Expression of the mouse α1(II) collagen gene is not restricted to cartilage during development

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Summary

The mouse α1(II) collagen gene has been isolated and a 5' portion of the gene which has low homology to other collagen genes was used to study the pattern of expression during mouse embryogenesis. In situ hybridization studies show that in the mouse, like the chick, α1(II) collagen is expressed in chondrogenic tissues in advance of chondrocyte differentiation. The gene is expressed early in embryogenesis at 9.5 days both in the cranial mesenchyme destined for the chondrocranium, and the sclerotome of the somites, and at 12.5 days in the primordia of the hyoid and the laryngeal cartilage. Type II collagen gene transcripts were found in all the chondrogenic tissues of the axial and appendicular skeleton until the onset of endochondral ossification. Expression of α1(II) collagen mRNA was also observed in non-chondrogenic tissues such as the notochord which may be responsible for inducing chondrogenesis in somitic mesoderm, neural retina, the corneal and conjunctival epithelia and sclera of the developing eye. Expression in the tail tendon was late, at 16.5-18.5 days.

Transient expression was also found in the heart at 9.5-12.5 days, the epidermis at 10.5-14.5 days, the calvarial mesenchyme at 12.5-16.5 days, the inner ear at 14.5 days and the fetal brain from 9.5-14.5 days. Within the neural tube, α1(II) collagen mRNA was localized in the proliferative ventricular cells of the forebrain and midbrain of 9.5- to 10.5-day embryos. Subsequently, transcription of the α1(II) collagen gene was confined to restricted areas of the rhombencephalic basal plate, the ventricular layer of the hindbrain and the cervical spinal cord. These examples of expression of the type II collagen gene in the developing nervous system seem to suggest that active transcription of this gene might be associated with early stages of neuroblast differentiation. Type II collagen may therefore have additional roles in development unrelated to chondrogenesis.

Key words: α1(II) collagen gene, mouse embryo, in situ hybridization, chondrogenesis, neurogenesis.

Introduction

Collagens are major structural proteins of the extracellular matrix and the correct temporal and tissue-specific expression of the collagen genes is crucial for the proper development of form during morphogenesis (Adamson, 1982; Reddi, 1984). The critical role of the expression of some collagen genes in mammalian development is illustrated by the perinatal lethality caused by the intergration of a retrovirus in the 5' region of the mouse α1(I) collagen gene (Schniecke et al. 1983; Harbers et al. 1984).

In vertebrates, there are at least 13 types of collagens made up of 25 genetically distinct α chains which are expressed in various combinations and amounts in different tissues (Miller and Gay, 1987; Vuorio and deCrombrugghe, 1990). Type II collagen has been classically recognized as the major collagenous component of cartilage. Because type II collagen is synthesized in mesenchymal cells in advance of overt chondrogenesis (Kravis and Upholt, 1985; Kosher et al. 1986; von der Mark and von der Mark, 1977; Swalla et al. 1988), it has been suggested that the expression of the α1(II) collagen gene may define and determine the sites and timing of chondrogenesis (Thorogood et al. 1986).

Apart from chondrocytes and their precursor cells, the presence of type II collagen has also been found by biochemical and immunohistochemical staining methods in non-chondrogenic tissues in avian embryos (chick and quail). Such tissues include the notochord (von der Mark et al. 1976), neural retina (Newsome et al. 1976; Smith et al. 1976; Von der Mark et al. 1977; Linsenmeyer and Little, 1978; Oettinger et al. 1985; Thorogood et al. 1986), corneal epithelium (Linsenmeyer et al. 1977; Hayashi et al. 1988; Fitch et al. 1988, 1989), vitreous body (Trelstad and Kang, 1974), ventrolateral surface of the neural tube (Thorogood et

al. 1986) and in the basement membrane of the otocyst, surface ectoderm, gut endoderm, mesonephric duct, dorsal aorta and heart (Kosher and Solursh, 1989; Mallein-Gerin, 1990). These recent findings of widespread expression of the type II collagen gene in the early avian embryo are strongly suggestive of a function other than for chondrogenic development.

In contrast to the avian species, the temporal and spatial pattern of expression of the α1(II) collagen gene has not been studied in the mammalian embryo. We report here the isolation of the 5′ first exon of the mouse α1(II) collagen gene. DNA sequence comparisons between the mouse, rat and human α1(II) collagen genes show that the gene is highly conserved at both nucleotide and amino acid levels. Antisense RNA probes for mouse α1(II) collagen mRNA were used to determine, by *in situ* hybridization, whether the α1(II) collagen gene in the developing mouse is expressed in both chondrogenic and non-chondrogenic tissues. An understanding of the normal expression of type II collagen is also essential for the interpretation of future studies on the phenotypic consequences of genetic defects in the α1(II) collagen gene.
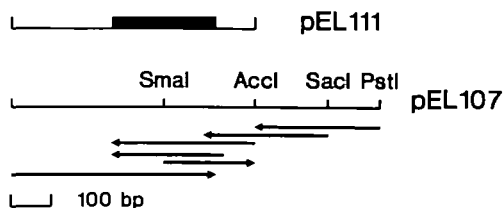
Materials and methods

Isolation of the mouse α1(II) collagen gene

A mouse genomic library constructed in the cosmid vector, Lorist2 (gift of P. Little, Imperial College, London), was screened by hybridization methods described by Cross and Little (1986), using as a probe, a 9.8 kb *Eco*RI fragment from the human α1(II) collagen gene (Cheah *et al.* 1985). Library filters were washed in 1×SSC, 0.1 % SDS at 65°C. From this screening, strongly hybridising clones were isolated and characterised. One clone was chosen for further study because of its strong hybridization to the 5′ and 3′ ends of the human α1(II) collagen gene (Cheah, *et al.* 1991). An 850 bp *Pst*I–*Sal*I DNA fragment which hybridised strongly to the 5′ first exon of the human gene was subcloned into Bluescript KS⁻ (Stratagene, USA) and sequenced (Fig. 1). This subclone, pEL107, and a shorter derivative, pEL111, containing the 600 bp *Acc*I–*Sal*I DNA fragment subcloned into pGEM3 (Promega, USA) were used for *in situ* hybridization studies.

DNA sequencing

The region covering the first exon of the mouse α1(II)

Fig. 1. Restriction enzyme map of DNA subclones used for DNA sequencing, RNAase protection assays and *in situ* hybridization. pEL111 and pEL107 are overlapping subclones containing the 5′ first exon of the mouse α1(II) collagen gene. The position of exon 1 is represented as a black box. The portions of pEL107 sequenced and the direction of DNA sequencing are shown by the arrows.

collagen gene was sequenced on both strands by the dideoxy chain termination method (Sanger *et al.* 1977) on double-stranded DNA using Sequenase 2.0 (United States Biochemical, USA) following the manufacturer's protocol. The complete DNA fragment was sequenced using the exonuclease/mungbean nuclease method of generating overlapping clones (Henikoff, 1987). Sequence comparison analyses were performed using the UWGCG computer programs of Devereux *et al.* (1984).

RNA isolation and RNAase protection assays

Total RNA was isolated from 16.5-day mouse fetuses using the lithium chloride–urea method (Lovell-Badge, 1987). pEL111 was used to generate ^{32}P-labelled antisense RNA probes for RNAase protection assays using T7 RNA polymerase as described by Melton *et al.* (1984). Hybridization was in 80 % formamide, 40 mM Pipes pH 6.7, 400 mM NaCl, 1 mM EDTA at 50°C for 18 h. Non-hybridized RNA was removed by digestion with RNAase A (10 μg ml^{-1}) and RNAase T1 (0.5 μg ml^{-1}) for 15 min at 37°C and the protected RNA fragments electrophoresed on 6 % polyacrylamide/8M urea gels as described by Lovell-Badge (1987).
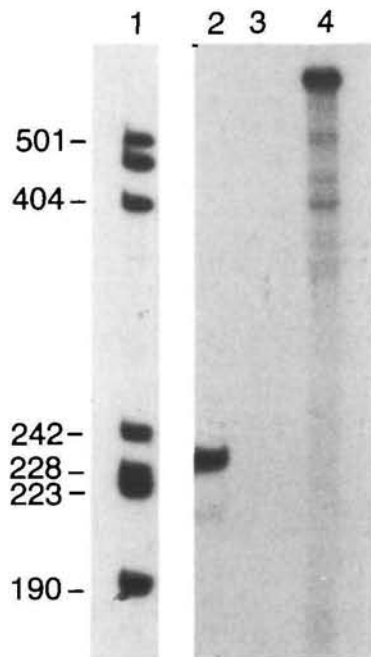
In situ hybridization

CBA/n mouse fetuses at 9.5 to 18.5 days *p.c.* (plug day=0.5 *p.c.*) were collected and processed for *in situ* hybridization as described by Wilkinson *et al.* (1987). Single-stranded ^{35}S-labelled sense and antisense RNA probes were generated from pEL107 using T3 and T7 RNA polymerase, respectively, and from pEL111 using SP6 and T7 RNA polymerase, respectively. Antisense ^{35}S-labelled RNA probes for mouse α1(I) collagen were generated from a subclone containing exon 2 of the gene (gift of R. Lovell-Badge, National Institute for Medical Research, London). LM-1 (Amersham) or K5 (Ilford) photographic emulsion was used for autoradiography. Slides were developed in Phenisol (Ilford) and stained with Harris haematoxylin and eosin or toluidine blue. Photographs of sections were taken using Kodak Ektachrome ASA 50 film on a Zeiss microscope with dark-field illumination alone or by a combination of dark-field illumination followed by bright-field microscopy with a red filter to produce superimposed images.
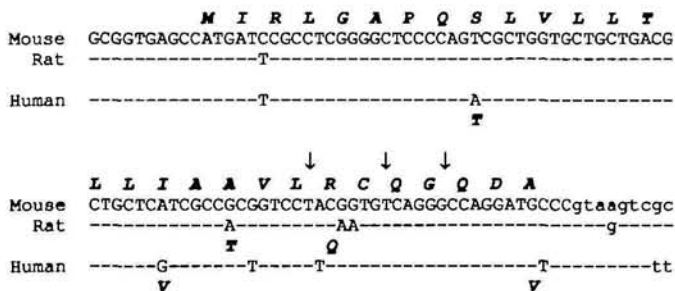
Results

The 5′ end of mouse α1(II) collagen gene is highly conserved

The DNA fragment encoding the 5′ first exon of the mouse α1(II) collagen gene was identified by strong cross-hybridization to the equivalent region of the human COL2A1 gene previously isolated (Cheah *et al.* 1985) and by nucleotide sequence analysis. DNA sequencing of pEL107 showed that it contained the 5′ first exon of the α1(II) collagen gene, which codes for the 5′ untranslated region, the signal peptide and the start of the amino-propeptide of the pro-α1(II) chain. The size of the 5′ first exon as determined by RNAase protection (Fig. 2) is approximately 235 bp and is therefore similar in size to the equivalent part of the rat and human genes (240 bp) (Kohno *et al.* 1985; Nunez *et al.* 1986). Comparison of the coding sequence of this region of the mouse α1(II) collagen gene with that of the human and rat genes show homologies of 94 % and 95 %, respectively, at the nucleotide level and 93 % at

Fig. 2. The 5' first exon of the mouse α1(II) collagen gene. Autoradiogram showing RNA fragments from 16.5-day fetuses protected from RNAase digestion after hybridization to ^{32}P-labelled antisense RNA transcripts synthesized from pEL111. Samples were: lane 1: DNA molecular weight markers; 2: 16.5-day mouse fetal RNA hybridised to antisense pEL111; 3: No RNA control; 4: Intact antisense RNA transcript.

Fig. 3. DNA and amino acid sequence comparisons between exon 1 of the mouse, rat and human α1(II) collagen genes. The sequence of part of the 5' untranslated region, initiation codon, the signal peptide and the start of the NH₂-propeptide are shown. Amino acid residues are in the standard single-letter code and shown in italics and bold type. Only base differences are shown, sequence identity is denoted by a dash. Intron sequences are in lower case, exon sequences in upper case. The arrows denote the potential signal peptide cleavage sites. Sequences for the rat and human α1(II) collagen genes are taken from Kohno *et al.* 1985, and Nunez *et al.* 1986 respectively.

the amino acid level (Fig. 3). This exon is therefore highly conserved in the three mammals, especially between mouse and rat.

Homologies of up to 60 % exist between different

collagens, particularly in domains such as the triple-helical and carboxy terminal portions (Dion and Myers, 1987; Ramirez *et al.* 1985). The absence of other lower molecular weight protected RNA fragments in the RNAase protection assays with 16.5 day fetuses suggest that there were no other mRNA transcripts in the mouse embryo with any significant homology to the probe. Nucleotide comparisons of this portion of the mouse α1(II) collagen gene with available sequences of other collagen genes [α1(I), α1(IV), α2(IV), α2(I)] showed no homology (data not shown). This region of the gene therefore provides a probe specific for α1(II) collagen mRNA for this *in situ* hybridization study. Similar RNAase protection results were obtained with 10.5- and 13.5-day fetuses, confirming the specificity of the probe at earlier times of development (Cheah and Lau, unpublished). The use of this same RNA probe for *in situ* hybridization combined with RNAase treatment of sections, post-hybridization, is equivalent to RNAase protection assays, *in situ*, and also reduces the possibility of generating artifactual positive signals.

Type II collagen mRNA expression in chondrogenic tissues

Transcripts of the type II collagen gene were screened for in tissues of fetuses at 8.5 to 18.5 days *p.c.* by *in situ* hybridization with antisense RNA probes transcribed from pEL107 and pEL111. Both probes produced similar results.

A low level of hybridization was observed in the 8.5-day conceptus over the extraembryonic yolk sac (data not shown). Significant hybridization was first detected in the sclerotome of the somites and the notochord in the trunk of 9.5-day embryos (Fig. 4A). Transcripts were not found in the dermamyotome, the newly segmented somites and the presomitic mesoderm. A similar but stronger pattern of hybridization was found in the perinotochordal sclerotome (Fig. 4B), the pre-vertebral and vertebral mass and in the paraxial mesoderm (Fig. 4C,D,E) of the 10.5- to 12.5-day fetuses. Specific hybridization was also detected as early as 9.5 days *p.c.* in the cranial mesenchyme associated with the mid- and hindbrain. Progressively more type II collagen mRNA was found in the cranial mesenchyme as the embryo developed (Fig. 4D). Expression was observed in mesenchymal cells well before any overt chondrogenesis could be detected. Other examples of an early expression of the α1(II) collagen gene are the primordia of the hyoid and thyroid cartilages located at the root of the tongue of the 12.5-day fetus (Fig. 4F). An early expression of α1(II) collagen mRNA was therefore observed in mesenchymal cells well ahead of any overt chondrogenesis which is normally first detected at 12.5–14 days *p.c.* (Rugh, 1968). By 14.5 to 16.5 days *p.c.* prominent expression of the α1(II) transcripts was found in all cartilaginous structures such as the vertebral column, the chondrocranium (Fig. 4G,H), otic capsule, nasal septum, jaw, limb cartilage, tracheal rings and ribs (Fig. 4H). In contrast, hybridization with the probe for α1(I) collagen mRNA showed a very different pattern of expression (Fig. 4I).

Type I collagen mRNA was not expressed in any chondrogenic tissue but was found at high levels in the skin, calvarium, fascial and peritoneal membranes surrounding internal organs, ossified bones and the perichondrium.

Expression of α1 (II) collagen mRNA in non-chondrogenic tissues

Type II collagen gene transcripts were confined not just to cartilaginous tissues and their precursors. Expression of α1(II) collagen mRNA was also found in the calvarial mesenchyme, the notochord, the heart, the epidermis, the tail tendon and the fetal brain. Type II collagen mRNA was detected in the ectomesenchyme beneath the surface ectoderm in the 12.5- (Fig. 4D) to 14.5-day fetus (Fig. 4G,H). The level of transcript was high up to 16.5 days p.c. by which time the mesenchymal cells had differentiated to form osteoblasts of the calvarium during intramembraneous ossification. The developing calvarial bones were one of the few sites at which α1(II) and α1(I) collagen genes were co-expressed. This is in contrast to the other bones of the skull which form differently by endochondral ossification of a cartilaginous anlage. The levels of expression of α1(II) collagen mRNA in the developing calvaria was relatively lower than in the cartilage and lower than the expression of the α1(I) collagen gene.

The notochord of 9.5- to 12.5-day fetuses showed a strongly positive hybridization with the type II collagen RNA probes (Fig. 5A). In normal development, with the exception of the primordia of the nucleus pulposus of the intervertebral disc, most parts of the notochord regress during the formation of the cartilaginous vertebral body. There was a concomitant diminution of the intensity of hybridization in the notochordal tissues of the 14.5 and 16.5-day fetuses. The α1(II) collagen gene was transiently expressed in the epicardium and the myocardium of the heart in 9.5 to 12.5-day fetuses (Fig. 5B). Type II collagen mRNA was not detected in the endocardium or the pericardium, and was absent at later stages of cardiac development. However, type I collagen mRNA was expressed in the pericardium instead. Expression of α1(II) collagen mRNA was also detected transiently in the surface ectoderm at 10.5 to 14.5 days p.c. (Fig. 4C,E,H and 5A) but was absent by 16.5 days p.c. In contrast to the early expression in the heart and surface ectoderm, type II collagen mRNA was found much later in the tail tendon of the 16.5- to 18.5 day fetuses (Fig. 5C).
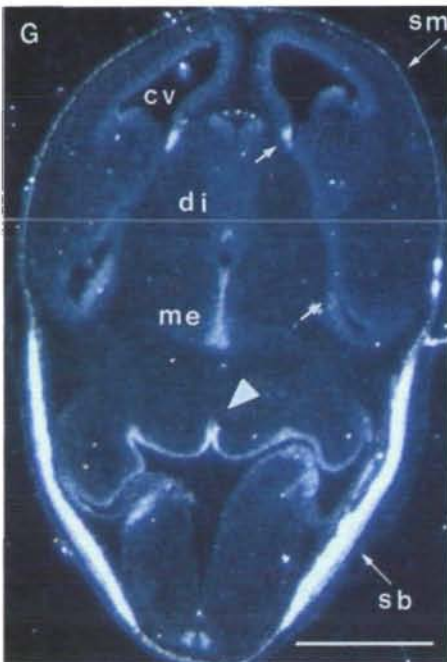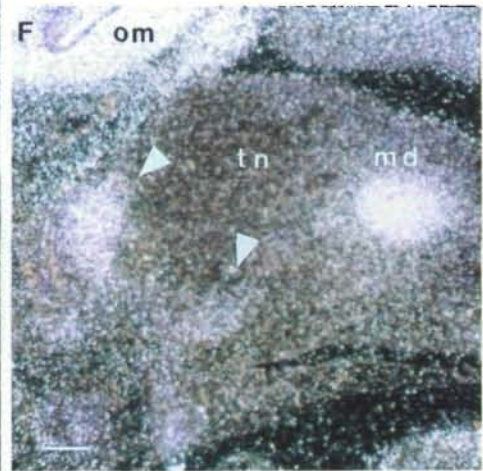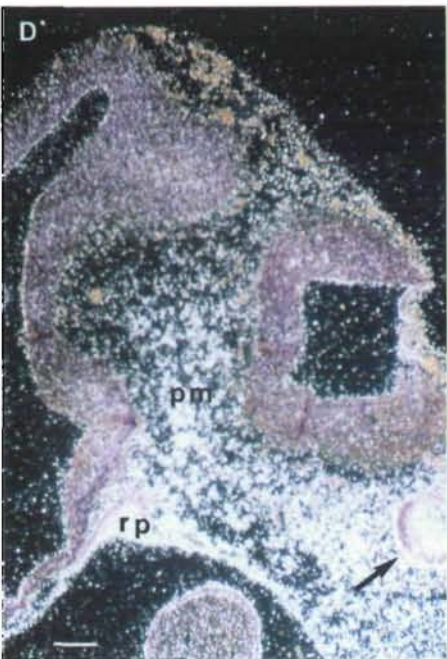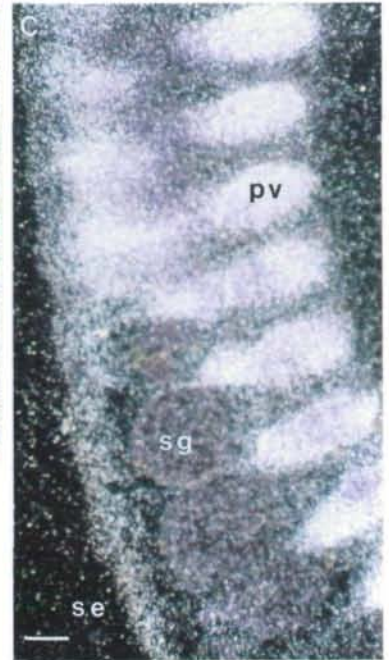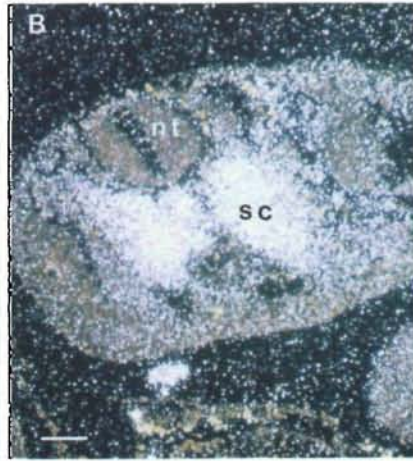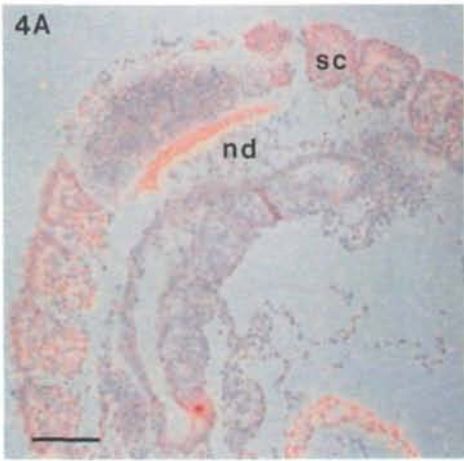
By far the most unexpected expression of the α1(II) collagen gene was that observed in the developing brain of 9.5- to 14.5-day fetuses. Type II collagen mRNA was first detected at low levels in the telencephalic neuroepithelium at 9.5 days p.c. and became more prominent from 11.5 days onwards. Type II collagen mRNA was initially uniformly distributed in the neuroepithelium of the fore- and midbrain vesicles but was subsequently more localised in the tissue on the floor of the mesencephalon at 12.5 days p.c. (Fig. 5D). By 14.5 days p.c., a high level of transcripts was found in the ventricular layer of the cerebral vesicles and the
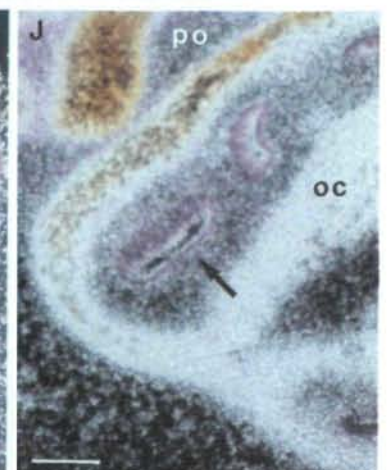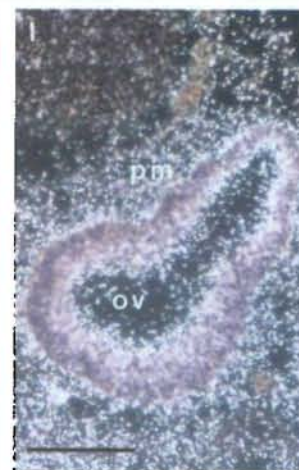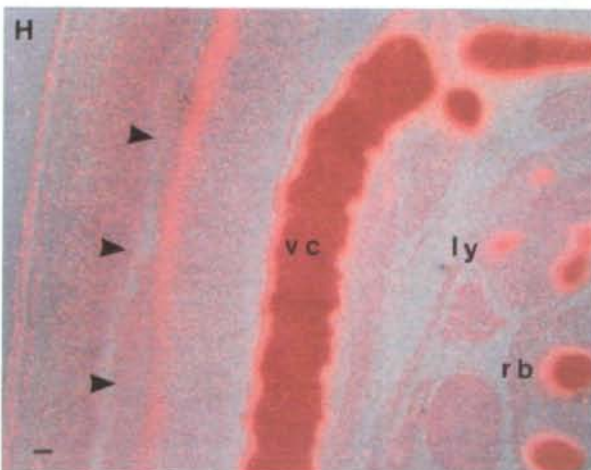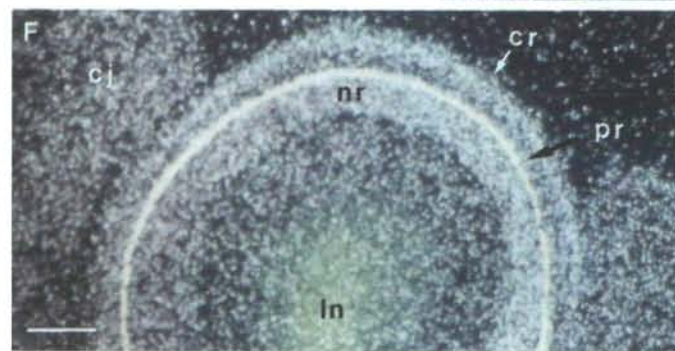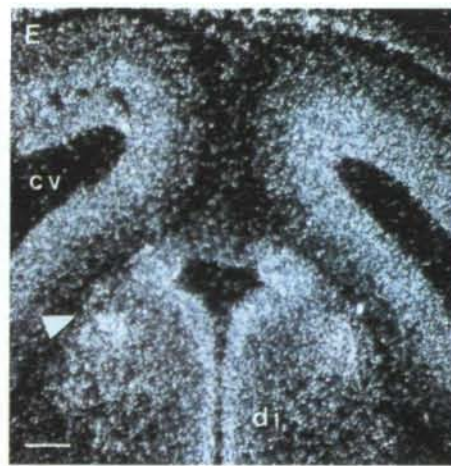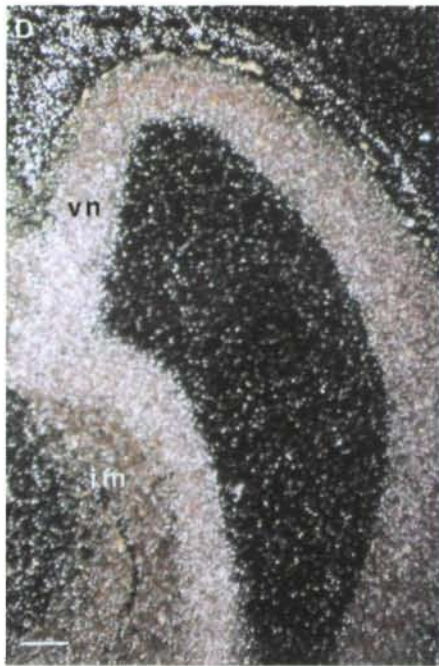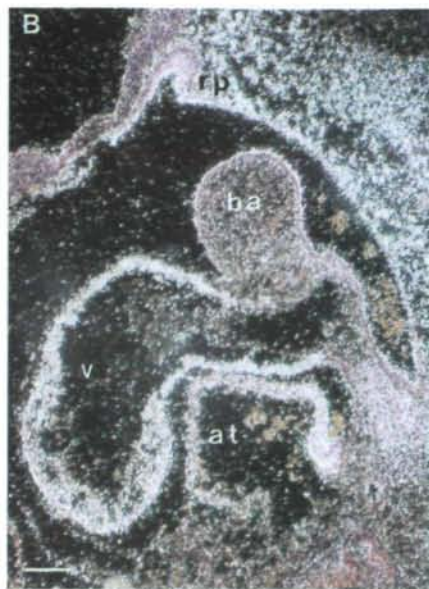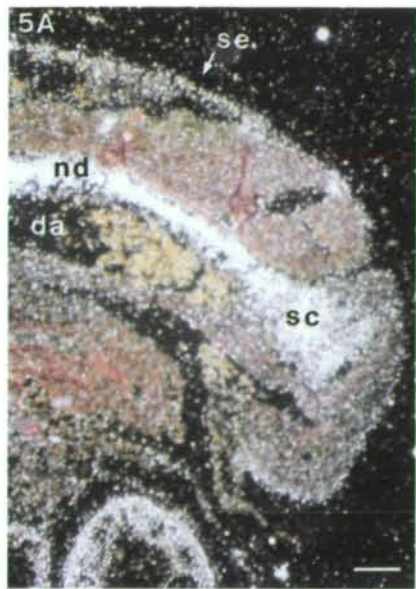
Fig. 4. The localization of type II collagen mRNA in the tissues of mouse fetuses by in situ hybridization. Accumulation of transcripts are found in the following. (A) The sclerotome of the differentiating somites (sc) and the notochord (nd) of a 9.5-day embryo, (B) The perinotochordal condensation of the sclerotome (sc) in the caudal region of a 12.5-day fetus, (C) The pre-vertebrae (pv) in the upper trunk region of a 12.5-day fetus but no hybridization in the adjacent spinal ganglia (sg), (D) The cranial mesenchyme (pm) ventral to the developing brain vesicles, with a much stronger hybridization in the mesenchyme adjacent to the hypophyseal pouch (rp) and the otocyst (arrow) in a 10.5-day embryo, (E) The chondrocytes in the developing centrum (cn) of the vertebrae of a 12.5-day fetus, (F) The mesenchymal cell masses (arrowheads) found at the root of the tongue (tn) which are destined to form the hyoid bone and the thyroid cartilage and the periotic mesenchyme (om) of a 12.5-day fetus, (G) The superfical mesenchyme (sm) over the cerebral vesicles which is destined for the frontal bone and part of the parietal bone and that of the supraoccipital bone (sb) covering the rhombencephalon, cells in the hippocampal cortex (arrows), the ventricular cell layer in the cerebral vesicles (cv), the diencephalon (di), the mesencephalon (me) and on the floor of the fourth ventricles in the rhombencephalon (arrowhead). (H) and (I) provide a comparison of the pattern of localization of the transcripts of α1(II) and α1(I) collagen genes on two sagittal sections of 14.5-day fetuses. With the exception of the calvaria and the differentiating epidermis, the localization of the transcripts for the two collagen types appears to be mutually exclusive. nt, neural tube; md, mesenchyme for mandible; om, periotic mesenchyme; se, surface ectoderm. Bar 100 μm except for G,H and I where it represents 1 mm.

diencephalon (Fig. 4G, 5E). Type II collagen mRNA was also detected in the neural retina, the corneal and conjunctival epithelium and the sclera of the developing eye (Fig. 5F). Cells in the hippocampal cortex (Fig. 4G) and the thalamus showed a particularly strong hybridization at this stage (Fig. 5E). In the midbrain and hindbrain regions, the transcripts were more localised in the ventricular layer of the basal plate. Expression was strongest in two longitudinal bands of the basal ventricular cells nearest to the floor plate (Fig. 5G). A low level of transcript was also detected in the basal plate ventricular layer of the medulla oblongata and the cervical spinal cord (Fig. 5H), while none were found in the mature tissue of the spinal cord or the spinal ganglia. A much diminished level of expression was observed in the ventricular and the intermediate cell layers of the 16.5-day fetal brain. Type II collagen mRNA was also detected in the epithelium of the otic vesicles of the 9.5- and 10.5-day fetus (Fig. 5I) and in the differentiating otic epithelium of the inner ear (Fig. 5J) of 14.5-day fetuses.

Among the non-chondrogenic tissues, the α1(I) and α1(II) collagen genes were co-expressed transiently in the epidermis (at 10.5 days), and later in the calvaria (14.5-16.5 days) and the tail tendon (at 16.5-18.5 days). However, no expression of type I collagen mRNA was found in the brain at any stage of
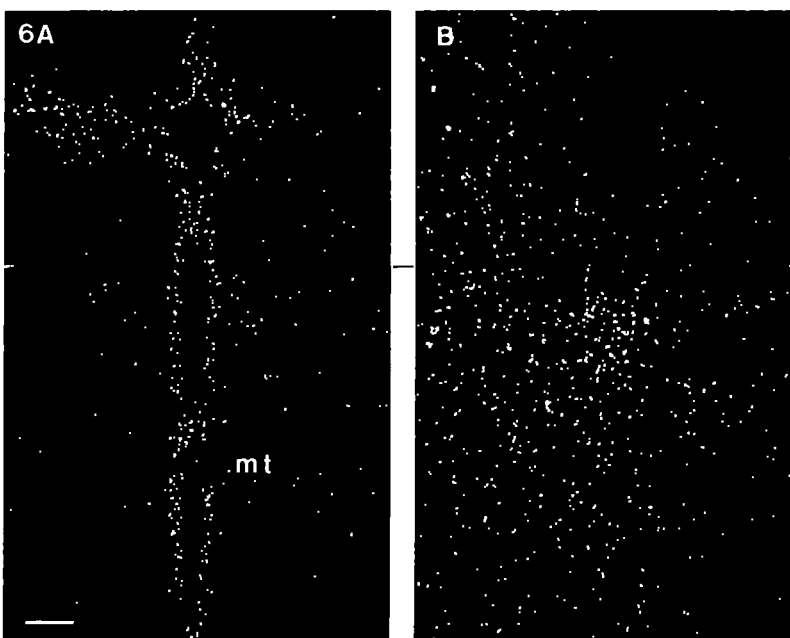
Fig. 5. Localization of type II collagen mRNA by *in situ* hybridization. (A) The notochord (nd) and the sclerotome (sc) in the trunk of a 10.5-day embryo, (B) The myocardium and epicardium of the ventricle (v) and atrium (at) of the developing heart of the 10.5-day embryo same as Fig. 4D, (C) The tendon (tn) in the tail of a 16.5-day fetus, (D) The ventricular cell layer (vn) of the diencephalon with a concentration of the transcript to the floor of the brain vesicle and a much diminished level of transcripts in the intermediate cell layer (im) of a 13.5-day fetus, (E) The ventricular cell layer of the cerebral vesicles (cv), the diencephalon (di) and the neuroblasts of the thalamic nuclei (arrowhead) of a 14.5-day fetus, (F) The neural retina (nr), the conjunctival epithelium (cj) and the cornea (cr) of the developing eye in a 14.5-day embryo. The pigment retina (pr) and the lens (ln) show no hybridization. The mesenchyme of the eyelids expressed a low level of transcripts. (G) Two longitudinal bands (cut in cross section) of neuroepithelial cells in the basal plate of the upper rhombencephalon of a 14.5-day fetus; a strong hybridization was seen in the mesenchyme of the prospective occipital bone (ob). (H) The ventricular cell layer (arrowheads) on the basal side of the cervical spinal cord of a 14.5-day fetus, the cervical vertebral column (vc) showed an intense hybridization to the probe, (I) The epithelium of the otocyst (ov) with a concentration of the hybridization at the apical cytoplasm, and the surrounding mesenchymal cells of a 10.5-day embryo. (J) The periotic mesenchyme (po), the differentiating cochlear epithelium (arrow) of the inner ear and the investing cartilage (oc) of a 14.5-day fetus. ba, branchial arch; da, dorsal aorta; ly, laryngeal cartilage; pm, paraxial mesenchyme; se, surface ectoderm; rp, hypophyseal pouch; rb, ribs. Bar 100 μm.

embryogenesis (data not shown) transcripts being restricted to the neighbouring meninges (Fig. 4I). No significant signal was seen in the brain when sections were hybridized with sense α1(II) collagen mRNA probes (Fig. 6A,B), confirming the specificity of hybridization and excludes the possibility of non-specific sticking of the RNA probes to non-chondrogenic sites.

Discussion

Type II collagen is a major extracellular component of cartilage and is actively synthesized by chondrocytes during cartilage formation (von der Mark, 1980). However, several recent studies have demonstrated that the gene encoding this collagen is expressed early in the pre-chondrogenic limb mesenchyme well in advance of overt cartilage differentiation (Kravis and Upholt, 1985; Kosher *et al.* 1986) and the formation of mesenchymal condensation results in an elevated expression of the type II collagen transcript (Swalla *et al.* 1988). Similar results were obtained from the present *in situ* hybridization study in the mouse embryo. Detectable levels of α1(II) collagen mRNA were observed early in development in (1) the cranial paraxial mesenchyme, destined for the formation of the chondrocranium, (2) the sclerotome, which later forms the vertebrae, (3) the mesenchymal primordia of the hyoid and laryngeal cartilage, (4) the thoracic mesenchyme for the ribs and (5) the prechondrogenic limb bud mesenchyme. The early expression of α1(II) collagen transcripts in the prechondrogenic mesenchyme may therefore be interpreted as activity related to the establishment of the extracellular microenvironment for inducing chondrocyte differentiation.

Recent immunohistochemical studies in avian embryos have revealed that type II collagen is found not only in the prechondrogenic mesenchyme but also at the epithelial–mesenchymal interface at prospective sites of cartilage formation (Kosher and Solursh, 1989; Thorogood, 1988). Our *in situ* hybridization studies and more recent immunohistochemical studies by Wood *et al.* 1991 show that the type II collagen gene is similarly expressed in prechondrogenic and non-chondrogenic tissues in the developing mouse. Type II collagen is found on the basal surface of embryonic epithelia such as the neural tube, the optic and auditory vesicles in the craniofacial region of the embryo and the notochord.

Fig. 6. *In situ* hybridization of coronal sections of a 14.5-day fetus with antisense and sense pEL111 RNA probes. (A) Positive hybridization of antisense pEL111 RNA transcripts to the neuroepithelium of the mesencephalon (mt). (B) Absence of signal in the neuroepithelium with sense pEL111 RNA transcripts. Bar=100 μm.

The distribution of type II collagen at these sites matches closely with the pattern of chondrocranium development (Thorogood, 1988; Wood et al. 1991). Taken together, these observations are consistent with the hypothesis that type II collagen is involved in the specification of both the temporal and spatial pattern of chondrogenesis during the development of the skull. The mouse auditory vesicle (otocyst) in particular has been shown to be capable of inducing chondrogenesis in the otic capsule at about 11–13 days p.c. but not earlier (Van de Water and Ruben, 1974; McPhee and Van de Water, 1986). This ability to induce chondrogenesis may be correlated with an increase in the synthesis of glycosaminoglycans, which like type II collagen are major matrix components of cartilage. However, type II collagen is expressed in the otic vesicle as early as 9.5 days (this study and Wood et al. 1991), which may serve a function related to more than a simple promotion of chondrocyte differentiation at the time of otic capsule formation.

In the trunk region, type II collagen is- found abundantly in the notochord and the sclerotomal mesenchyme. The appearance of type II collagen mRNA in this tissue therefore heralds the inductive influence of the notochord and the concomitant chondrogenic activity of the somitic mesoderm (Lash, 1968; Cohen and Hay, 1971; Kosher and Lash, 1975). Our DNA sequence data indicate that there is a high degree of interspecies homology of the $\alpha1(II)$ collagen gene in vertebrates. This sequence homology, together with the conservation of the expression of type II collagen in the notochord of the sturgeon (Miller and Mathews, 1974), lamprey (Eikenberry et al. 1984; Sheren et al. 1986), Xenopus (Ramirez, personal communication) as well as in the bird and the mouse, imply that this matrix molecule serves a basic and common developmental role such as the determination of the sites of chondrogenesis and the maintenance of tissue architecture (Trelstad and Birk, 1984; Hay, 1984).

The present in situ hybridization study suggests that there is active expression of type II collagen mRNA in the early neuroepithelia of the brain, the eye, the spinal cord, in the notochord and the otic vesicle of the mouse embryos at about the stage of type II collagen deposition in the basal aspect of the embryonic epithelia. This, therefore, strongly suggests an epithelial origin of the newly synthesized type II collagen, although contribution by the mesenchyme could not be ruled out because $\alpha1(II)$ collagen transcripts are also expressed in the adjacent paraxial mesenchyme and the peri-otic mesenchyme.

The functional significance of expression of type II collagen in other non-chondrogenic tissues is unclear. In addition to the prechondrogenic primordia and the embryonic epithelia associated with sites of chondrogenesis, type II collagen has also been detected in other embryonic tissues apparently unrelated to cartilage development. In both the chick and the mouse (Kosher and Solursh, 1989; Wood et al. 1991), type II collagen is found in the basement membrane of epithelia or tissues

such as the dorsal and lateral surface ectoderm, the lateral and ventral gut endoderm, the mesonephric duct, the splanchnic mesoderm and the interface between the epimyocardium and the endocardium. With the exception of the gut, the splanchnic mesoderm, the pharyngeal endoderm and the mesonephric duct, the present study shows that, in the mouse, type II collagen mRNA was detected in similar tissues, e.g. heart and the surface ectoderm. The timing of expression of $\alpha1(II)$ collagen mRNA in non-chondrogenic tissues in general paralleled the deposition of the protein in the extracellular matrix in avians and the mouse (Thorogood et al. 1986; Fitch et al. 1988; Kosher and Solursh, 1989; Wood et al. 1991) indicating control of type II collagen gene expression during these stages of development at the level of transcription rather than at translation.

Of particular significance is the localization of $\alpha1(II)$ collagen mRNA in the proliferative ventricular cells of the embryonic brain at 9.5 days and its subsequent localization to restricted areas in the rhombencephalic basal plate and the ventricular cells of the cervical spinal cord. Here, type II collagen may serve to maintain the organization of the neuroepithelium during active neurogenesis and neuroblast migration either by acting as an adhesive molecule between epithelial cells or by contributing to the basement membrane.

Type II collagen mRNA was also detected in the neural retina of the eye. This could be related to the appearance of type II collagen in the internal limiting membrane and the vitreous humor and may have some role in guiding the axonal processes of the retinal ganglion cells as they traverse the retinal surface and converge to form the optic nerve bundle. The localization of type II collagen transcripts in the basal plate of the rhombencephalon and the basal ventricular layer of the spinal cord is particularly interesting. This region also expresses high levels of alkaline phosphatase between 10.5 and 15.5 days during which time the $\alpha1(II)$ collagen gene is also transcribed. This part of the neural tube has been identified as the site of active production and migration of the motor neuroblasts of the cranial nerves and the spinal nerves (Kwong and Tam, 1984; Tam and Kwong, 1987). The biological role of alkaline phosphatase, a membrane-bound enzyme, is unclear, but it has been proposed that it may participate in processes such as the mineralization of bone and cartilage, in Pi-transport, and in the regulation of cell division and signal transduction (Wuthier and Register, 1985). The significance of co-expression of alkaline phosphatase and type II collagen in this region is unknown. In addition to a mechanical support role, type II collagen may promote, in concert with other molecules, the migration of the differentiating neuroblasts. The early onset and transience of expression, between 9.5 and 15.5 days, in the neuroepithelium is consistent with a stage-specific role in neural ontogeny. The early expression of the $\alpha1(II)$ collagen gene in the neuroepithelium at 9.5 days is particularly interesting because this coincides with the period of extensive morphogenesis of the neural tube. Further studies are

in progress to investigate the early expression of the α1(II) collagen in developing neural tissue.

Type II collagen gene transcripts were also found in supporting/interstitial tissues such as the epimyocardium, the surface ectoderm before full thickness of the epidermis is formed, the tail tendon and the extra-embryonic membrane (amnion and yolk sac). It is not possible from the detection of mRNA alone to predict the final extracellular destination of the protein product. The immuno-localization of type II collagen to the basement membrane of the neural tube and other embryonic organs (Wood *et al.* 1991) indicates that, after synthesis, the protein is secreted and transported to sites near to the expressing cells. Expression of α1(II) collagen mRNA in the mouse epimyocardium would be consistent with a final accumulation of the protein at the interface of the epimyocardium and the endocardium (Kosher and Solursh, 1989; Wood *et al.* 1991).

It is of interest that the mRNAs for type I and type II collagen were expressed together in developing mouse calvarial bones. The *in situ* hybridization results confirm previous studies using RNAase protection assays (Lovell-Badge *et al.* 1987). Intramembraneous bone, unlike endochondral bone, does not go through a cartilage phase in development and have been classically thought to synthesize only type I collagen (Jackson and Randall, 1956; Glimcher, 1976). Our results and recent findings in the chick (MacDonald and Tuan, 1989) show that this hypothesis is no longer valid and that type I and type II collagen are co-transcribed in the formation of calvaria.

The expression of the α1(II) collagen gene in non-chondrogenic tissue is intriguing and points to a role apart from chondrogenesis. However, there is the possibility that the gene product of the α1(II) collagen gene expressed in non-chondrogenic tissues is not or only partly incorporated into the type II collagen molecules. Type XI collagen, a fibril-forming collagen, is a quantitatively minor collagen component of hyaline cartilage and consists of molecules composed of a heterotrimer of three different polypeptide subunits, α1(XI), α2(XI), and α3(XI). The α3(XI) chain is generally thought to be the product of the α1(II) collagen gene whereas the α1(XI) and α2(XI) chains are distinct gene products (Eyre and Wu, 1987; Burgeson and Hollister, 1979; Vuorio and DeCrombrugghe, 1990). Recent studies have shown that expression of type XI collagen is not confined to cartilage (Bernard *et al.* 1988; Niyibizi and Eyre, 1989; Yoshioka and Ramirez, 1990) and the mRNAs for the three α chains of type XI collagen are not always co-expressed in every tissue (Cheah and Lui, unpublished results). In the immunohistochemical studies in the chick and quail (Thorogood *et al.* 1986; Kosher and Solursh, 1989) and mouse (Wood *et al.* 1991), type II collagen antibodies were used but the possibility of recognizing a common epitope conferred on types II and XI collagen by the α1(II)/α3(XI) chains needs to be excluded. The α1(II) collagen mRNAs detected in non-chondrogenic tissues may therefore represent either type II or type XI collagen or a mixture of both.

The function of type XI collagen is not fully understood. Recent reports indicate that fibrils in cartilage contain mixtures of types II, IX and XI collagen (Mendler *et al.* 1989). The presence of type V and I collagens in the collagen fibrils of non-cartilage tissues is thought to be important in regulating the diameter of these fibrils in the matrix (Eyre and Wu, 1987; Birk *et al.* 1988). Co-expression of types II and XI collagen may reflect a similar role of type XI collagen in cartilage. The co-expression of α1(II)/α3(XI) and α1(I) collagen mRNAs in the developing mouse heart, skin, tendon and calvaria may reflect such a role. *In situ* hybridization and immunohistochemical studies on the developmental pattern of expression of α1(XI) and α2(XI) collagen should help resolve these possibilities.

Although the developmental role of transient type II collagen expression in tissue other than cartilage still remains to be defined, it is interesting that, in many types of inherited human chondrodysplasias, an association of congenital heart defects, abnormalities in hippocampal formation and skull abnormalities has been reported (Wynne-Davies *et al.* 1985; Knisely and Ambler, 1988). The transient expression of type II collagen in the developing mouse heart and brain may therefore have some relevance to the non-skeletal defects found in human skeletal dysplasias. Experiments aimed at studying the phenotypic effects of interfering with α1(II) collagen gene expression should yield a better insight into the full function of type II collagen during development.

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