})$ and $\alpha 1(\text{II})$ mRNAs were detected in cartilage, but, using long exposure times, Northern hybridization revealed the presence of the ~ 5.3 kb procollagen $\alpha 1(\text{II})$ mRNA in most tissues analysed: calvarial and diaphyseal bone, striated and cardiac muscle, skin, brain, lung, kidney, liver, small intestine and colon. Both alternatively spliced forms of the mRNA were present in these tissues. In cartilage, the short form of the procollagen $\alpha 1(\text{II})$ mRNA (without exon 2 sequences) was clearly more abundant, whereas in most of the non-cartilaginous tissues the long form was the predominant one. Low levels of

procollagen $\alpha 2(\text{XI})$ mRNA were also seen in non-cartilaginous tissues: calvarial and diaphyseal bone, kidney, skin, muscle, intestine, liver, brain, and lung. In all the other positive tissues except brain cortex, both collagen II and XI transcripts were observed. The localization of collagen II and XI signals was identical in cartilage, kidney and skin. However, in cartilage the signal with collagen II probe was much higher than that with the collagen $\alpha 2(\text{XI})$ probe. In epidermis the situation was reversed. Our results show considerable co-expression and co-localization of procollagen $\alpha 1(\text{II})$ and $\alpha 2(\text{XI})$ mRNAs in many tissues of developing human fetuses. Since the collagen $\alpha 1(\text{II})$ gene also codes for the $\alpha 3(\text{XI})$ chain of collagen XI we propose that some, but not all, of the expression of the collagen II gene in non-cartilaginous tissues relates to collagen XI production.

INTRODUCTION

Collagen II is the major collagenous component of cartilage and has traditionally been considered very specific for cartilage. With more sensitive detection methods this concept has changed as more and more tissues have been found to contain small amounts of the protein or its mRNA. The list includes the notochord (von der Mark et al., 1976; Swiderski and Solursh, 1992), neural retina (Thorogood et al., 1986), nucleus pulposus (Eyre and Muir, 1977), vitreous humor (Trelstad and Kang, 1974), ventrolateral surface of the neural tube (Thorogood et al., 1986), spinal ganglia (Sandell et al., 1991; Swiderski and Solursh, 1992), and the basement membrane of the otocyst, surface ectoderm, gut endoderm, mesonephric duct, dorsal aorta and heart of the early avian embryo (Koshler and Solursh, 1989; Mallein-Gerin, 1990). Co-polymers of collagens I and II have also been suggested to be present in developing avian cornea (Linsenmayer et al., 1985). Recently, collagen II and its transcripts have been localized in developing mouse by immunohistochemistry and *in situ* hybridization in the cranial mesenchyme, otic and optic vesicles, olfactory conchi, endocardial and mesocardial tissues, pharyngeal endoderm, notochord, neural retina, the corneal and conjunctival epithelia, sclera of developing eye and tail tendon (Cheah et al., 1991; Wood et al., 1991). Transient expression has also been found in the heart, epidermis, calvarial mesenchyme, inner ear and brain of mouse embryos (Cheah et al., 1991).

Another recent aspect of collagen II biochemistry is an alternative splicing step observed in the 5'-end (exon 2) of human, mouse, chick and *Xenopus laevis* pro $\alpha 1(\text{II})$ collagen mRNA (Ryan and Sandell, 1990; Nah and Upholt, 1991; Sandell et al., 1991; Su et al., 1991; Metsäranta et al., 1991). At protein level this codes for a globular domain within the N-terminal propeptide. In human vertebral development the form of collagen

II mRNA which includes exon 2 was observed in prechondrogenic 'fibroblastic' cells, whereas the form without exon 2 was localized in the cells having a chondrocyte phenotype (Sandell et al., 1991). However, it is not known whether this structural modulation, caused by alternative splicing, is associated with expression of collagen II in other tissues or at other developmental stages.

Collagen XI, another fibril-forming collagen, has been found in small quantities in cartilage. It is a heterotrimer composed of three different polypeptide subunits, $\alpha 1(\text{XI})$, $\alpha 2(\text{XI})$ and $\alpha 3(\text{XI})$ (see Kühn, 1986; Vuorio and de Crombrughe, 1990). The $\alpha 3$ chain is not unique to collagen XI, but it is a product of the $\alpha 1(\text{II})$ gene and differs from the $\alpha 1(\text{II})$ chain only by elevated levels of hydroxylysine-linked carbohydrate moieties (Furoto and Miller, 1983). The $\alpha 1(\text{XI})$ and $\alpha 2(\text{XI})$ are distinct gene products (Eyre and Wu, 1987; Burgeson and Hollister, 1979). The first cDNA clones for pro $\alpha 1(\text{XI})$ chain were identified in placental cDNA libraries made for RNA from simian-virus-40-transformed placenta fibroblasts (Bernard et al., 1988). This and the demonstration of $\alpha 1(\text{XI})$ chains in association with $\alpha 1(\text{V})$ and $\alpha 2(\text{V})$ chains in bone (Niyibizi and Eyre, 1989) suggest that the expression of collagen XI is not restricted to cartilaginous tissues.

In the present study we addressed the following questions: How widely is collagen II expressed in non-cartilaginous tissues during human fetal development? Does collagen II expression in these tissues involve alternative splicing of exon 2 or not? Is either form of collagen II mRNA co-expressed with collagen XI?

EXPERIMENTAL

RNA extraction and northern blotting

Total RNAs were extracted as described previously (Chirgwin et al., 1979) from 15–19-week-old human fetuses from therapeutic

abortions. All specimens were obtained with permission of the joint ethical committee of the University Central Hospital and University of Turku (Turku, Finland). Aliquots (15 µg) of total RNA were fractionated on 1%-agarose gels containing 2 M formaldehyde, blotted on to GeneScreen Plus transfer membranes (New England Nuclear) and hybridized with ³²P-labelled random primed cDNA inserts at 42 °C for 20 h. The hybridization mixture and washes were done as suggested by the supplier. The bound probe was detected by autoradiography at -70 °C using X-ray films and intensifying screens.

Construction of cDNA clones

To obtain a probe specific for the alternatively spliced exon 2 of the human collagen II gene, the sequence was amplified by PCR. An *EcoRI* 7.2 kb genomic subclone was used as the template (Strom and Upholt, 1984; Cheah et al., 1985) and two specific oligonucleotides (5'-GAGGCTGGCAGCTGTGTGCA-3') and (5'-CACTGGCAGTGGCGAGGTCA-3'), corresponding to the ends of the exon 2 (from Ryan et al., 1990), were used as primers in the amplification by PCR (Gene Amp; Perkin-Elmer/Cetus).

For cDNA cloning of the collagen α2(XI), total RNA (10 µg) from developing human growth plates was used as the template for cDNA synthesis by Moloney-murine-leukaemia-virus reversible transcriptase under conditions suggested by the supplier (New England Biolabs). Oligo(dT) was used as the primer. Aliquots of cDNA were used for amplification by PCR using specific 21-mer oligonucleotide primers corresponding to amino acids 35-41 (5'-GGCAGTCCTGGGGGGCTGGAG-3') and 127-178 (5'-GGCCTCTGATCGGGGATACAG-3') (from Kimura et al., 1989) of the C-propeptide of the human α2(XI) procollagen.

The PCR reactions were cycled by denaturing at 94 °C for 1 min, annealing at 45 °C for 2 min, and extension at 72 °C for 2 min. After 30 amplification cycles, aliquots of the reaction mixtures were fractionated by electrophoresis on 1.5%-agarose gels, and the specific fragments were purified and cloned into the *EcoRV* site of the Bluescript KS⁺ (Stratagene) vector. The clones were identified by sequencing the double-stranded DNA using the Sequenase reagent kit (U.S. Biochemicals) and [³⁵S]deoxy-(thio)ATP.

For RNAase protection assays, the 224 bp *PvuII-EcoRI* fragment of clone pHCAR1 (Elima et al., 1985) corresponding to the C-propeptide region of collagen II was cloned into the pGEM1 vector between the *SmaI* and *EcoRI* sites. This clone was named 'pMSCol2A1'.

PCR and Southern blotting

The tissue distribution of exon 2 sequences in collagen II mRNA was studied by reverse-transcriptase (RT)-PCR. For this purpose total RNA (10 µg) from the tissues positive by Northern hybridization were used as the template for cDNA synthesis as described above, and a specific oligonucleotide corresponding to exon 8 sequences of collagen II gene as the primer. Aliquots of cDNA were used for amplification by PCR using specific oligonucleotide primers corresponding to the end of exon 1 (5'-GCTGCTCGT-CGCCGCTGT-3') and three last nucleotides of exon 4A and the start of exon 4B (5'-TTGGGTCCTACAATATCCT-3') of collagen II gene (from Su et al., 1989). In the negative-control sample the template DNA was omitted in the PCR reactions. The PCR reactions were performed as described above, but the annealing was at 52 °C and 45 cycles were run. Aliquots of each reaction mixture were fractionated by electrophoresis in 1.5%-agarose gels, with *MspI*-digested pBR322 as size standard, blotted to Pall Biotyne membrane (Pall Process Filtration) under

denaturing conditions and hybridized with the probe for exon 2 of collagen II under conditions suggested by the supplier.

Hybridization probes

Selected fragments of clones for human collagens I, II, III and XI exhibiting the lowest extent of sequence similarity to other collagens were used as probes in Northern and *in situ* hybridization. Specifically, a 372-bp *PvuII-PstI* fragment of cDNA pHCAL1U (Sandberg and Vuorio, 1987) corresponding to the C-propeptide region of the collagen I mRNA and a 400 bp *EcoRI-DraI* fragment of cDNA clone pHCAR3 (Elima et al., 1987) corresponding to the 3'-untranslated region of the collagen II mRNA were used. The entire 207 bp fragment of clone pCol2ex2, corresponding to exon 2 of collagen II, was used. The 295 bp *PstI-PstI* fragment of clone pHFS3 (Sandberg et al., 1989) corresponding to the triple helical and C-telopeptide regions of the collagen III mRNA were used. Likewise the 444 bp fragment of clone pCol11a2, corresponding to the C-propeptide region of α2(XI) procollagen chain, was used. The probes were labelled with [³²P]dCTP by the random priming method for the Northern hybridizations and with [³⁵S]deoxy-(thio)ATP for the *in situ* hybridizations. *BglII* generated 100-790 bp fragments of bacteriophage λDNA were labelled similarly and used as a negative control probe in the *in situ* hybridizations.

For RNAase protection assays the clone pMSCol2a1 was linearized with *HindIII* and the T7 polymerase was used to generate 258 nt antisense cRNA. This cRNA is protected as a 224 nt fragment by collagen II mRNA. The clone pCol2ex2 was used to generate 287 nt antisense cRNA from *HindIII*-linearized template by T7 polymerase. The protected fragment by exon 2 of collagen II is 207 nt. The clone pCol11a2 was linearized with *EcoRI*, and the 504 nt cRNA was generated by T3 polymerase. This cRNA is protected as a 444 nt fragment by α2(XI) procollagen mRNA. Clone pRb233bs (Fung et al., 1987; Hirvonen et al., 1991) linearized with *SacI* was used to generate a 322 nt retinoblastoma (RB) antisense cRNA using T3 polymerase. This cRNA is protected as a 233 nt fragment by retinoblastoma mRNA.

RNAase protection assays

For RNAase protection assays, the cRNA probes were generated from linearized templates (see above) using commercial transcription kits (Transprobe STP and T; Pharmacia, Uppsala, Sweden) with [³²P]UTP (1000 Ci/mmol, Amersham) as the labelled nucleotide. The full-length antisense transcripts were eluted from 4.5%-polyacrylamide gels and hybridized overnight to 15 mg of total cellular RNAs in 80% formamide/400 mM NaCl/40 mM Pipes (pH 6.4)/1 mM EDTA at 56 °C. The hybridizations were terminated by digestion with RNAase A and RNAase T₁, followed by proteinase K digestion, purified by phenol/chloroform extractions, ethanol-precipitated and electrophoresed in 4%-polyacrylamide/8M urea gels with molecular-mass ladders composed of end-labelled *MspI*-generated fragments of pBR322. For each experiment, hybridization with yeast tRNA (15 µg) was performed as a negative control.

In situ hybridization

In situ hybridization for collagens II and XI was carried out as described previously (Sandberg and Vuorio, 1987).

RESULTS AND DISCUSSION

The present study demonstrates a widespread, low-level, expression of mRNAs for collagens II and XI in several human

Figure 1 Northern-blot analysis of total RNAs from developing human fetal tissues

The filters were hybridized with cDNA probes for (a) $\alpha 1(I)$ collagen, (b) $\alpha 1(II)$ collagen, (c) $\alpha 1(III)$ collagen and for $\alpha 2(XI)$ collagen. The position of 28 S rRNA marker is indicated on the left. In (b) the difference in the signals between kidney samples 1 and 2 is due to unequal loading of the total kidney RNAs in the corresponding gel.

fetal issues. However, high-level expression of the $\alpha 1(II)$ gene coding for the homotrimeric collagen II is still very cartilage-specific. Similarly, the highest levels of collagen $\alpha 2(XI)$ mRNA were seen in cartilage. The presence of mRNA transcripts and/or immunoreactive collagens II and XI in non-chondrogenic tissues during development presents interesting questions about the role of the two alternatively spliced forms of the collagen $\alpha 1(II)$ mRNA and their possible correlation with the expression of collagen XI mRNAs. We have attempted to approach these questions by searching for differences and similarities in the expression patterns of these three mRNA species in several human tissues.

New hybridization probes

To obtain the necessary hybridization probes we first constructed two short cDNA clones, pCol2ex2 for exon 2 of collagen II, and pCol11a2 for the C-propeptide of $\alpha 2(XI)$ procollagen. The clones were identified by nucleotide sequencing and found to be identical with those described by Ryan et al. (1990) and Kimura et al. (1989) for exon 2 of collagen II and for the C-propeptide of the human $\alpha 2(XI)$ procollagen (amino acids 35–178) respectively.

Northern hybridizations

Northern analysis of several tissues was performed with probes specific for human collagen I, II, III and XI mRNAs. All tissues studied were found to contain mRNAs for collagens I and III, except liver and lens. However, due to sparsity of material, lens was reliably analysed only with the collagen III probe (Figures 1a and c). In short exposures of approx. 1–2 days, the Northern analyses revealed collagen II mRNA only in total RNA isolated from growth plate and epiphyseal cartilages. However, using longer exposure times (approx. 6 days), hybridization to the ~5.3 kb collagen II mRNA was seen in several other tissues: calvaria, diaphyseal bone, striated and cardiac muscle, skin, brain, lung, kidney, liver, small intestine and colon (Figure 1b). Tissues which did not contain detectable levels of collagen II

mRNA were placenta, testis, spleen and thymus. Since the alternative splicing of exon 2 changes the size of the collagen $\alpha 1(II)$ mRNA only by 207 nt, the two mRNAs cannot be resolved into separate bands in Northern analysis.

The highest expression of collagen $\alpha 2(XI)$ mRNA was detected in the growth plate and epiphyseal cartilages. In Northern analysis low levels were also seen in RNA from calvaria, diaphysis, muscle, skin and brain, but not in placenta, small intestine or colon (Figure 1d).

Table 1 Summary of the estimated protection signal intensities with collagen II, exon 2 of collagen II and collagen $\alpha 2(XI)$ probes in different human fetal tissues

The retinoblastoma (RB) probe was included in the hybridizations as internal standard to verify the amount of RNA used. Symbols: —, no signal; +/—, less than RB signal; +, as intense as RB signal; ++, somewhat more than RB signal; + + +, much more than RB signal; + + + +, the highest signal of all.

Tissue	Probe ...	Signal intensity			RB
		$\alpha 1(II)$ 3'	exon2 of $\alpha 1(II)$	$\alpha 2(XI)$	
Kidney		+++	+++	++	+
Skin		+	+	++	+
Muscle		++	++	++	+
Intestine		++	++	+	+
Liver		+++	+++	+/-	+
Meninges		++	++	+/-	+
Brain cortex		—	—	+	+
Calvaria		+++	+++	+++	+
Diaphysis		++	+	++	+
Lung		+	+	+/-	+
Epiphysis*		++++	+++	+++	+
Growth plate*		++++	+++	+++	+

* A different scale (analysed from a shorter exposure than in Figure 2) was used for the cartilaginous tissues, owing to considerably higher mRNA levels throughout.

Figure 2 RNAase protection analysis of developing human tissues

The four antisense cRNA probes used were: for collagen II (lane 1), for exon 2 sequences of collagen II (lane 2), for $\alpha 2$ chain of collagen XI (lane 3) and for retinoblastoma (RB) (lane 4). The sizes of the protected fragments are 224, 207, 444 and 233 nt respectively. The RB cRNA probe was included in the hybridizations as internal standard to verify the amount of RNA used. 'MW' indicates molecular-mass markers.

RNAase protection assays

RNAase protection assays were performed to ensure that the signal obtained by Northern hybridization with collagen II and XI probes was not due to cross-reaction with other collagen mRNAs. All cRNA probes were designed to contain vector-specific sequences, to allow demonstration of complete digestion of the probes by the RNAases. The retinoblastoma cRNA probe was included in the hybridizations as an internal standard to verify the amount of RNA used. Since the specific activities and sizes of probes were known some quantitation of the specific mRNAs was also possible (Table 1).

Tissues which were positive for collagen II mRNA in Northern hybridization were chosen for RNAase protection analysis with all four probes. Several different expression patterns were observed (Figure 2 and Table 1).

Expression of the longer and shorter forms of collagen II mRNA

On the basis of RNAase protection assays and RT-PCR analyses both the shorter and the longer form of collagen II mRNA were detected in all the tissues analysed, except the cortical plate of brain, where neither signal was observed (Figures 2 and 3).

Southern hybridization of the PCR products with the exon-2-specific probe confirmed that exon 2 was present in mRNA from all the tissues except cortical plate of brain, even in those samples (growth plate, epiphysis and diaphysis) where only weak bands corresponding to the longer mRNA form in PCR were seen (Figure 3). The probe corresponding to exon 2 of collagen II gave only a very faint signal in cartilage (results not shown) in the *in situ* hybridizations whereas the 3'-end probe of collagen II gave a very strong signal as shown previously (Sandberg & Vuorio, 1987; Sandberg et al., 1988). These observations are in accordance with the results of Ryan and Sandell (1990), Sandell et al. (1991) and Nah and Upholt (1991) who suggested that the shorter form of collagen II mRNA is a marker for the chondrocyte phenotype. However, our results indicate that both forms of

collagen II mRNA are expressed to some extent in all the tissues positive with 3'-end probe of collagen II, thus suggesting that neither of the two forms is strictly specific to any tissue. Ryan and Sandell (1990) also found both forms of the collagen II mRNA in fetal skeletal tissues.

In all non-skeletal tissues studied with RT-PCR and RNAase protection assays the longer form of collagen II seemed to be expressed at a higher level than the shorter form (Table 1). The RNAase protection assays, which are more reliable for quantification, gave signals of approximately similar intensity for non-

Figure 3 PCR analysis of alternative splicing of exon 2 of collagen II

(b) PCR products obtained with oligonucleotides corresponding to exons 1 and 4A/4B of collagen II demonstrating the alternatively spliced forms of collagen II. (a) Southern blot of the PCR products with probe for exon 2 of collagen II showing the α -chains which contain exon 2. In diaphysis, epiphysis and growth plate the alternatively spliced form of collagen II without exon 2 was more abundant than in the other tissues. All the other tissues studied, except cortical plate of brain, also seemed to express collagen II mRNA containing exon 2 sequences (b).

Figure 4 *In situ* hybridization of the $\alpha 2$ -chain of collagen XI in developing human long bone

The dark-field image on the right (b) corresponds to the phase-contrast micrograph on the left (a). The bar in (a) represents 100 μm .

skeletal tissues with the 3'-end probe and exon 2 probe, suggesting that a majority of the procollagen $\alpha 1(\text{II})$ mRNA in non-skeletal tissues includes exon 2 sequences. The importance of this observation remains unclear. Since association of the α -chains begins in the C-propeptide, it is unlikely that the globular domain of the N-propeptide coded for by exon 2 would have a role in chain selection. Therefore it is possible that the two resultant procollagen chains form heterotrimers. After removal of the N-propeptide, both types of pro $\alpha 1(\text{II})$ -chains will yield identical mature $\alpha 1(\text{II})$ -chains. This suggests that the alternative splicing event does not affect the building blocks of the cartilage collagen fibril unless it affects chain selection between collagens II and XI. The alternatively spliced domain of collagen II corresponds to a region in procollagens $\alpha 1(\text{I})$ and $\alpha 1(\text{III})$ which has been implicated in fibril formation (Fleischmajer et al., 1981) and feedback control of collagen biosynthesis (Wiestner et al., 1979; Wu et al., 1986). The fact that exon-2-containing mRNAs are more abundant in non-chondrogenic tissues suggests that sequences encoded by this exon could play a regulatory role in the biosynthesis and fibrillar organization of collagen II in these tissues. Finally, a possibility exists that a signalling function is invested in the domain involved in the alternative splicing which could direct the pro α -chains into collagen II or XI molecules.

Collagen II and XI mRNAs in skeletal tissues

As expected, the highest levels of the two $\alpha 1(\text{II})$ and the $\alpha 2(\text{XI})$ collagen mRNAs were seen in epiphyseal and growth-plate cartilages (Figure 2). In RNAase protection assays the strongest signal was obtained with the probe corresponding to the 3'-end of collagen II. This was expected, since this probe detects both forms of collagen II mRNA, including the message coding for those α -chains which will be modified into $\alpha 3(\text{XI})$ chains. At the level of individual chondrocytes the chains of collagens II and XI are not always synthesized simultaneously (Swoboda et al., 1989). Our present *in situ*-hybridization data on growth cartilages agrees with these results, as the expression of the collagen $\alpha 2(\text{XI})$ mRNA was less evenly distributed than that of collagen $\alpha 1(\text{II})$ mRNA, although both signals were similarly accentuated in the zone of hypertrophic chondrocytes (Figure 4).

Our observation of collagen $\alpha 2(\text{XI})$ mRNA in human di-

aphyseal bone is in accordance with the findings of collagen XI chains in bovine bone, apparently forming heterotrimers with collagen V chains (Niyibizi and Eyre, 1989). In diaphyseal bone samples, RNAase protection experiments suggested that the expression of collagen $\alpha 2(\text{XI})$ mRNA was about equal to total collagen $\alpha 1(\text{II})$ mRNA measured with the 3'-probe. The low signal obtained with exon 2 probe both in RNAase protection experiments and in *in situ* hybridizations shows that the shorter form of procollagen II is the predominant one in bone, and suggests that this processing variant serves as the $\alpha 3$ -chain of collagen XI in diaphyseal bone. According to *in situ* hybridization, osteoblasts were shown to synthesize mRNA for collagen $\alpha 2(\text{XI})$ in bone (Figure 4).

Since intramembranous bone formation does not proceed via a cartilage intermediate calvarial bones have been thought not to synthesize the collagens found in cartilage, e.g. collagens II and XI. However, our present Northern and RNAase protection results show expression of collagen II and XI genes in the developing human calvaria. This is in accordance with the recent findings in the chick (McDonald and Tuan, 1989; Nah et al., 1992) and the mouse (Cheah et al., 1991), indicating some synthesis of collagen II in developing calvarial bones. The collagen $\alpha 1(\text{II})$ mRNA is certainly present in occipital calvarial bone of mouse, which is cartilaginous at birth and undergoes endochondral ossification (Rintala et al., 1993).

Collagen II and XI mRNAs in non-chondrogenic tissues

Several different patterns of short and long forms of collagen $\alpha 1(\text{II})$ mRNA and collagen $\alpha 2(\text{XI})$ mRNA were observed in the tissues studied (Table 1). The intensities of the RNAase protection signals suggested similar levels of procollagen II (long form) mRNA and procollagen $\alpha 2(\text{XI})$ mRNA in muscle and calvarial bones. We propose that the expression of the long form of the collagen $\alpha 1(\text{II})$ mRNA in these tissues is connected to the expression of collagen XI.

In kidney the *in situ*-hybridization signals with collagen II (Figures 5a-e) and XI (Figure 5f) probes were identically localized along the periphery of the developing glomeruli. However, the level of collagen $\alpha 1(\text{II})$ mRNA (mainly long form) in RNAase protection assays was higher than that of $\alpha 2(\text{XI})$

Figure 5 *In situ* hybridization with probes for collagen II (a–d), exon 2 of collagen II (e) and for $\alpha 2$ -chain of collagen XI (f) in developing human kidney

The dark-field images on the right correspond to the phase-contrast micrographs on the left or above. The bar represents 100 μm in (a) (for a and b) and 100 μm in c (for c–f).

mRNA. This suggests that only a fraction of $\alpha 1(\text{II})$ chains are used as $\alpha 3(\text{XI})$ chains.

In situ hybridization of skin samples also revealed colocalization of collagen II and XI mRNAs in epidermis (Figure 6). However, the ratio of the two mRNAs in RNAase protection experiments is different from kidney: there appears to be more $\alpha 2(\text{XI})$ mRNA than either form of $\alpha 1(\text{II})$ mRNA. Epidermis must therefore be considered an example of a tissue where the number of $\alpha 3(\text{XI})$ chains is rate-limiting for production of

collagen XI. Our rather surprising findings of collagen II and XI mRNAs in skin are in agreement with earlier data on chick and mice, where collagen II or its mRNA has also been detected in epidermis (Thorogood et al., 1986; Fitch et al., 1989; Koshler and Solursh, 1989).

In total RNA from kidney, intestine, liver, meninges and lung the signal intensity in the RNAase protection assay with $\alpha 2(\text{XI})$ probe was lower than that with collagen II probes, the latter giving approximately equal signals. Accordingly it seems that

Figure 6 Expression of pro α 1(II) collagen (a and b) and pro α 2(XI) collagen (c) in the epidermis of developing human fetal skin

The dark-field images correspond to the phase-contrast micrograph in (a). The bar represents 100 μ m and applies to (b) and (c) as well as (a).

both collagens II and XI are synthesized in these tissues and that most of the collagen II mRNA is of the longer variety, that is, it contains exon 2 sequences. In the lung the *in situ*-hybridization signals with the procollagen α 1(II) and α 2(XI) probes were observed only in the cartilaginous structures of developing bronchi, demonstrating that most, if not all, of the mRNAs are present in chondrocytes of the bronchial cartilages. In the other

tissues the *in situ* hybridization with the collagen II probes or with the collagen XI probe gave only weak signals throughout the tissue sections. In Northern analysis of liver RNA, very faint signals could be detected with collagen I and III probes (compared with the other tissues studied; Figure 1), whereas a more intense band was seen with collagen II probe in Northern and RNAase protection analyses. This suggests that probes for collagens II and XI should be included in studies on liver metabolism and hepatic fibrosis.

By Northern analysis, brain was observed to express both collagen II and XI genes. For RNAase protection experiments, brain cortex was carefully separated from the meninges for RNA extraction. In brain cortex RNA, no signal was observed with the collagen II probes, whereas mRNA for collagen α 2(XI) could be detected. This finding suggests that collagen XI molecules do not always contain α 3(XI) chains. On the basis of structural analyses of the C-propeptide of the pro α 2(XI) chains Kimura et al. (1989) have also suggested that these chains could form both homo- and hetero-trimeric molecules. In RNA from the meninges, both probes for collagen II mRNA gave signals in the RNAase protection assay; a weak signal was also seen with the α 2(XI) probe. These results suggest that the Northern signal in the brain sample with collagen II probe is due to contaminating meninges. On the other hand, transient expression of collagen II has been found in developing fetal mouse brain (Cheah et al., 1991) and in total RNA from embryonic chick brain (Nah et al., 1992). Thus the difference in the signal with collagen II probes in Northern and RNAase protection analyses could be partly due to differences in the age of the brain samples used or between species.

In summary, our results based on Northern analyses, RNAase protection assays, RT-PCR and *in situ* hybridization revealed several different expression patterns for the two different forms of the collagen II mRNA and for the collagen α 2(XI) mRNA. In all the samples analysed except brain cortex, all three transcripts were observed, suggesting some co-ordination in their regulation in many, but not in all tissues. In most of the non-skeletal tissues the α 1(II) transcripts contained exon 2 regardless of the level of collagen α 2(XI) transcripts, whereas in cartilaginous tissues a majority of the collagen α 1(II) mRNA was without exon 2 sequences.

We conclude that the presence of collagen α 1(II) mRNAs in non-chondrogenic tissues can only partially be explained by production of α 3-chains for collagen XI. The role of widespread collagen II and XI expression remains unknown. One possible explanation could be 'illegitimate transcription' as described by Chan and Cole (1991) for α 1(II) gene in fibroblasts and lymphoblastoid cells. However, the fact that the phenomenon is seen during chick, mouse and human development suggests that it serves some specific function(s). We propose that the expression patterns of collagen II and XI during development may have relevance to the non-skeletal defects found in many human chondrodysplasias, e.g. congenital abnormalities of hippocampal formation and heart defects (Wynne-Davies et al., 1985; Knisely and Ambler, 1988). Detailed analyses of transgenic mice carrying mutated collagen II genes may also be useful for elucidating the role of collagen II in non-chondrogenic tissues.

Merja Lakkisto and Tuula Oivanen are gratefully acknowledged for expert technical assistance. This study was financially supported by the Medical Research Council of the Finnish Academy.

REFERENCES

- Bernard, M., Yoshioka, H., Rodriguez, E., van der Rest, M., Kimura, T., Ninomiya, Y., Olsen, B. R. and Ramirez, F. (1988) *J. Biol. Chem.* **263**, 17159–17166

- Burgeson, R. E. and Hollister, D. W. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1124–1131
- Chan, D. and Cole, W. G. (1991) *J. Biol. Chem.* **266**, 12487–12494
- Cheah, K. S. E., Stoker, N. G., Griffin, J. R., Grosveld, F. G. and Solomon, E. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2555–2559
- Cheah, K. S. E., Lau, E. T., Au, P. K. C. and Tam, P. P. L. (1991) *Development* **111**, 945–953
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- Elima, K., Mäkelä, J., Vuorio, T., Kauppinen, S., Knowles, J. and Vuorio, E. (1985) *Biochem. J.* **229**, 183–188
- Elima, K., Vuorio, T. and Vuorio, E. (1987) *Nucleic Acids Res.* **15**, 9499–9504
- Eyre, D. and Wu, J. J. (1987) in *Structure and Function of Collagen Types* (Mayne, R. and Burgeson, R. E., eds.), pp. 261–281, Academic Press, New York
- Eyre, D. R. and Muir, H. (1977) *Biochim. Biophys. Acta* **492**, 29–42
- Fitch, J. M., Mentzer, A., Mayne, R. and Linsenmayer, T. F. (1989) *Development* **105**, 85–95
- Fleischmajer, R., Timpl, R., Tuderman, L., Raisher, L., Wiestner, M., Perlsh, J. S. and Graves, P. N. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7360–7364
- Fung, Y.-K. T., Murphree, A. L., T'Ang, A., Qian, J., Hinrichs, S. H. and Benedict, W. F. (1987) *Science* **236**, 1657–1661
- Furoto, D. K. and Miller, E. J. (1983) *Arch. Biochem. Biophys.* **226**, 604–611
- Hirvonen, H., Hukkanen, V., Salmi, T. T., Mäkelä, T. P., Pelliniemi, T.-T., Knuutila, S. and Alitalo, R. (1991) *Blood* **78**, 3012–3020
- Kimura, T., Cheah, K. S. E., Chan, S. D. H., Lui, V. C. H., Mattei, M.-G., van der Rest, M., Ono, K., Solomon, E., Ninomiya, Y. and Olsen, B. R. (1989) *J. Biol. Chem.* **264**, 13910–13916
- Knisely, A. S. and Ambler, M. W. (1988) *Pediatr. Neurosci.* **14**, 169–176
- Koshler, R. A. and Solursh, M. (1989) *Dev. Biol.* **131**, 558–566
- Kühn, K. (1986) *Rheumatology* **10**, 29–69
- Linsenmayer, T. F., Fitch, J. M., Gross, J. and Mayne, R. (1985) *Ann. N.Y. Acad. Sci.* **440**, 232–245
- Mallein-Gerin, F. (1990) *Roux's Arch. Dev. Biol.* **198**, 363–369
- McDonald, S. A. and Tuan, R. S. (1989) *Dev. Biol.* **133**, 221–234
- Metsäranta, M., Toman, D., de Crombrughe, B. and Vuorio, E. (1991) *J. Biol. Chem.* **266**, 16862–16869
- Nah, H.-D. and Upholt, W. B. (1991) *J. Biol. Chem.* **266**, 23446–23452
- Nah, H.-D., Barembaum, M. and Upholt, W. B. (1992) *J. Biol. Chem.* **267**, 22581–22586
- Niyibizi, C. and Eyre, D. R. (1989) *FEBS Lett.* **242**, 314–318
- Rintala, M., Metsäranta, M., Garofalo, S., de Crombrughe, B., Vuorio, E. and Rönning, O. (1993) *J. Craniofac. Genetics Dev. Biol.*, in the press
- Ryan, M. C. and Sandell, L. J. (1990) *J. Biol. Chem.* **265**, 10334–10339
- Ryan, M. C., Sieraski, M. and Sandell, L. J. (1990) *Genomics* **8**, 41–48
- Sandberg, M. and Vuorio, E. (1987) *J. Cell. Biol.* **104**, 1077–1084
- Sandberg, M., Vuorio, T., Hirvonen, H., Alitalo, K. and Vuorio, E. (1988) *Development* **102**, 461–470
- Sandberg, M., Mäkelä, J. K., Multimäki, P., Vuorio, T. and Vuorio, E. (1989) *Matrix* **9**, 82–91
- Sandell, L. J., Morris, N., Robbins, J. R. and Goldring, M. B. (1991) *J. Cell Biol.* **114**, 1307–1319
- Strom, C. M. and Upholt, W. B. (1984) *Nucleic Acids Res.* **12**, 1025–1038
- Su, M.-W., Benson-Chanda, V., Vissing, H. and Ramirez, F. (1989) *Genomics* **4**, 438–441 (1989)
- Su, M.-W., Suzuki, H. R., Bieker, J. J., Solursh, M. and Ramirez, F. (1991) *J. Cell Biol.* **115**, 565–575
- Swiderski, R. E. and Solursh, M. (1992) *Dev. Dynam.* **194**, 118–127
- Swoboda, B., Holmdahl, R., Stöb, H. and von der Mark, K. (1989) *J. Cell Biol.* **109**, 1363–1369
- Thorogood, P., Bee, D. E. and von der Mark, K. (1986) *Dev. Biol.* **116**, 497–509
- Trelstad, R. L. and Kang, A. H. (1974) *Exp. Eye Res.* **18**, 395–406
- von der Mark, H., von der Mark, K. and Gay, S. (1976) *Dev. Biol.* **48**, 237–249
- Vuorio, E. and de Crombrughe, B. (1990) *Annu. Rev. Biochem.* **59**, 837–872
- Wiestner, M., Krieg, T., Hörlein, D., Glanville, R. W., Fietzek, R. and Müller, P. K. (1979) *J. Biol. Chem.* **254**, 7016–7023
- Wood, A., Ashurst, D., Corbett, A. and Thorogood, P. (1991) *Development* **111**, 955–968
- Wu, C. H., Donovan, C. B. and Wu, G. Y. (1986) *J. Biol. Chem.* **261**, 10482–10484
- Wynne-Davies, R., Hall, C. M. and Apley, A. G. (1985) *Atlas of Skeletal Dysplasias*, Churchill Livingstone, Edinburgh
- Yoshioka, H. and Ramirez, F. (1990) *J. Biol. Chem.* **265**, 6423–6426